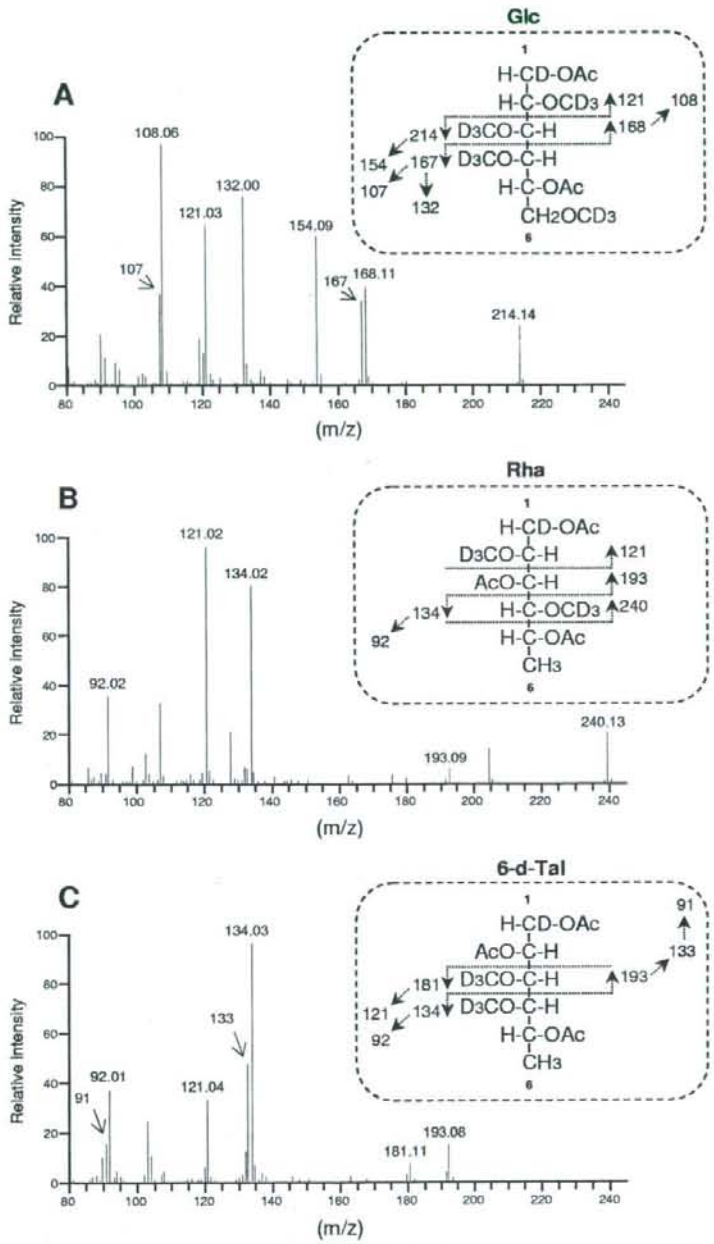

FIG. 4. GC-MS spectra and fragment ion assignments for Glc (A), Rha (B), and 6-d-Tal (C), which were derived from alditol acetates of sugars released from deuteriomethylated GPL-SG-U. Ac, acetate; D, deuterium.

clarified that the *gtfB-drrC* regions of serovar 2-, 7-, and 16-specific GPL-producing strains contain the genes involved in the formation of the specific sugar residues that are transferred to the Rha residue of serovar 1-specific GPL (18, 19, 30). Thus, this region could play an important role in generating the structural diversity of ssGPLs. As shown in this study, the specific functions for formation of sugar moieties of serovar 8-specific GPL were due to the genes present in the *gtfB-drrC*

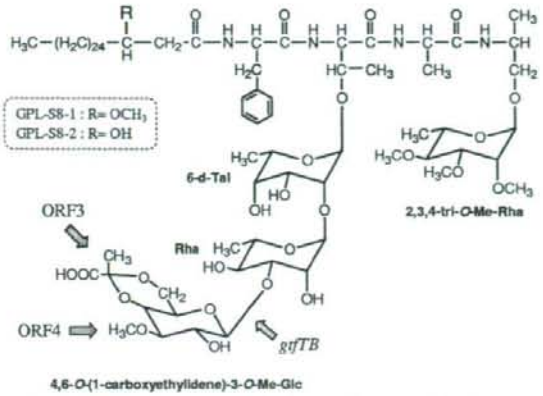

FIG. 5. Proposed structure and biosynthetic genes of GPL-S8 (serovar 8-specific GPL). Me, methyl.

region, suggesting that focusing on this region might provide clues for elucidating the characteristics of other ssGPLs whose biosynthesis is still not known.

It has been reported previously that the *gtfTB* gene in *M. avium* strains 104 and A5 was not likely to be associated with GPL biosynthesis because its ancestral homologue, Rv1516c (61% identity with the GtfTB gene), was the gene of *M. tuberculosis*, which produces no GPLs (28). Thus, it was interesting that *gtfTB* encodes a glycosyltransferase that does participate in GPL biosynthesis in which a Glc residue is transferred to serovar 1-specific GPL, yielding the serovar 8-specific GPL. *M. avium* strains 104 and A5 synthesize serovar 1-specific GPL as a final product and intermediate, respectively, while it has been recognized that neither of these strains produces serovar 8-specific GPL in spite of the presence of *gtfTB* in the GPL biosynthetic gene cluster (28). These observations raised the possibility that the transcription of *gtfTB* is inefficient in both strains due to the upstream sequences. Actually, in *M. avium* strain 104, a transposase sequence was observed upstream of *gtfTB*, indicating that this strain might be deficient in glucosylation, and consequently a serovar 1-specific GPL-producing strain is obtained (28). On the other hand, it has been shown that the biosynthetic gene cluster for serovar 7-specific GPL in *M. intracellulare* strain ATCC 35847 contains a putative glycosyltransferase gene which encodes amino acid sequences that are similar to the amino acid sequences encoded by *gtfTB* (59% identity) (18). Structural analysis of sugar moieties in serovar 7-specific GPL indicated that this GtfTB homologue may serve as a glycosyltransferase during formation of the terminal amidohexose residue that structurally resembles Glc (18).

The deduced amino acid sequences encoded by ORF3 and ORF4 showed that these genes putatively encode polysaccharide pyruvyltransferase and *O*-methyltransferase, respectively. Expression of ORF3 and ORF4 together with *gtfTB* led to structural alterations in which Glc was modified with both 4,6-*O*-(1-carboxyethylidene) and 3-*O*-methyl groups. Based on these observations, it is strongly suggested that ORF3 is associated with the formation of the 4,6-*O*-(1-carboxyethylidene) group that is synonymous with the cyclic pyruvate ketal and that ORF4 is associated with the 3-*O*-methylation of the Glc residue (Fig. 5). In mycobacteria, homologues of ORF3 and ORF4 were found only in *M. smegmatis*, as MSMEG_4736 (for ORF3), MSMEG_4737 (for ORF3), and MSMEG_4739 (for ORF4). *M. smegmatis* also produces glycolipids containing 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl-Glc as a sugar moiety (25, 34), which suggests that both homologues participate in the synthesis of these glycolipids. Sugar residues with a 4,6-*O*-(1-carboxyethylidene) group substitution have been found in carbohydrates such as extracellular polysaccharide and N-linked glycan, which are produced by some bacteria and yeasts (1, 15, 20, 22, 26). It has been shown that an increase in 4,6-*O*-(1-carboxyethylidene)-containing sugar residues leads to enhanced viscosity of extracellular polysaccharide from *Xanthomonas* sp., which alters the cell surface properties related to cellular attachment and protection from environmental stress (10). Accordingly, in terms of the properties of serovar 8-specific GPL, the presence of the 4,6-*O*-(1-carboxyethylidene) group might influence the pathogenicity of MAC serovar 8.

With regard to the antibody reactivity, it is unclear whether serovar 8-specific antibodies react with GPL-S8 because there are minor structural differences in the methylated positions of fatty acids and the terminal Rha residue linked to the tetrapeptide between GPL-S8 and serovar 8-specific GPL of MAC. Evaluation of the antibody response to GPL-S8 using serovar 8-specific antibodies would facilitate understanding the immunoreactivity mediated by ssGPLs.

In this study, we proved that *gtfTB* and adjacent genes in the GPL biosynthetic gene cluster in MAC serovar 8 strain are responsible for the formation of a unique glucose residue in serovar 8-specific GPL (Fig. 5). In particular, *gtfTB* encodes the glucosyltransferase that plays a critical role in the pathway leading from serovar 1-specific GPL to serovar 8-specific GPL. Through further study, including generation of *gtfTB* knockout mutants of MAC serovar 8 strains, results relevant to the biosynthesis of serovar 8-specific GPL might help clarify the biological function of ssGPLs and their role in the host-pathogen relationships of MAC.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Science and Technology of Japan and Research on Emerging and Re-Emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1. **Aman, P., M. McNeil, L.-E. Franzen, A. G. Darvill, and P. Albersheim.** 1981. Structural elucidation, using HPLC-MS and GLC-MS, of the acidic exopolysaccharide secreted by *Rhizobium meliloti* strain Rm1021. Carbohydr. Res. **95**:263–282.
2. **Aspinall, G. O., D. Chatterjee, and P. J. Brennan.** 1995. The variable surface glycolipids of mycobacteria: structures, synthesis of epitopes, and biological properties. Adv. Carbohydr. Chem. Biochem. **51**:169–242.
3. **Barrow, W. W., T. L. Davis, E. L. Wright, V. Labrousse, M. Bachelet, and N. Rastogi.** 1995. Immunomodulatory spectrum of lipids associated with *Mycobacterium avium* serovar 8. Infect. Immun. **63**:126–133.
4. **Belisle, J. T., K. Klaczkiewicz, P. J. Brennan, W. R. Jacobs, Jr., and J. M. Inamine.** 1993. Rough morphological variants of *Mycobacterium avium*. Characterization of genomic deletions resulting in the loss of glycopeptidolipid expression. J. Biol. Chem. **268**:10517–10523.
5. **Bhatnagar, S., and J. S. Schorey.** 2007. Exosomes released from infected macrophages contain *Mycobacterium avium* glycopeptidolipids and are proinflammatory. J. Biol. Chem. **282**:25779–25789.
6. **Bjorndal, H., C. G. Hellerqvist, B. Lindberg, and S. Svensson.** 1970. Gas-liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. Angew. Chem. Int. Ed. Engl. **9**:610–619.

7. **Brennan, P. J., G. O. Aspinall, and J. E. Shin.** 1981. Structure of the specific oligosaccharides from the glycopeptidolipid antigens of serovars in the *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex. J. Biol. Chem. **256:**6817–6822.
8. **Brennan, P. J., H. Mayer, G. O. Aspinall, and J. E. Nam Shin.** 1981. Structures of the glycopeptidolipid antigens from serovars in the *Mycobacterium avium/Mycobacterium intracellulare/Mycobacterium scrofulaceum* serocomplex. Eur. J. Biochem. **115:**7–15.
9. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
10. **Casas, J. A., V. E. Santos, and F. Garcia-Ochoa.** 2000. Xanthan gum production under several operational conditions: molecular structure and rheological properties. Enzyme Microb. Technol. **26:**282–291.
11. **Chatterjee, D., and K. H. Khoo.** 2001. The surface glycopeptidolipids of mycobacteria: structures and biological properties. Cell. Mol. Life Sci. **58:** 2018–2042.
12. **Ciucanu, I., and F. Kerek.** 1984. A simple and rapid method for the permethylation of carbohydrates. Carbohydr. Res. **131:**209–217.
13. **Daffe, M., and P. Draper.** 1998. The envelope layers of mycobacteria with reference to their pathogenicity. Adv. Microb. Physiol. **39:**131–203.
14. **Daffe, M., M. A. Laneelle, and G. Puzo.** 1983. Structural elucidation by field desorption and electron-impact mass spectrometry of the C-mycosides isolated from *Mycobacterium smegmatis*. Biochim. Biophys. Acta **751:**439–443.
15. **D'Haeze, W., J. Glushka, R. De Rycke, M. Holsters, and R. W. Carlson.** 2004. Structural characterization of extracellular polysaccharides of *Azorhizobium caulinodans* and importance for nodule initiation on *Sesbania rostrata*. Mol. Microbiol. **52:**485–500.
16. **Eckstein, T. M., J. T. Belisle, and J. M. Inamine.** 2003. Proposed pathway for the biosynthesis of serovar-specific glycopeptidolipids in *Mycobacterium avium* serovar 2. Microbiology **149:**2797–2807.
17. **Eckstein, T. M., F. S. Silbaq, D. Chatterjee, N. J. Kelly, P. J. Brennan, and J. T. Belisle.** 1998. Identification and recombinant expression of a *Mycobacterium avium* rhamnosyltransferase gene (*rtfA*) involved in glycopeptidolipid biosynthesis. J. Bacteriol. **180:**5567–5573.
18. **Fujiwara, N., N. Nakata, S. Maeda, T. Naka, M. Doe, I. Yano, and K. Kobayashi.** 2007. Structural characterization of a specific glycopeptidolipid containing a novel *N*-*acyl*-deoxy sugar from *Mycobacterium intracellulare* serotype 7 and genetic analysis of its glycosylation pathway. J. Bacteriol. **189:**1099–1108.
19. **Fujiwara, N., N. Nakata, T. Naka, I. Yano, M. Doe, D. Chatterjee, M. McNeil, P. J. Brennan, K. Kobayashi, M. Makino, S. Matsumoto, H. Ogura, and S. Maeda.** 2008. Structural analysis and biosynthesis gene cluster of an antigenic glycopeptidolipid from *Mycobacterium intracellulare*. J. Bacteriol. **190:**3613–3621.
20. **Gemmill, T. R., and R. B. Trimble.** 1996. *Schizosaccharomyces pombe* produces novel pyruvate-containing N-linked oligosaccharides. J. Biol. Chem. **271:**25945–25949.
21. **Horgen, L., E. L. Barrow, W. W. Barrow, and N. Rastogi.** 2000. Exposure of human peripheral blood mononuclear cells to total lipids and serovar-specific glycopeptidolipids from *Mycobacterium avium* serovars 4 and 8 results in inhibition of TH1-type responses. Microb. Pathog. **29:**9–16.
22. **Jansson, P. E., L. Kenne, and B. Lindberg.** 1975. Structure of extracellular polysaccharide from *Xanthomonas campestris*. Carbohydr. Res. **45:**275–282.
23. **Jeevarajah, D., J. H. Patterson, M. J. McConville, and H. Billman-Jacobe.** 2002. Modification of glycopeptidolipids by an *O*-methyltransferase of *Mycobacterium smegmatis*. Microbiology **148:**3079–3087.
24. **Julander, I., S. Hoffner, B. Petrini, and L. Ostlund.** 1996. Multiple serovars of *Mycobacterium avium* complex in patients with AIDS. APMIS **104:**318–320.
25. **Kamisango, K., S. Saadat, A. Dell, and C. E. Ballou.** 1985. Pyruvylated glycolipids from *Mycobacterium smegmatis*. Nature and location of the lipid components. J. Biol. Chem. **260:**4117–4121.
26. **Kojima, N., S. Kaya, Y. Araki, and E. Ito.** 1988. Pyruvic-acid-containing polysaccharide in the cell wall of *Bacillus polymyxa* AHU 1385. Eur. J. Biochem. **174:**255–260.
27. **Krzywinska, E., S. Bhatnagar, L. Sweet, D. Chatterjee, and J. S. Schorey.** 2005. *Mycobacterium avium* 104 deleted of the methyltransferase D gene by allelic replacement lacks serotype-specific glycopeptidolipids and shows attenuated virulence in mice. Mol. Microbiol. **56:**1262–1273.
28. **Krzywinska, E., and J. S. Schorey.** 2003. Characterization of genetic differences between *Mycobacterium avium* subsp. *avium* strains of diverse virulence with a focus on the glycopeptidolipid biosynthesis cluster. Vet. Microbiol. **91:**249–264.
29. **Li, Z., G. H. Bai, C. F. von Reyn, P. Marino, M. J. Brennan, N. Gine, and S. L. Morris.** 1996. Rapid detection of *Mycobacterium avium* in stool samples from AIDS patients by immunomagnetic PCR. J. Clin. Microbiol. **34:**1903–1907.
30. **Miyamoto, Y., T. Mukai, Y. Maeda, N. Nakata, M. Kai, T. Naka, I. Yano, and M. Makino.** 2007. Characterization of the fucosylation pathway in the biosynthesis of glycopeptidolipids from *Mycobacterium avium* complex. J. Bacteriol. **189:**5515–5522.
31. **Miyamoto, Y., T. Mukai, N. Nakata, Y. Maeda, M. Kai, T. Naka, I. Yano, and M. Makino.** 2006. Identification and characterization of the genes involved in glycosylation pathways of mycobacterial glycopeptidolipid biosynthesis. J. Bacteriol. **188:**86–95.
32. **Miyamoto, Y., T. Mukai, F. Takeshita, N. Nakata, Y. Maeda, M. Kai, and M. Makino.** 2004. Aggregation of mycobacteria caused by disruption of fibronectin-attachment protein-encoding gene. FEMS Microbiol. Lett. **236:**227–234.
33. **Patterson, J. H., M. J. McConville, R. E. Haites, R. L. Coppel, and H. Billman-Jacobe.** 2000. Identification of a methyltransferase from *Mycobacterium smegmatis* involved in glycopeptidolipid synthesis. J. Biol. Chem. **275:**24900–24906.
34. **Saadat, S., and C. E. Ballou.** 1983. Pyruvylated glycolipids from *Mycobacterium smegmatis*. Structures of two oligosaccharide components. J. Biol. Chem. **258:**1813–1818.
35. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.
36. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature **351:**456–460.
37. **Sweet, L., and J. S. Schorey.** 2006. Glycopeptidolipids from *Mycobacterium avium* promote macrophage activation in a TLR2- and MyD88-dependent manner. J. Leukoc. Biol. **80:**415–423.
38. **Tassell, S. K., M. Pourshafie, E. L. Wright, M. G. Richmond, and W. W. Barrow.** 1992. Modified lymphocyte response to mitogens induced by the lipopeptide fragment derived from *Mycobacterium avium* serovar-specific glycopeptidolipids. Infect. Immun. **60:**706–711.
39. **Tsang, A. Y., J. C. Denner, P. J. Brennan, and J. K. McClatchy.** 1992. Clinical and epidemiological importance of typing of *Mycobacterium avium* complex isolates. J. Clin. Microbiol. **30:**479–484.
40. **Vergne, I., and M. Daffe.** 1998. Interaction of mycobacterial glycolipids with host cells. Front. Biosci. **3:**d865–876.
41. **Yakrus, M. A., and R. C. Good.** 1990. Geographic distribution, frequency, and specimen source of *Mycobacterium avium* complex serotypes isolated from patients with acquired immunodeficiency syndrome. J. Clin. Microbiol. **28:**926–929.

RESEARCH ARTICLE

GM-CSF-mediated T-cell activation by macrophages infected with recombinant BCG that secretes major membrane protein-II of *Mycobacterium leprae*

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Received 14 July 2008; revised 18 October 2008; accepted 23 October 2008.
First published online 8 December 2008.

DOI:10.1111/j.1574-695X.2008.00495.x

Editor: Patrick Brennan

Keywords
macrophage; *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG); *Mycobacterium leprae*; GM-CSF; T-cell activation; interleukin-10.

Abstract

The potential of *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) needs to be augmented to efficiently activate $CD4^+$ T cells through macrophages. *Mycobacterium leprae*-derived recombinant major membrane protein (MMP)-II induced GM-CSF production from macrophages. A recombinant BCG-SM that secretes MMP-II more efficiently produced GM-CSF and activated interferon (IFN)-γ-producing $CD4^+$ T cells than did vector control BCG when infected with macrophages. The T-cell activation by BCG-SM was dependent on the GM-CSF production by macrophages. Interleukin (IL)-10 production by macrophages stimulated with *M. leprae* was inhibited in a GM-CSF-dependent manner when the precursor monocytes were infected with BCG-SM. BCG inducing GM-CSF production was effective in macrophage-mediated T-cell activation partially through IL-10 inhibition.

Introduction

Leprosy is a chronic infectious disease induced by parasitic infection with *Mycobacterium leprae* (Stoner, 1979). Despite the marked reduction in the number of both registered leprosy cases and new cases, a significant number of new cases (254 525 for the year 2007) are still detected each year (World Health Organization, 2008). The emergence of multidrug-resistant *M. leprae* (Kai *et al.*, 2004), although still few in number, and the complexity of leprosy reactions are distressing (Moschella, 2004). These observations indicate the urgent need to develop an efficacious vaccine against leprosy. *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) has been known to provide partial protection against the development of leprosy (Ponnighaus *et al.*, 1992). However, meta-analyses conducted by Setia *et al.* (2006) demonstrated an overall protective effect of only 26% against leprosy. There seem to be several reasons why BCG is not as effective as previously predicted. One of them may be that the human immune cells most susceptible to BCG infection are macrophages (Grode *et al.*, 2005). On entry into macrophages, mycobacteria inhibit phagosome–lysosome fusion, which results in a less efficient stimulation of interferon (IFN)-γ-producing type 1 $CD4^+$ T cells (Ridley & Jopling, 1966; Frehel & Rastogi, 1987). Further, BCG as well as pathogenic mycobacteria can induce the production of an abundant amount of interleukin (IL)-10 from macrophages (Yamamura *et al.*, 1991), which inhibits activation of $CD4^+$ T cells (Jonuleit *et al.*, 2001; Granelli-Piperno *et al.*, 2004). Moreover, it has been demonstrated in a murine study that BCG primarily infects macrophages *in vivo*, and the active proliferation of T cells *in vivo* needs the enrolment of dendritic cells (DC). Further, DC are known to be the most professional antigen-presenting cells (APC) in terms of T-cell activation. Thus, the transfer to DC of antigens produced by the processing of intracellular BCG or of proteins secreted from the mycobacteria in macrophages, seems to be important (Winau *et al.*, 2006).

We previously identified major membrane protein (MMP)-II (gene name, bfrA or ML2038), which is originally identified as bacterioferritin (Pessolani *et al.*, 1994) and localized in the cell membrane, as one of the dominant antigen of *M. leprae* (Maeda *et al.*, 2005; Makino *et al.*, 2005). Recombinant (r) MMP-II-pulsed DC activate naïve $CD4^+$ T cells to produce IFN-γ in an antigen-specific manner, and also stimulate T cells from not only paucibacillary leprosy, a representative clinical leprosy at one pole of the clinical spectrum, but also multibacillary leprosy, a representative leprosy at the opposite pole (Makino *et al.*, 2005). The activation of type 1 $CD4^+$ T cells is closely associated with the inhibition of the spread of *M. leprae in vivo* as observed in paucibacillary leprosy (Sieling *et al.*, 1999). In this respect, it was interesting to find that T cells from some paucibacillary leprosy patients seemed to be primed with MMP-II antigen *in vivo* (Makino *et al.*, 2005). Therefore, MMP-II was considered to be an immunodominant antigen of *M. leprae*. We constructed an rBCG strain (BCG-SM) that secretes MMP-II of *M. leprae* (Makino *et al.*, 2006). BCG-SM-infected DC stimulated quite efficiently both human naïve $CD4^+$ T cells and naïve $CD8^+$ T cells *in vitro*, and MMP-II-specific memory T cells were produced in mice inoculated with BCG-SM (Makino *et al.*, 2006).

Macrophages are heterogeneous in various aspects (Randolph *et al.*, 1999), and their differentiation is largely influenced by the cytokine milieu (Nakata *et al.*, 1991; Akagawa, 2002). Previously, we analysed the characteristics of two distinct macrophage subsets: rGM-CSF-mediated macrophages (GM-Mϕ) and rM-CSF-mediated macrophages (M-Mϕ) (Makino *et al.*, 2007). Both macrophages were equally susceptible to mycobacterial infection *in vitro*, but M-Mϕ infected with *M. leprae* did not activate $CD4^+$ T cells even after activation using both CD40 ligand and exogenous IFN-γ. Likewise, *Mycobacterium tuberculosis*-infected M-Mϕ failed to stimulate T cells (Verreck *et al.*, 2004). Further, a large amount of IL-10 was produced from M-Mϕ on stimulation with mycobacteria. Therefore, the fact that mycobacteria are highly susceptible to phagocytosis by M-Mϕ and poorly stimulate T cells through M-Mϕ, may be closely associated with the affinity of mycobacteria to macrophages, the induction of a latent infection and, in some cases, the development of disease. Likewise, M-Mϕ is one of the major target immune cells of BCG infection. However, to control the subsequently invading pathogenic mycobacteria, such as *M. leprae*, by producing memory T cells, modified BCG including the newly developed recombinant BCG-SM is required to be able to fully stimulate T cells even if M-Mϕ are the initial target host cells.

In this report, we examined the T-cell-stimulating ability of BCG-SM-infected M-Mϕ, and further assessed the influence of BCG-SM on the IL-10-producing activity of Mϕ upon a challenge with *M. leprae*.

Materials and methods

Preparation of cells and bacteria

Peripheral blood was obtained from healthy purified protein derivative (PPD)-positive individuals with informed consent. In Japan, most healthy individuals are PPD-positive due to a compulsory BCG vaccination for children (0–4 years old). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use as previously described (Makino & Baba, 1997). For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from freshly isolated heparinized blood or from cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antiboby (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates and nonadherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (Makino & Baba, 1997). Macrophages were generated by culturing monocytes in the presence of 20% foetal calf serum and either rM-CSF (R and D Systems, Abingdon, UK) (M-Mϕ) or rGM-CSF (PeproTech EC Ltd, London, UK) (GM-Mϕ) (Makino *et al.*, 2007). Both GM-Mϕ and M-Mϕ were pulsed with rBCGs on day 3 or 5 of culture, and were used as a stimulator of T cells on day 5 or 7 (Makino *et al.*, 2007). A recombinant BCG that secretes *M. leprae*-derived MMP-II was constructed as described previously (Makino *et al.*, 2006). In brief, a shuttle vector, pMV-261, having a kanamycin resistance gene and origins of replication for *Escherichia coli* and mycobacteria was used to construct pMV-SM (Secreting MMP-II) having the MMP-II cDNA fragment. The BCG substrain Pasteur was cultured *in vitro* using Middlebrook 7H9 broth (BD Biosciences Pharmingen, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin–dextrose–catalase (BD). Expression vectors were introduced into BCG by electroporation (Snapper *et al.*, 1988). Transformants were selected on Middlebrook 7H10 agar (BD) plates supplemented with 10% OADC (BD) and 25 $\mu g\,mL^{-1}$ kanamycin. Mycobacteria were subsequently grown in Middlebrook 7H9 broth containing 25 $\mu g\,mL^{-1}$ of kanamycin. BCG containing pMV-SM as an extrachromosomal plasmid is referred to as BCG-SM, and BCG containing pMV-261 is referred to as BCG-pMV (vector control BCG). *Mycobacterium leprae* (Thai-53) was isolated from the footpads of BALB/*c-nu/nu* mice (McDermott-Lancaster *et al.*, 1987). The isolated bacteria were counted using Shepard's method (McDermott-Lancaster *et al.*, 1987). The multiplicity of infection (MOI) was determined based on the assumption that macrophages were equally susceptible to infection with BCG or *M. leprae* (Hashimoto *et al.*, 2002). A recombinant MMP-II protein was produced as reported previously (Maeda *et al.*, 2005). Briefly,

the MMP-II gene (ML2038) was inserted into the expression plasmid pET28 (Novagen, Madison, WI) and transformed into *E. coli* strain ER2566 (New England BioLabs, Ipswich, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA). As a control for *M. leprae* antigen, we have purified hsp18 (ML1795) in *E. coli* using the PET expression system. The cytosolic fraction of the parental BCG was obtained as described previously (Maeda *et al.*, 2003).

Antigen-presenting function of rBCG-infected macrophages

The ability of rBCG-infected macrophages to stimulate T cells was assessed using an autologous macrophage-T cell coculture system as previously described (Wakamatsu *et al.*, 1999; Hashimoto *et al.*, 2002). The responder $CD4^+$ T cells were purified from freshly thawed PBMC using a CD4-negative isolation kit (Dynabeads 450) (Wakamatsu *et al.*, 1999). The purity of $CD4^+$ T cells was > 95% as assessed by FACS. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and macrophages were added to give the indicated macrophage : $CD4^+$ T-cell ratio. Supernatants of macrophage–T cell cocultures were collected on day 4. To identify molecules restricting T-cell activation, the following purified mAbs were used: anti-HLA-DR Ab (L243) and anti-CD86 Ab [IT2.2, Becton Dickinson (BD), San Jose, CA]. The concentration of IFN-γ produced by $CD4^+$ T cells was quantified using an enzyme assay kit, OptEIA Human enzyme-linked immunosorbent assay(ELISA) Set (BD). In some cases, M-Mϕ were pulsed with BCG-SM in the presence of 10 μg mL^{-1} of either normal rat IgG or neutralizing mAb to GM-CSF (rat IgG2a) (BD).

Production of IL-10 and GM-CSF by macrophages

The ability of M-Mϕ to produce IL-10 on stimulation with *M. leprae* was assessed. The monocytes were pretreated with the indicated dose of rBCG and subsequently made to differentiate into M-Mϕ by culturing for 5 days in the presence of rBCG and M-CSF. These macrophages were stimulated with *M. leprae* at the indicated MOI for 24 h. In some cases, monocytes were infected with BCG-SM in the presence of 10 μg mL^{-1} of neutralizing mAb to GM-CSF. Also, the ability of M-Mϕ to produce GM-CSF on stimulation with rBCG for 24 h was assessed. The concentration of these cytokines was quantified using OptEIA Human ELISA Set (BD).

Statistical analysis

Student's *t*-test was applied to determine statistical differences.

Results

Effect of rBCG-infected macrophages on T-cell-stimulating activity

We analysed the T-cell-stimulating activity of rBCG-infected GM-Mϕ and M-Mϕ (Fig. 1). GM-Mϕ infected with either BCG-SM or BCG-pMV significantly stimulated $CD4^+$

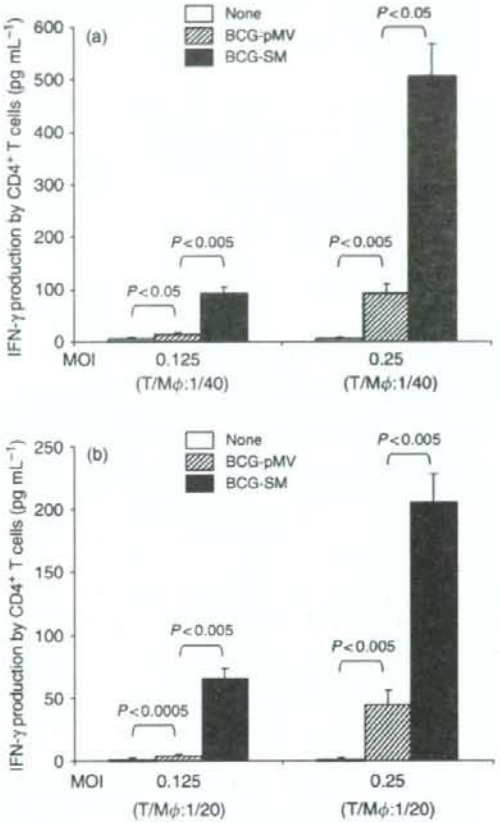

Fig. 1. Production of IFN-γ by $CD4^+$ T cells. (a) GM-Mϕ, differentiated by 3 days of culture with rGM-CSF from monocytes, were infected with BCG-pMV (vector control BCG) or BCG-SM (rBCG that secretes MMP-II) at the indicated MOI, and cultured for another 2 days. These GM-Mϕ were used as a stimulator of $CD4^+$ T cells (1×10^5 cells per well) at a T cell : GM-Mϕ ratio of 40 : 1 in a 4-day culture. (b) M-Mϕ, differentiated by 5 days of culture with rM-CSF from monocytes, were infected with BCG-pMV or BCG-SM at the indicated MOI, and cultured for another 2 days. M-Mϕ were then used as a stimulator of $CD4^+$ T cells (1×10^5 cells per well) at a T cell : M-Mϕ ratio of 20 : 1 in a 4-day culture. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

T cells. However, a larger amount of IFN-γ was produced by the T cells when GM-Mϕ were infected with BCG-SM (Fig. 1a), although BCG vaccination did not prime for MMP-II-specific T-cell response (not shown). We then analysed the T-cell-stimulating activity of BCG-infected M-Mϕ (Fig. 1b). Again, M-Mϕ infected with BCG-SM induced a higher amount of IFN-γ production by T cells than did BCG-pMV-infected M-Mϕ, although the IFN-γ production was less efficient than that induced by rBCG-infected GM-Mϕ even though higher doses of BCG-infected M-Mϕ were used as a stimulator.

Factors associated with the induction of the T-cell-stimulating activity of M-Mϕ

To define the factors associated with the $CD4^+$ T-cell activation by BCG-SM-infected M-Mϕ, we phenotypically analysed M-Mϕ infected with either BCG-pMV or BCG-SM. There was no significant difference between BCG-pMV-infected M-Mϕ and BCG-SM-infected M-Mϕ in the expression of HLA-DR, CD86 or CD40 molecules (not shown). The cytokines produced by M-Mϕ stimulated with rBCGs, including GM-CSF and IL-23, were examined. Both rBCGs induced GM-CSF production, but BCG-SM did so more efficiently than BCG-pMV (Fig. 2). However, IL-23 was not produced by M-Mϕ on stimulation with either BCG-pMV or BCG-SM. We also assessed whether rMMP-II protein can induce GM-CSF production in macrophages. Whereas *M. leprae*-derived cytosolic protein (not shown), other mycobacterial proteins such as BCG-derived cytosolic protein (5–10 μg mL^{-1}), control recombinant *M. leprae* antigen (hsp18), and lipopolysaccharide (amount present with rMMP-II protein) did not stimulate Mϕ, MMP-II induced GM-CSF production in a concentration-dependent manner (Fig. 3). rMMP-II also efficiently induced the production of other cytokines including tumour necrosis factor (TNF)α and IL-12p40 from Mϕ (not shown).

We examined the influence of surface antigens on M-Mϕ. The T-cell-stimulating activity of BCG-SM-infected M-Mϕ was significantly inhibited when the infected M-Mϕ were pretreated with the mAb to HLA-DR or CD86 antigens, whereas the control IgG did not affect IFN-γ production by T cells (Fig. 4a). However, IFN-γ production was partially inhibited when BCG-SM-infected M-Mϕ were treated with the mAb to MMP-II (not shown). Next, we examined the effect on T-cell activation of GM-CSF produced by M-Mϕ stimulated with BCG-SM (Fig. 4b). When M-Mϕ were infected with BCG-SM in the presence of the neutralizing mAb to GM-CSF, IFN-γ production by $CD4^+$ T cells was significantly inhibited. The T-cell-stimulating activity of BCG-SM-infected M-Mϕ was not affected by normal rat IgG.

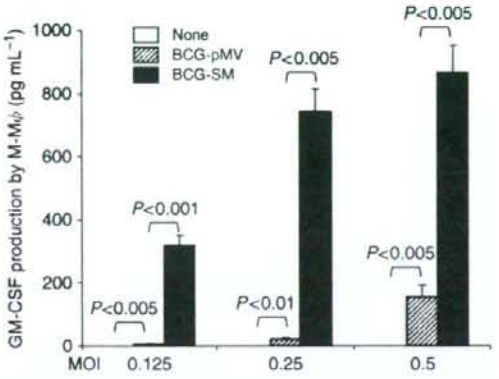

Fig. 2. Production of GM-CSF by M-Mϕ. M-Mϕ differentiated by 5 days of culture with rM-CSF from monocytes, were stimulated with BCG-pMV or BCG-SM for 24 h at the indicated MOI. A representative experiment based three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

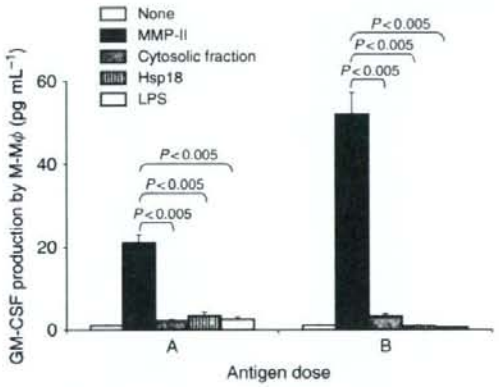

Fig. 3. GM-CSF production by M-Mϕ. M-Mϕ obtained after 5 days of culture with rM-CSF were stimulated for 24 h with rMMP-II, the BCG-derived cytosolic fraction or *Mycobacterium leprae*-derived hsp18 antigen [dose of antigen in (a) 5 μg mL^{-1} and that in (b) 10 μg mL^{-1}]. Lipopolysaccharide, assumed to be present with rMMP-II protein (660 ng mg^{-1} MMP-II protein), was used as a negative control (lipopolysaccharide in (a) 3.3 ng mL^{-1} and that in (b) 6.6 ng mL^{-1}). A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

Effect of infection of monocytes with BCG on IL-10 production by M-Mϕ

Macrophages are one of the cells most sensitive to *M. leprae* infection and M-Mϕ produce abundant IL-10 when infected with the bacteria (Makino *et al.*, 2007). As precursor

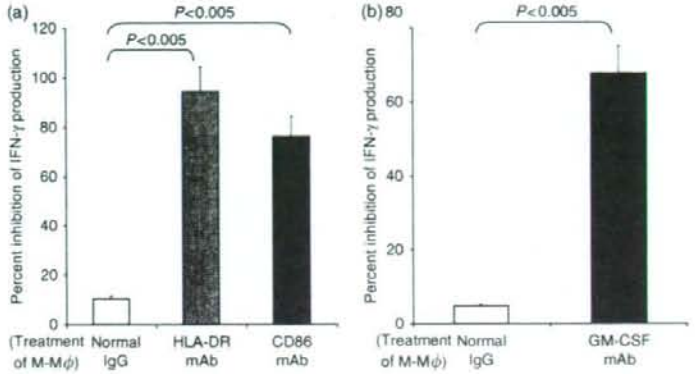

Fig. 4. (a) Inhibition of IFN-γ production by $CD4^+$ T cells by pretreatment of BCG-SM-infected M-Mϕ with mAb to HLA-DR or CD86. M-Mϕ differentiated from monocytes using rM-CSF were infected with BCG-SM at an MOI of 0.25 on day 5 of culture and cultured for another 2 days. The BCG-SM-infected M-Mϕ were treated with mAb to HLA-DR or CD86, or isotype-matched control IgG (10 μg mL $^{-1}$), and used as a stimulator of $CD4^+$ T cells, at a T cell : M-Mϕ ratio of 20 : 1 and cultured for another 4 days. The optimal concentration of mAb was determined in advance. Non-pretreated BCG-SM-infected M-Mϕ induced the production of 220.8 pg mL $^{-1}$ of IFN-γ by $CD4^+$ T cells. This titre was taken as 0% inhibition. (b) Inhibition of IFN-γ production by $CD4^+$ T cells by neutralizing GM-CSF produced from BCG-SM-infected M-Mϕ. M-Mϕ, differentiated from monocytes by culturing for 5 days with rM-CSF, were infected with BCG-SM (MOI 0.25) in the presence of neutralizing mAb to GM-CSF or isotype-matched control IgG (10 μg mL $^{-1}$). These M-Mϕ were used as a stimulator of $CD4^+$ T cells as in (a). The optimal concentration of mAb was determined in advance. M-Mϕ infected with BCG-SM in the absence of any Ab induced the production of 168.3 pg mL $^{-1}$ of IFN-γ by $CD4^+$ T cells. This titre was taken as 0% inhibition. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

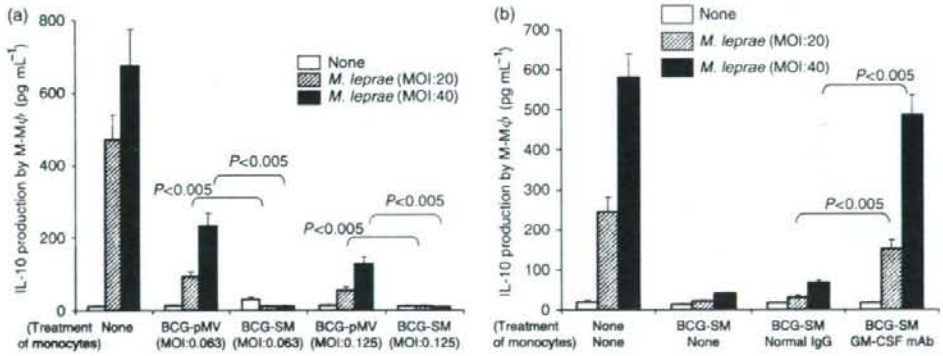

Fig. 5. (a) Production of IL-10 by M-Mϕ. Monocytes were infected with the indicated dose of BCG-pMV or BCG-SM, and subsequently differentiated into M-Mϕ by culturing for 5 days in the presence of M-CSF and rBCGs. These rBCG-preinfected M-Mϕ were stimulated with *Mycobacterium leprae* at the indicated MOI for 24 h. (b) Recovery of IL-10 production by M-Mϕ. Monocytes were infected with BCG-SM (MOI 0.063) in the presence of neutralizing GM-CSF mAb or isotype-matched control IgG and were subsequently differentiated into M-Mϕ by culturing for 5 days. These BCG-SM-preinfected M-Mϕ were stimulated with *M. leprae* at the indicated MOI for 24 h. The optimal concentration of mAb was determined in advance. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is show. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

monocytes also produced GM-CSF on stimulation with BCG in a BCG-SM-predominant fashion (not shown), we examined the effect of infection with rBCGs in monocytes on IL-10 production by M-Mϕ challenged with *M. leprae* (Fig. 5). M-Mϕ differentiated from monocytes untreated with any bacteria produced $> 400\,pg\,mL^{-1}$ of IL-10 on stimulation with *M. leprae*; however, the production of cytokine by M-Mϕ pretreated with rBCGs was significantly inhibited (Fig. 5a). The inhibition was more significant when BCG-SM was used as a stimulator of monocytes, and IL-10 production by M-Mϕ was almost completely inhibited. The inhibition was dependent on the dose of BCGs

used for pretreatment. In addition, pretreatment of monocytes with BCG-SM inhibited the IL-10 production induced even by lipopolysaccharide (not shown).

Furthermore, M-Mϕ differentiated from monocytes infected with BCG-SM in the presence of normal IgG did not produce IL-10 on stimulation with *M. leprae* (Fig. 5b). However, a significant level of IL-10 was produced when monocytes were infected with BCG-SM in the presence of the neutralizing mAb to GM-CSF. These results indicate that endogenously produced GM-CSF can inhibit IL-10 production.

Discussion

The host defence against intracellular parasitic pathogens such as *M. leprae* is closely associated with the activation of IFN-γ-producing type 1 T cells (Hashimoto *et al.*, 2002). In fact, in patients with paucibacillary leprosy, the activation of $CD4^+$ T cells results in inhibition of the intracellular multiplication and intercellular spread of *M. leprae* (Sieling *et al.*, 1999). The T-cell activation largely depends on the extent of the activation of APC, in which DC play an extremely important role, as DC are the most powerful T-cell stimulators among the APC (Hashimoto *et al.*, 2002). However, if T cells are not efficiently activated due to poor participation of DC, *M. leprae* may be predominantly retained in macrophages. In fact, multibacillary leprosy patients retain numerous *M. leprae* in their macrophages which, in some cases, allow the multiplication and intercellular spread of the bacteria (Ridley & Jopling, 1966).

The tissue resident macrophages, represented by GM-Mϕ and M-Mϕ, are heterogeneous in terms of function (Nakata *et al.*, 1991; Randolph *et al.*, 1999; Akagawa, 2002), despite being similarly susceptible to mycobacterial infection (Makino *et al.*, 2007). GM-Mϕ infected with *M. tuberculosis* or *M. leprae* significantly stimulated $CD4^+$ T cells, whereas M-Mϕ failed to stimulate $CD4^+$ T cells (Verreck *et al.*, 2004; Makino *et al.*, 2007). In this study, we found that, similar to those pathogenic mycobacteria, vector control BCG (BCG-pMV)-infected GM-Mϕ significantly stimulated $CD4^+$ T cells, whereas the BCG-pMV-infected M-Mϕ were less efficient in stimulating these cells. These results indicate the possibility that parental BCG may long reside in M-Mϕ and stimulate T cells inadequately, like the *M. leprae*-infected resident macrophages in multibacillary leprosy. In contrast to BCG-pMV, rBCG that secretes MMP-II (BCG-SM) has the ability to enlist not only GM-Mϕ, but also M-Mϕ, for T-cell activation. Further, the production of IFN-γ by $CD4^+$ T cells stimulated with BCG-SM-infected M-Mϕ was significantly inhibited by pretreatment of the M-Mϕ with the mAb to HLA-DR or CD86 antigens. In addition, the pretreatment of M-Mϕ infected with both BCG-SM and BCG-pMV effectively inhibited $CD4^+$ T-cell activation (not shown). Therefore, the BCG-SM-infected M-Mϕ seemed to stimulate $CD4^+$ T cells in an antigen-specific manner. Furthermore, there was a striking difference between BCG-pMV and BCG-SM in the induction of GM-CSF production. Not only from M-Mϕ, but also from GM-Mϕ, BCG-SM more efficiently induced GM-CSF production than BCG-pMV, and, further, rMMP-II protein, though less efficient, induced significant GM-CSF production. Previously, we reported that rMMP-II is highly immunogenic and induces production of various cytokines, including IL-12 and TNF-α, from APCs such as macrophages and DC (Maeda *et al.*, 2005). These findings indicate that the enhanced production of GM-CSF on stimulation by BCG-SM was at least partially associated with the secretion of MMP-II from BCG-SM.

As the activation of T cells by BCG-SM-infected M-Mϕ was largely inhibited when endogenously produced GM-CSF was neutralized by the mAb to GM-CSF, the endogenously produced GM-CSF may be closely associated with the enhanced T-cell activation by BCG-SM. Although we could not identify the most relevant antigen for T-cell activation, GM-CSF may change the activation status of macrophages or may at least partially transform the BCG-SM-infected M-Mϕ to GM-Mϕ (Makino *et al.*, 2007). Therefore, BCG-SM seems to be a unique rBCG capable of producing GM-CSF and utilizing M-Mϕ for T-cell stimulation.

Another important characteristic of mycobacteria which contributes to the inhibition of T-cell activation is the abundant production of IL-10 by M-Mϕ (Jonuleit *et al.*, 2001; Mochida-Nishimura *et al.*, 2001; Granelli-Piperno *et al.*, 2004). The major purpose of a vaccination is the production of memory T cells which can rapidly respond to subsequently invading pathogenic mycobacteria. However, IL-10 inhibits the re-activation of memory T cells *in vitro*. We found that the ability of BCG-SM to induce production of GM-CSF is useful to inhibit IL-10 production. Monocytes were quite sensitive in the production of GM-CSF, and both BCG-pMV and BCG-SM induced cytokine production by monocytes, although BCG-SM predominated at lower doses (not shown). Thus, even BCG-pMV inhibited IL-10 production at higher doses. However, macrophages differentiated from monocytes which were infected with a small dose of BCG-SM completely inhibited IL-10 production upon subsequent challenge with *M. leprae*, and the inhibitory activity was at least partially cancelled out by the neutralization of endogenously produced GM-CSF. Further, heat-killed BCG-SM, which does not secrete MMP-II (Makino *et al.*, 2006), did not inhibit IL-10 production. These observations indicate that macrophages treated with GM-CSF endogenously diminished the ability to produce IL-10 upon stimulation with *M. leprae*. Previously, we observed that addition of GM-CSF exogenously blocked the ability to produce IL-10 (Makino *et al.*, 2007), which agrees with the

present data. The benefit of inhibition of IL-10 production for host defence has been previously demonstrated *in vivo*. IL-10-deficient mice displayed increased anti-mycobacterial immune responses and decreased bacterial burden (Murray & Young, 1999). In the absence of IL-10, antigen-specific memory T cells, which are efficiently produced by vaccination with BCG-SM for instance, may be fully activated for elimination of *M. leprae*. Although these are still preliminary findings, in one experiment BCG-SM more efficiently inhibited the multiplication of *M. leprae* in footpads of mice than in parent BCG. Therefore, BCG-SM may wipe out favourable conditions for the survival of *M. leprae*. The molecules that are present in the parental BCG and are associated with GM-CSF production remain undefined in the present study, but identification of these molecules may be useful to further enhance the T-cell-stimulating activity of BCG-SM. Also, the identification of such molecules may contribute greatly to the control of the pathogenic mycobacterial diseases using modified BCG.

In this study, we demonstrated that BCG-SM which can induce abundant GM-CSF production, may be more potent than parent BCG in immunostimulation and in the inhibition of IL-10 production, for preventing the survival of *M. leprae*.

Acknowledgements

We acknowledge the contribution of Ms N. Makino to the preparation of the manuscript. We also thank Ms Y. Harada for technical support, and the Japanese Red Cross Society for kindly providing PBMCs from healthy donors.

This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour, and Welfare of Japan.

References

Akagawa KS (2002) Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages. *Int J Hematol* **76**: 27–34.

Frehel C & Rastogi N (1987) *Mycobacterium leprae* surface components intervene in the early phagosome–lysosome fusion inhibition event. *Infect Immun* **55**: 2916–2921.

Granelli-Piperno A, Golebiowska A, Trumpfheller C, Siegal FP & Steinman RM (2004) HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation. *P Natl Acad Sci USA* **101**: 7669–7674.

Grode L, Seiler P, Baumann S *et al.* (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* Bacille Calmette–Guérin mutants that secrete listeriolysin. *J Clin Invest* **115**: 2472–2479.

Hashimoto K, Maeda Y, Kimura H, Suzuki K, Masuda A, Matsuoka M & Makino M (2002) Infection of *M. leprae* to monocyte derived dendritic cells and its influence on antigen presenting function. *Infect Immun* **70**: 5167–5176.

Jonuleit H, Schmitt E, Steinbrink K & Enk AH (2001) Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends Immunol* **22**: 394–400.

Kai M, Maeda Y, Maeda S, Fukutomi Y, Kobayashi K, Kashiwabara Y, Makino M, Abbasi MA, Khan MZ & Shah PA (2004) Active surveillance of leprosy contacts in country with low prevalence rate. *Int J Leprosy* **72**: 50–53.

Maeda Y, Gidoh M, Ishii N, Mukai C & Makino M (2003) Assessment of cell mediated immunogenicity of *Mycobacterium leprae*-derived antigens. *Cell Immunol* **222**: 69–77.

Maeda Y, Mukai T, Spencer J & Makino M (2005) Identification of immunomodulating agent from *Mycobacterium leprae*. *Infect Immun* **73**: 2744–2750.

Makino M & Baba M (1997) A cryopreservation method of human peripheral blood mononuclear cells for efficient production of dendritic cells. *Scand J Immunol* **45**: 618–622.

Makino M, Maeda Y & Ishii N (2005) Immunostimulatory activity of major membrane protein-II from *Mycobacterium leprae*. *Cell Immunol* **233**: 53–60.

Makino M, Maeda Y & Inagaki K (2006) Immunostimulatory activity of recombinant *Mycobacterium bovis* BCG that secretes Major Membrane Protein II of *Mycobacterium leprae*. *Infect Immun* **74**: 6264–6271.

Makino M, Maeda Y, Fukutomi Y & Mukai T (2007) Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages. *Microbes Infect* **9**: 70–77.

McDermott-Lancaster RD, Ito T, Kohsaka K, Guelpa-Lauras CC & Grosset JH (1987) Multiplication of *Mycobacterium leprae* in the nude mouse, and some applications of nude mice to experimental leprosy. *Int J Leprosy* **55**: 889–895.

Mochida-Nishimura K, Akagawa KS & Rich EA (2001) Interleukin-10 contributes development of macrophage suppressor activities by macrophage colony-stimulating factor, but not by granulocyte-macrophage colony-stimulating factor. *Cell Immunol* **214**: 81–88.

Moschella SL (2004) An update on the diagnosis and treatment of leprosy. *J Am Acad Dermatol* **51**: 417–426.

Murray PJ & Young RA (1999) Increased antimycobacterial immunity in interleukin-10-deficient mice. *Infect Immun* **67**: 3087–3095.

Nakata K, Akagawa KS, Fukayama M, Hayashi Y, Kadokura M & Tokunaga T (1991) Granulocyte-macrophage colony-stimulating factor promotes the proliferation of human alveolar macrophages *in vitro*. *J Immunol* **147**: 1266–1272.

Pessolani MC, Smith DR, Rivoire B, McCormick J, Hefta SA, Cole ST & Brennan PJ (1994) Purification, characterization, gene sequence, and significance of a bacterioferritin from *Mycobacterium leprae*. *J Exp Med* **180**: 319–327.

Ponnighaus JM, Fine PE, Sterne JA, Wilson RJ, Msosa E, Gruer PJ, Jenkins PA, Lucas SB, Liomba NG & Bliss L (1992) Efficacy of BCG vaccine against leprosy and tuberculosis in northern Malawi. *Lancet* **14**: 636–639.

Randolph GJ, Inaba K, Robbiani DF, Steinman RM & Muller WA (1999) Differentiation of phagocytic monocytes into lymph node dendritic cells *in vivo*. *Immunity* **11**: 753–761.

Ridley DS & Jopling WH (1966) Classification of leprosy according to immunity. A five-group system. *Int J Leprosy* **34**: 255–273.

Setia MS, Steinmaus C, Ho CH & Rutherford GW (2006) The role of BCG in prevention of leprosy: a meta-analysis. *Lancet Infect Dis* **6**: 162–170.

Sieling PA, Jullien D, Dahlem M, Tedder TF, Rea TH, Modlin RL & Porcelli SA (1999) CD1 expression by dendritic cells in human leprosy lesions: correlation with effective host immunity. *J Immunol* **162**: 1851–1858.

Snapper SB, Iugosi L, Jekkel A, Melton RE, Kieser T, Bloom BR & Jacobs WR Jr (1988) Lysogeny and transformation in mycobacteria: stable expression of foreign genes. *P Natl Acad Sci USA* **85**: 6987–6991.

Stoner GL (1979) Importance of the neural predilection of *Mycobacterium leprae* in leprosy. *Lancet* **2**: 994–996.

Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, Kastelein R, Kolk A, de Waal-Malefyt R & Ottenhoff TH (2004) Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *P Natl Acad Sci USA* **101**: 4560–4565.

Wakamatsu S, Makino M, Tei C & Baba M (1999) Monocyte-driven activation-induced apoptotic cell death of human T-lymphotropic virus type I-infected T cells. *J Immunol* **163**: 3914–3919.

Winau F, Weber S, Sad S, de Diego J, Hoops SL, Breiden B, Sandhoff K, Brinkmann V, Kaufmann SHE & Schaible UE (2006) Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity* **24**: 105–117.

World Health Organization (2008) Global leprosy situation, 2008. *Wkly Epidemiol Rec* **83**: 293–300.

Yamamura M, Uyemura K, Deans RJ, Weinberg K, Rea TH, Bloom BR & Modlin RL (1991) Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* **254**: 277–279.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2008, p. 2992–2993 Vol. 52, No. 8
0066-4804/08/$08.00+0 doi:10.1128/AAC.01686-07

Coprevalence of Plasmid-Mediated Quinolone Resistance Determinants QepA, Qnr, and AAC(6′)-Ib-cr among 16S rRNA Methylase RmtB-Producing *Escherichia coli* Isolates from Pigs[▽]

Plasmid-mediated quinolone resistance determinants, including Qnr peptides and AAC(6′)-Ib-cr, are increasingly identified worldwide among various clinical isolates of *Enterobacteriaceae* (7, 9, 10). Very recently, a novel plasmid-mediated fluoroquinolone-resistant determinant, QepA (quinolone efflux pump), which showed a considerable similarity to the major facilitator superfamily-type efflux pumps, was first identified in an *Escherichia coli* clinical isolate from Japan (13) and later found also in an *E. coli* isolate in Belgium (6). Interestingly, both of the two *qepA*-harboring *E. coli* isolates also contained the *rmtB* gene encoding a 16S rRNA methyltransferase, an emerging new molecular mechanism responsible for high-level pan-aminoglycoside resistance among gram-negative pathogens (3, 4, 6, 13, 14).

Our previous study showed that *rmtB* was highly prevalent among *E. coli* isolates from pigs in China (1). The aim of this study was to investigate the prevalence of plasmid-mediated quinolone resistance determinants among *rmtB*-producing *E. coli* isolates from pigs in China and to identify the association of the *qepA* gene with *rmtB*.

One hundred fifty-one *E. coli* isolates were obtained from pig feces sampled at two pig farms. These isolates were collected from 2005 to 2006, and 48 of them were identified as producers of RmtB. (Some of these data were published previously [1].) Screening for *qepA*, *qnrA*, *qnrB*, *qnrS*, and *aac(6′)-Ib-cr* genes was carried out by PCR amplification among the 48 *rmtB*-positive isolates. For *qepA*, the following primers were used to produce a 218-bp amplicon: *qepA*-F (5′-GCAGGTCC AGCAGCGGGTAG-3′) and *qepA*-R (5′-CTTCCTGCCCGA GTATCGTG-3′). Positive results were confirmed by direct sequencing of PCR products. *qnrA*, *qnrB*, *qnrS*, and *aac(6′)-Ib-cr* genes were detected by PCR using specific primers (the used *qnrB* primers were able to detect almost all known *qnrB* alleles except *qnrB8*), as previously described (5, 8, 11), and were finally confirmed by sequencing of each PCR product.

Overall, *qepA*, *qnrB*, *qnrS*, and *aac(6′)-Ib-cr* were detected in 28 (58.3%), 1 (2.1%), 9 (18.8%), and 6 (12.5%) of 48 RmtB-producing *E. coli* isolates, respectively (Table 1). The *qnrB* genes were identified as *qnrB6* alleles by sequencing. The *qnrS* genes were confirmed as *qnrS1* (four isolates) and *qnrS2* (five isolates) alleles by sequencing. Four isolates with uniform pulsed-field gel electrophoresis (PFGE) patterns harbored *qepA*, *qnrS2*, and *aac(6′)-Ib-cr* genes concurrently.

To investigate the association of *rmtB* and *qepA*, *rmtB*-positive *E. coli* transconjugants described previously (1) were subjected to PCR amplification of *qepA*, and all transconjugants

TABLE 1. Characteristics of *E. coli* isolates and transconjugants harboring *rmtB*, as well as *qnr*, *qepA*, and/or *aac(6′)-Ib-cr*

Isolate(s)[a]	PFGE type	Resistance gene detected	MIC (μg/ml) of enrofloxacin	Fold increase in quinolone MIC for transconjugant vs recipient[b]				
				NOR	ENR	CIP	NAL	LEV
GZ3	A1	*qepA*	64	16	4	8	8	2
GZ4	A2	*qepA*	32	4	4	8	2	2
GZ5, GZ6	B	*qepA*	16	16,8	8, 2	8, 16	1	2, 4
GZ8	C	*qepA*	64	8	16	4	4	1
GZ9	D	*qepA*	32	16	2	8	4	2
GZ11	E	*qepA*	128	4	4	4	2	2
GZ12, GZ13, GZ14	F	*qepA*	16, 8, 16	16, 8, 16	4, 2, 8	8, 16, 4	1, 4, 1	8, 2, 4
GZ15	G	*qepA*	8	4	8	4	1	2
GZ16	H	*qepA*	>128	8	2	8	2	4
CQ15	I1	*qepA*	2	8	16	16	1	1
CQ18, CQ2, CQ5	I2	*qepA*	2, 2, 4	8, 16, 16	1, 16, 2	1, 3, 2, 8	1	1, 4, 1
CQ4[c]	J1	*qepA*	0.03	2	1	1	2	1
CQ20	J1	*qepA*	0.03	4	4	2	1	2
CQ26	K	*qepA*	0.5	4	2	4	1	1
CQ10	L	*qepA*	0.25	32	2	8	1	2
CQ14	M	*qepA*	16	32	2	4	1	2
GZ7	N	*qepA*, *qnrS1*	32	16	1	2	16	1
GZ1	O	*qnrS1*	4	16	32	16	4	4
GZ2[c]	O	*qnrS1*	2	2	2	4	4	2
CQ22[c]	P	*qnrS1*	4	2	2	1	1	1
CQ13	K	*qnrS2*	0.5	4	8	4	4	4
CQ6, CQ7, CQ12, CQ16	Q	*qepA*, *qnrS2*, *aac(6′)-Ib-cr*	2	16	2, 2, 4, 2	16, 4, 8, 8	16, 1, 1, 2	4, 1, 2, 2
GZ10	R	*qepA*, *aac(6′)-Ib-cr*	16	16	4	16	16	4
CQ19	S	*qepA*, *aac(6′)-Ib-cr*	2	16	4	4	1	1
CQ1[c]	U	*qnrB6*	0.25	2	1	1	2	1

[a] Isolates with the same letters were isolated from the same farm.
[b] The quinolone MICs of the recipient strains were 4 μg/ml for nalidixic acid (NAL); 0.015 μg/ml for ciprofloxacin (CIP); and 0.03 μg/ml for norfloxacin (NOR), enrofloxacin (ENR), and levofloxacin (LEV).
[c] RmtB-positive transconjugants not containing any plasmid-mediated quinolone resistance determinants.

that originated from the 28 *qepA*-positive isolates selected with aminoglycoside resistance were positive for the *qepA* gene except one, suggesting an strong linkage of *qepA* with *rmtB*. Two *rmtB*-positive transconjugants also harbored *qnrS1* or *qnrS2*.

MICs of ciprofloxacin, enrofloxacin, levofloxacin, nalidixic acid, and norfloxacin for the 27 *qepA*-positive and 2 *qnrS*-positive transconjugants were determined by the agar dilution method according to CLSI guidelines (2). The increase (fold) in quinolone MICs for transconjugants compared with those of recipients is shown in Table 1. The MICs for transconjugants strongly indicated that *qepA* as well as *qnrS* conferred quinolone resistance, with a 4- to 32-fold increase in norfloxacin MICs and 1- to 32-fold increase in enrofloxacin and ciprofloxacin MICs. However, variations in the quinolone MICs for different transconjugants suggested that the QepA may be expressed at variable levels. Xu et al. (12) recently reported that different promoter strengths may cause the differences in *qnrA* expression levels and in ciprofloxacin MICs of different transconjugants. Further studies are needed to find out whether the wide range of MICs of quinolones for different *qepA*-harboring transconjugants depends on the diversities in *qepA* expression levels due to different promoter strengths. MICs of enrofloxacin for all isolates were also determined by the agar dilution method according to CLSI guidelines. As indicated in Table 1, most isolates were resistant to enrofloxacin (MIC, $\geq$2 μg/ml), but six isolates were susceptible to enrofloxacin.

This study shows the high prevalence of plasmid-mediated quinolone resistance determinants among *E. coli* isolates recovered from food-producing animals. A total of 58.3% (28/48) of *rmtB*-positive *E. coli* isolates harbored *qepA* gene, indicating a close relationship between *qepA* and *rmtB*, which has been reported in the previous studies (6, 13). This is also the first time three different plasmid-mediated quinolone resistance determinants (QepA, Qnr, and AAC(6′)-Ib-cr) were identified in an *E. coli* strain. Coproduction of QepA, Qnr, AAC(6′)-Ib-cr, and RmtB may well facilitate the survival of bacteria under selective pressure of antimicrobial agents in both veterinary and human clinical environments, and the resistance determinants in food-producing animals could be transmitted to humans via the food chain. Further spread of these resistance determinants among pathogenic microbes may occur in the near future. Thus, it is necessary to monitor and minimize the spread of such resistance determinants among hazardous bacteria in both humans and animals.

We are grateful to Ming-Gui Wang (Division of Infectious Diseases, Huashan Hospital, Fudan University) and Sheng Chen (Department of Microbiology and Molecular Genetics, Medical College of Wisconsin) for critically reading the manuscript.

This work was supported in part by research grants from the National Natural Science Foundation of China (30500373 and 30130140) and National Key Technology R&D Program of China (2006BAK02A03-5).

REFERENCES

1. **Chen, L., Z. L. Chen, J. H. Liu, Z. L. Zeng, J. Y. Ma, and H. X. Jiang.** 2007. Emergence of RmtB methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. J. Antimicrob. Chemother. **59:**880–885.
2. **Clinical and Laboratory Standards Institute.** 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. Approved standard, 2nd ed. Document M31-A2. CLSI, Wayne, PA.
3. **Doi, Y., and Y. Arakawa.** 2007. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clin. Infect. Dis. **45:**88–94.
4. **Doi, Y., K. Yokoyama, K. Yamane, J.-I. Wachino, N. Shibata, T. Yagi, K. Shibayama, H. Kato, and Y. Arakawa.** 2004. Plasmid-mediated 16S rRNA methylase in *Serratia marcescens* conferring high-level resistance to aminoglycosides. Antimicrob. Agents Chemother. **48:**491–496.
5. **Park, C. H., A. Robicsek, G. A. Jacoby, D. Sahm, and D. C. Hooper.** 2006. Prevalence in the United States of *aac(6′)-Ib-cr* encoding a ciprofloxacin-modifying enzyme. Antimicrob. Agents Chemother. **50:**3953–3955.
6. **Périchon, B., P. Courvalin, and M. Galimand.** 2007. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrob. Agents Chemother. **51:**2464–2469.
7. **Robicsek, A., G. A. Jacoby, and D. C. Hooper.** 2006. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect. Dis. **6:**629–640.
8. **Robicsek, A., J. Strahilevitz, D. F. Sahm, G. A. Jacoby, and D. C. Hooper.** 2006. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. Antimicrob. Agents Chemother. **50:**2872–2874.
9. **Robicsek, A., J. Strahilevitz, G. A. Jacoby, M. Macielag, D. Abbanat, C. H. Park, K. Bush, and D. C. Hooper.** 2006. Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. Nat. Med. **12:**83–88.
10. **Tran, J. H., and G. A. Jacoby.** 2002. Mechanism of plasmid-mediated quinolone resistance. Proc. Natl. Acad. Sci. USA **99:**5638–5642.
11. **Wu, J.-J., W.-C. Ko, S.-H. Tsai, and J.-J. Yan.** 2007. Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of *Enterobacter cloacae* in a Taiwanese hospital. Antimicrob. Agents Chemother. **51:**1223–1227.
12. **Xu, X., S. Wu, X. Ye, Y. Liu, W. Shi, Y. Zhang, and M. Wang.** 2007. Prevalence and expression of the plasmid-mediated quinolone resistance determinant *qnrA1*. Antimicrob. Agents Chemother. **51:**4105–4110.
13. **Yamane, K., J. Wachino, S. Suzuki, K. Kimura, N. Shibata, H. Kato, K. Shibayama, T. Konda, and Y. Arakawa.** 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob. Agents Chemother. **51:**3354–3360.
14. **Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa.** 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet **362:**1888–1893.

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▽ Published ahead of print on 19 May 2008.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2008, p. 1564–1566
0066-4804/08/$08.00+0 doi:10.1128/AAC.01137-07

Vol. 52, No. 4

Plasmid-Mediated *qepA* Gene among *Escherichia coli* Clinical Isolates from Japan▽

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Received 29 August 2007/Returned for modification 9 September 2007/Accepted 30 January 2008

Seven hundred fifty-one *Escherichia coli* clinical isolates collected from 140 Japanese hospitals between 2002 and 2006 were screened for the *qepA* and *qnr* genes. Two *E. coli* isolates (0.3%) harbored *qepA*, but no *qnr* was identified. The results suggested a low prevalence of *E. coli* harboring *qepA* or *qnr* in Japan.

The most common chromosomal mechanism of resistance to fluoroquinolones (FQs) in pathogenic bacteria is amino acid substitutions in the quinolone resistance-determining regions of DNA gyrase (GyrA) and/or topoisomerase IV (ParC), which are the main target molecules of FQs (7, 8). Efflux pumps and alteration in the outer membrane proteins also contribute to chromosomal FQ resistance (6). Plasmid-mediated mechanisms of resistance to FQs such as Qnr and AAC(6′)-Ib-cr have also been described (13). We recently identified *qepA*, a new plasmid-mediated gene responsible for reduced FQ susceptibility from *Escherichia coli* C316, which was isolated in 2002 from the urine of an inpatient in Japan (21), and *qepA* was also reported from *E. coli* 1450, which was isolated in a Belgian hospital (12). *qepA* encodes an efflux pump belonging to the major facilitator subfamily (MSF). The MICs of norfloxacin, enrofloxacin, and ciprofloxacin were 32- to 64-fold higher for the experimental strains expressing QepA compared with the host strain (21). The MICs of ampicillin, erythromycin, kanamycin, tetracycline, and chemical substances such as carbonyl cyanide *m*-chlorophenylhydrazone, acriflavine, rhodamine 6G, crystal violet, and sodium dodecyl sulfate were not affected, however, indicating that FQs are the specific substrates of QepA. Moreover, a norfloxacin accumulation assay with or without carbonyl cyanide *m*-chlorophenylhydrazone, an efflux pump inhibitor, showed that QepA is an FQ-specific MSF-type efflux pump (21).

qnrA was the first plasmid-mediated gene that conferred resistance to quinolones such as nalidixic acid and increased MICs of FQs, originally reported in *Klebsiella pneumoniae* clinical isolates from the United States (11, 17). Subsequently, two other groups of *qnr* genes, *qnrB* (9) and *qnrS* (5), as well as their variants, have been reported. Qnrs belong to the pentapeptide repeat family and mimic DNA fragments bound to the DNA gyrase (17). The *qnr* genes have been identified in various bacterial species belonging to the family *Enterobacteriaceae* in many countries (13). In Japan, *qnrS* was first identified in *Shigella flexneri* (5) and *qnrA* was also identified recently (15, 16). Clinically, *E. coli* is the most frequent cause of urinary tract infections and FQs are some of the preferred antimicrobial agents for treatment (19). In this study, we investigated the prevalence of *qepA*, as well as *qnrA*, *qnrB*, and *qnrS*, among *E. coli* clinical isolates collected from Japanese medical facilities.

A total of 751 nonduplicate *E. coli* isolates isolated from patients admitted to 140 medical facilities in Japan between 2002 and 2006 were submitted to our reference laboratory for characterization of the genetic determinants responsible for antimicrobial resistance, as well as their genetic relatedness. All of the isolates were suspended in Luria-Bertani (LB) broth supplemented with 25% glycerol and stored in a −80°C deep freezer until analysis. The isolates were initially screened by growth on LB agar plates containing 0.025 μg/ml norfloxacin. PCR analyses for *qepA* and the three *qnr* genes were performed for all of the isolates that grew on the norfloxacin-containing plates. DNA templates for the PCR were prepared by the standard boiling method. The primer sets used for detection of *qnrA*, *qnrB*, and *qnrS* have been described by Cattoir et al. (1) and Robicsek et al. (14). The pairs of primers designed by Cattoir et al. (1) were able to amplify internal fragments with *qnrA1* to *qnrA6*, *qnrB1* to *qnrB8*, and *qnrS1* to *qnrS2*, respectively. A 199-bp fragment of *qepA* was amplified by PCR with primers QEPA-F (5′-GCA GGT CCA GCA GCG GGT AG-3′) and QEPA-R (5′-CTT CCT GCC CGA GTA TCG TG-3′). The pair of primers used for detection of *rmtB* have been described by Doi and Arakawa (4). *rmtB* is a 16S rRNA methylase gene that confers resistance to aminoglycosides and was located in close proximity to *qepA* on a transferable plasmid in *E. coli* C316 (21). Positive control strains for *qnrA*, *qnrB*, and *qnrS* were *E. coli* J53(pMG252) (11), *E. coli* J53(pMG298) (9), and *E. coli* DH10B(pBC-H2.6) (5), respectively, and that for *qepA* was *E. coli* KAM32(pSTVqepA) (21). The PCR conditions used for *qepA* were as follows: initial denaturation at 96°C for 1 min, followed by 30 cycles of amplification at 96°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The final extension step was at 72°C for 5 min. The multiplex PCR condition for the *qnr* genes has been described previously (1, 14).

Of the 751 *E. coli* isolates tested, 325 grew on LB agar plates supplemented with 0.025 μg/ml norfloxacin. Two isolates (0.3%) were positive for *qepA* and *rmtB* (MRY04-1030 and MRY05-3283). The two isolates came from geographically distant hospitals. However, no *qnr* gene was detected among the *E. coli* isolates tested in this study.

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▽ Published ahead of print on 19 February 2008.

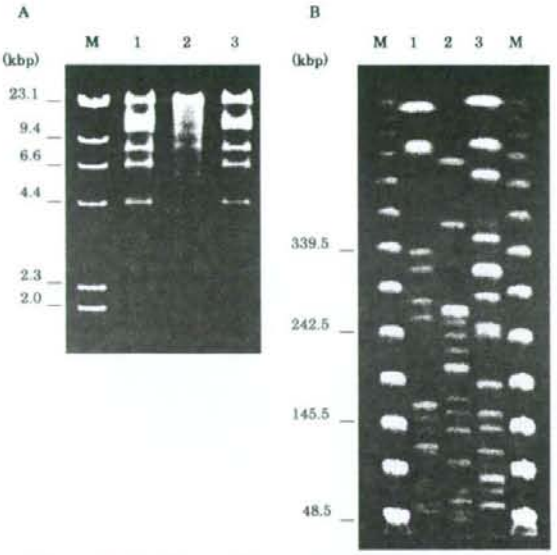

FIG. 1. (A) EcoRI restriction profiles of *qepA*-carrying plasmids from the transconjugants. Lane M, lambda HindIII marker; lane 1, pHPA from *E. coli* C316; lane 2, p041060 from *E. coli* MRY04-1060; lane 3, p05283 from *E. coli* MRY05-3283. (B) PFGE fingerprinting patterns of XbaI-digested total DNA preparations from three *E. coli* isolates. Lanes M, lambda ladder PFGE marker used as a molecular size marker. Lanes 1 to 3, *E. coli* C316, MRY04-1030, and MRY05-3283, respectively.

Transconjugation analysis was performed by the filter mating method with *E. coli* DH10B as the recipient (3). Transconjugants were selected on LB agar plates supplemented with streptomycin (50 μg/ml) and amikacin (50 μg/ml) because the plasmid carried *rmtB*, which confers resistance to amikacin. Plasmids were digested with EcoRI (New England BioLabs, Beverly, MA) and electrophoresed through a 1.0% agarose gel. FQ resistance was successfully transferred from the two *qepA*-positive *E. coli* isolates to *E. coli* DH10B at a frequency of 10^{-5} to 10^{-6} cells per recipient cell by conjugation. EcoRI restriction patterns for *qepA* carrying plasmids are shown in Fig. 1. The restriction patterns of pHPA from *E. coli* C316 and p05283 from *E. coli* MRY05-3283 were very similar. However, those of p05283 and p041060 from *E. coli* MRY04-1060 were completely different from the other two. Neither of the *qepA*-positive plasmids conferred resistance to ceftazidime and cefotaxime.

Pulsed-field gel electrophoresis (PFGE) was performed with the CHEF-Mapper system (Bio-Rad Laboratories, Hercules, CA). Genomic DNA preparations from *E. coli* C316, MRY04-1060, and MRY05-3283 were digested with XbaI (New England BioLabs) (Fig. 1). The PFGE fingerprinting patterns of the three *qepA*-positive strains were apparently different from each other.

Antimicrobial susceptibility testing of the *qepA*-positive isolates and their transconjugants was performed by the agar dilution method according to the guidelines recommended by the Clinical and Laboratory Standards Institute (2) (Table 1). The MICs of norfloxacin for the transconjugants of each *qepA*-positive isolate were four- to fivefold higher than that for the recipient strain. The two *qepA*-positive isolates were also highly resistant to all of the aminoglycosides tested, including amikacin, tobramycin, and gentamicin, but susceptible to the expanded-spectrum cephalosporins and imipenem.

In our previous study, *qepA* and *rmtB* were found to be encoded on the same transferable plasmid, and the analysis of the genetic environment of *qepA* in *E. coli* showed that *qepA* and *rmtB* were likely mediated by a composite transposon flanked by two copies of IS*26* (21). Interestingly, an *E. coli* strain positive for both *qepA* and *rmtB* has also been isolated in Belgium (12). The genetic organization of the region containing *qepA* and *rmtB* was very similar to that of *E. coli* C316, suggesting the *qepA*-harboring isolates demonstrating panresistance to aminoglycosides by production of RmtB may well have already spread worldwide.

Although *qnr* genes have been identified in *E. coli* and other members of the family *Enterobacteriaceae* isolated from other East Asian countries, such as China, Korea, and Taiwan (10, 18, 20), the results of our study indicate that *qnr*-harboring *E. coli* is still very rare in Japanese medical facilities.

We are grateful to George A. Jacoby for provision of the *qnrA*- and *qnrB*-positive control strains.

pBC-H2.6, deposited by Mami Hata, was provided by the DNA Bank, RIKEN BioResource Center (Tsukuba, Japan), with the support of the National Bio-Resources Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan (MEXT). Part

TABLE 1. Antimicrobial susceptibilities of the *qepA* donor, transconjugant, and recipient strains used in this study

Antimicrobial agent	MIC (μg/ml) for *E. coli* strain:				
	MRY04-1060	MRY05-3283	DH10B(p041060)[a]	DH10B(p05283)[b]	DH10B
Norfloxacin	>128	>128	0.25	0.25	≤0.008
Levofloxacin	64	128	0.008	0.015	≤0.008
Ciprofloxacin	>128	>128	0.015	0.015	≤0.008
Ceftazidime	0.5	0.5	0.5	0.5	0.25
Cefotaxime	0.13	0.13	0.06	0.06	0.06
Imipenem	0.13	0.13	0.25	0.25	0.25
Gentamicin	>128	>128	>128	>128	0.5
Amikacin	>128	>128	>128	>128	2
Tobramycin	>128	>128	>128	>128	0.5

[a] Transconjugant of *E. coli* MRY04-1060.
[b] Transconjugant of *E. coli* MRY05-3283.

of this work was funded by a grant from the Kurozumi Medical Foundation and Grant-in-Aid for Young Scientists (B) 19790400 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

REFERENCES

1. **Cattoir, V., L. Poirel, V. Rotimi, C. J. Soussy, and P. Nordmann.** 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. J. Antimicrob. Chemother. **60:**394–397.
2. **Clinical and Laboratory Standards Institute.** 2005. Performance standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI M100-S15. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
4. **Doi, Y., and Y. Arakawa.** 2007. 16S rRNA methylation: emerging resistance mechanism against aminoglycosides. Clin. Infect. Dis. **45:**88–94.
5. **Hata, M., M. Suzuki, M. Matsumoto, M. Takahashi, K. Sato, S. Ibe, and K. Sakae.** 2005. Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. Antimicrob. Agents Chemother. **49:** 801–803.
6. **Hooper, D. C.** 1999. Mechanisms of fluoroquinolone resistance. Drug Resist. Updates **2:**38–55.
7. **Hooper, D. C.** 2001. Emerging mechanisms of fluoroquinolone resistance. Emerg. Infect. Dis. **7:**337–341.
8. **Hopkins, K. L., R. H. Davies, and E. J. Threlfall.** 2005. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. Int. J. Antimicrob. Agents **25:**358–373.
9. **Jacoby, G. A., K. E. Walsh, D. M. Mills, V. J. Walker, H. Oh, A. Robicsek, and D. C. Hooper.** 2006. *qnrB*, another plasmid-mediated gene for quinolone resistance. Antimicrob. Agents Chemother. **50:**1178–1182.
10. **Jeong, J. Y., H. J. Yoon, E. S. Kim, Y. Lee, S. H. Choi, N. J. Kim, J. H. Woo, and Y. S. Kim.** 2005. Detection of *qnr* in clinical isolates of *Escherichia coli* from Korea. Antimicrob. Agents Chemother. **49:**2522–2524.
11. **Martínez-Martínez, L., A. Pascual, and G. A. Jacoby.** 1998. Quinolone resistance from a transferable plasmid. Lancet **351:**797–799.
12. **Périchon, B., P. Courvalin, and M. Galimand.** 2007. Transferable resistance to aminoglycosides by methylation of G1405 in 16S rRNA and to hydrophilic fluoroquinolones by QepA-mediated efflux in *Escherichia coli*. Antimicrob. Agents Chemother. **51:**2464–2469.
13. **Robicsek, A., G. A. Jacoby, and D. C. Hooper.** 2006. The worldwide emergence of plasmid-mediated quinolone resistance. Lancet Infect. Dis. **6:**629–640.
14. **Robicsek, A., J. Strahilevitz, D. F. Sahm, G. A. Jacoby, and D. C. Hooper.** 2006. *qnr* prevalence in ceftazidime-resistant *Enterobacteriaceae* isolates from the United States. Antimicrob. Agents Chemother. **50:**2872–2874.
15. **Saga, T., T. Akasaka, H. Takase, M. Tanaka, K. Sato, and M. Kaku.** 2007. First detection of the plasmid-mediated quinolone resistance determinant *qnrA* in *Enterobacteriaceae* clinical isolates in Japan. Int. J. Antimicrob. Agents **29:**738–739.
16. **Saito, R., W. Kumita, K. Sato, T. Chida, N. Okamura, K. Moriya, and K. Koike.** 2007. Detection of plasmid-mediated quinolone resistance associated with *qnrA* in an *Escherichia coli* clinical isolate producing CTX-M-9 β-lactamase in Japan. Int. J. Antimicrob. Agents **29:**600–602.
17. **Tran, J. H., and G. A. Jacoby.** 2002. Mechanism of plasmid-mediated quinolone resistance. Proc. Natl. Acad. Sci. USA **99:**5638–5642.
18. **Wang, M., J. H. Tran, G. A. Jacoby, Y. Zhang, F. Wang, and D. C. Hooper.** 2003. Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China. Antimicrob. Agents Chemother. **47:**2242–2248.
19. **Warren, J. W., E. Abrutyn, J. R. Hebel, J. R. Johnsin, A. J. Schaeffer, and W. E. Stamm.** 1999. Guidelines for antimicrobial treatment of uncomplicated acute bacterial cystitis and acute pyelonephritis in woman. Clin. Infect. Dis. **29:**745–758.
20. **Wu, J. J., W. C. Ko, S. H. Tsai, and J. J. Yan.** 2007. Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of *Enterobacter cloacae* in a Taiwanese hospital. Antimicrob. Agents Chemother. **51:**1223–1227.
21. **Yamane, K., J. Wachino, S. Suzuki, K. Kimura, N. Shibata, H. Kato, K. Shibayama, T. Konda, and Y. Arakawa.** 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. Antimicrob. Agents Chemother. **51:**3354–3360.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2008, p. 4194–4197 Vol. 52, No. 11
0066-4804/08/$08.00+0 doi:10.1128/AAC.01337-07

KHM-1, a Novel Plasmid-Mediated Metallo-β-Lactamase from a *Citrobacter freundii* Clinical Isolate▽

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Received 17 October 2007/Returned for modification 2 December 2007/Accepted 1 August 2008

A novel gene, bla_{KHM-1}, encoding a metallo-β-lactamase, KHM-1, was cloned from a clinical isolate of *Citrobacter freundii* resistant to most β-lactam antibiotics. *Escherichia coli* expressing bla_{KHM-1} was resistant to all broad-spectrum β-lactams except for monobactams and showed reduced susceptibility to carbapenems. Recombinant KHM-1 exhibited EDTA-inhibitable hydrolytic activity against most β-lactams, with an overall preference for cephalosporins.

Acquired metallo-β-lactamases (MBLs) produced by gram-negative bacteria, including *Pseudomonas aeruginosa*, *Acinetobacter* spp., and several enterobacteria, confer resistance to all β-lactams except the monobactams (2). Acquired MBLs are categorized on the basis of amino acid sequences into various types (2, 23). The IMP- and VIM-type enzymes are the most common and are found worldwide (2, 8, 23). Recently, four additional types, SPM, GIM, SIM, and AIM, have been found in Brazil (21), Germany (3), Korea (11), and Australia (24), respectively. We report here on the detection of a novel acquired MBL in a clinical isolate of *Citrobacter freundii* identified in Japan.

C. freundii strain KHM243 was isolated in 1997 from a patient with catheter-associated urinary tract infection at Kyorin University Hospital (Tokyo, Japan). *Escherichia coli* K-12 strain W1895 was used as the recipient in conjugation experiments. *E. coli* JM109 (Takara Bio, Shiga, Japan) was used as the host for recombinant plasmids. Plasmid pHSG396 (Takara Bio) was used for the cloning of bla_{KHM-1} fragments.

Susceptibility to β-lactams was determined by the microdilution method (4). The production of MBL was detected by a double-disk synergy test with disks containing sodium mercaptoacetic acid (MBL production test; Eiken Chemical Co. Ltd., Tokyo, Japan), as described by Arakawa et al. (1).

The transfer of resistance by conjugation was analyzed as described previously (7). *E. coli* transconjugants were selected on Penassay broth agar (antibiotic medium no. 3; Becton Dickinson, Franklin Lakes, NJ) containing rifampin (200 μg/ml; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and moxalactam (16 μg/ml; Shionogi & Co., Ltd., Osaka, Japan). Plasmid DNA was extracted by an alkaline lysis procedure (9). Plasmid R100 (94.5 kb) (13) from *E. coli* CSH2, plasmid R478 (275 kb) (6) from *E. coli* J53, and three cryptic plasmids (200, 60, and 2.4 kb) from *Salmonella enterica* serovar Enteritidis L119 (15) were used as molecular size markers.

PCR analysis specific for class 1 integrons was performed as described previously (12). DNA sequences flanking bla_{KHM-1} were determined by inverse PCR (16). Briefly, plasmid DNA extracted from *E. coli* transconjugant W1895(pCF243) was digested with EcoRV or XspI (Takara Bio). Self-ligated digests were used as the template for an inverse PCR. The upstream and downstream flanking regions of bla_{KHM-1} were amplified by inverse PCR with two sets of primers: primers 5′-CGATA TAACAAGAGCTATTTTCAT-3′ and 5′-GGTATGCGCTG ACGATTC-3′ for the upstream region and primers 5′-GGTG TACAGATAAACGCCG-3′ and 5′-TTTATTTGGTGGCTG TTTTGTC-3′ for the downstream region.

The KHM-1 MBL from *E. coli* JM109(pKHM-1) was purified with HiTrap Q HP and Superdex 200 columns (GE Healthcare Bio-Sciences KK, Tokyo, Japan), as described by Franceschini et al. (5). During the purification procedure, the presence of β-lactamase activity was monitored with 100 μM nitrocefin (Oxoid Ltd., Basingstoke, United Kingdom). The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Kinetic analysis was carried out in 50 mM phosphate buffer (pH 7.0) at 25°C with a UV-visible spectrophotometer (V-530; Jasco, Tokyo, Japan). The K_m and k_{cat} values and the k_{cat}/K_m ratio were determined by analyzing β-lactam hydrolysis under initial-rate conditions by use of the Lineweaver-Burk plot.

Antibiotic susceptibility testing showed that *C. freundii* KHM243 was resistant to most β-lactams and showed reduced susceptibility to carbapenems (Table 1). However, KHM243 was susceptible to monobactams (carumonam and aztreonam). The isolate was positive by the MBL production test (data not shown).

C. freundii KHM243 has two plasmids, one of approximately 70 kb and one of approximately 200 kb. A conjugation experiment was done with KHM243 and *E. coli* W1895. W1895 transconjugants that were resistant to β-lactams and that contained a 200-kb plasmid, designated pCF243, were obtained. The transconjugant exhibited a profile of susceptibility to β-lactams similar to that of KHM243, although the MICs for

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▽ Published ahead of print on 2 September 2008.

TABLE 1. MICs of β-lactams for *C. freundii* KHM243, *E. coli* W1895(pCF243) transconjugant, *E. coli* JM109(pKHM-1) expressing the KHM-1 MBL, and *E. coli* host strains

Antibiotic(s)[a]	MIC (μg/ml)				
	C. freundii KHM243	*E. coli* W1895 (pCF243)[b]	*E. coli* W1895	*E. coli* JM109 (pKHM-1)[c]	*E. coli* JM109
Ampicillin	256	64	8	16	1
Ampicillin-sulbactam	64	64	1	16	0.5
Ticarcillin	>512	>512	2	512	2
Ticarcillin-clavulanic acid	512	>512	4	512	2
Piperacillin	4	16	2	4	0.25
Cephaloridine	512	128	2	64	1
Cefuroxime	>512	>512	2	>512	8
Ceftazidime	>512	>512	0.125	>512	0.063
Cefotaxime	64	>512	0.008	128	0.004
Cefepime	32	>512	0.002	64	0.004
Cefozopran	16	256	0.016	64	0.008
Imipenem	2	4	0.063	0.5	0.063
Meropenem	4	4	0.004	4	0.004
Aztreonam	0.25	0.063	0.031	0.063	0.031
Carumonam	0.25	0.125	0.031	0.063	0.031
Cefoxitin	512	>512	8	>512	8
Cefmetazole	512	512	0.5	>512	0.25
Cefotetan	128	512	0.125	>512	0.031
Cefbuperazone	128	256	0.063	512	0.031
Cefminox	512	>512	0.125	512	0.25
Moxalactam	256	>512	0.063	>512	0.031
Flomoxef	64	256	0.031	128	0.031

[a] The ratio of the ampicillin to sulbactam was 2:1. The ratio of ticarcillin to clavulanic acid was 15:1.
[b] Natural plasmid carrying the bla_{KHM-1} gene.
[c] Recombinant plasmid constructed by insertion of DNA fragment containing the bla_{KHM-1} gene into the cloning vector pHSG396.

some cephalosporins, including cefotaxime, cefepime, and cefozopran, were significantly higher in the transconjugant than in KHM243 (Table 1).

EcoRI-digested fragments of pCF243 were subcloned into pHSG396 and were transformed into *E. coli* JM109 cells, and transformants were selected on agar medium containing moxalactam (1 μg/ml). Strain JM109 carrying the plasmid that conferred resistance to moxalactam, named pKHM-1, exhibited a profile of susceptibility to β-lactams similar to the susceptibility profiles of KHM243 and the *E. coli* W1895 transconjugant carrying pCF243 (Table 1). However, the MICs of some antibiotics, including cefotaxime and cefepime, were lower for the transformant carrying pKHM-1 than the transconjugant. This might be explained by insufficient expression of the gene due to insertion of the DNA fragment with a small 5′-flanking region.

pKHM-1 contained an 837-bp insert with a complete open reading frame (ORF) (data not shown). The 726-bp ORF encoded a putative protein of 241 amino acids. The protein was similar to MBLs, such as Uvs123 from an uncultured bacterium (82% identity) (22), IMP-1 (59% identity) (17), and SIM-1 (59% identity) (11) (Fig. 1). The protein was somewhat less similar to VIM-1 (38% identity) (10), GIM-1 (50% identity) (3), and SPM-1 (46% identity) (21) (Fig. 1). We named the ORF encoding the protein bla_{KHM-1} and designated the protein KHM-1 (Kyorin Health Science MBL 1). bla_{KHM-1} was different from the *Citrobacter freundii* genome in its GC contents (GC contents, 50.27% and 44.63%, respectively) and codon usage (data not shown). KHM-1 contained amino acid motifs conserved in MBL enzymes, including a zinc-binding motif (HXHXD, residues 97 to 101) and three other residues involved in zinc binding (residues 159, 178, and 217) (Fig. 2) (18, 23).

The DNA sequences flanking bla_{KHM-1} were determined from 774 bp upstream to 806 bp downstream of it. No sequence homologies for site-specific cointegration events, ORFs, or transmissible elements was detected within the 774-bp upstream of bla_{KHM-1}. A 360-bp ORF encoding a putative protein of 119 amino acids with 77% identity to hypothetical protein VP1798 of *Vibrio parahaemolyticus* (14) was located in the fragment 21 to 380 bp downstream of bla_{KHM-1}. Strain KHM243 carried a class 1 integron with an array of two gene cassettes, which carried the *aadA2* (20) and *aac(6′)-Iae* (19) aminoglycoside resistance determinants; however, bla_{KHM-1} was not detected in this integron.

Analysis of the purified KHM-1 protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed a single 25-kDa band. The activity of KHM-1 against various β-lactams was analyzed with the purified protein. It showed hydrolytic activity against all β-lactams tested except aztreonam (Table 2). Enzymatic activity against aztreonam was undetectable un-

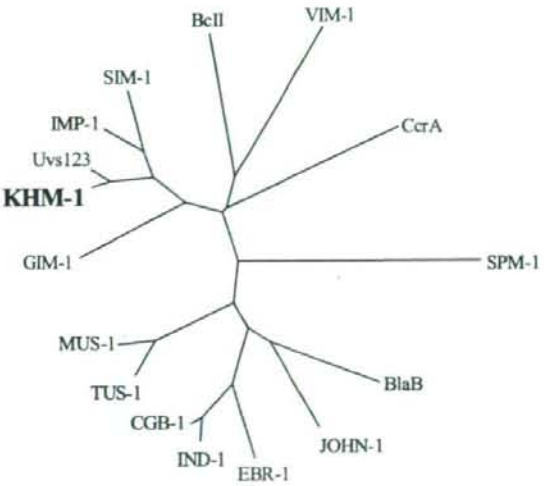

FIG. 1. Dendrogram showing the similarity of KHM-1 to other MBLs. KHM-1 and MBLs from a variety of organisms were tested. The dendrogram was created with the ClustalW program. Branch lengths correspond to the number of amino acid exchanges of the following MBL proteins (GenBank accession numbers, source organism) of BcII (P04190, from *Bacillus cereus*), BlaB (CAA65601, from *Elizabethkingia meningoseptica*), CcrA (P25910, from *Bacteroides fragilis*), CGB-1 (AAL55263, from *Chryseobacterium gleum*), EBR-1 (AAN32638, from *Empedobacter brevis*), GIM-1 (CAF05908, from *Pseudomonas aeruginosa*), IMP-1 (AAB30289, from *Serratia marcescens*), IND-1 (AAD20273, from *Chryseobacterium indologenes*), JOHN-1 (AAK38324, from *Flavobacterium johnsoniae*), MUS-1 (AAN63647, from *Myroides odoratimimus*), SIM-1 (AAX76774, from *Acinetobacter baumannii*), SPM-1 (CAD37801, from *P. aeruginosa*), TUS-1 (AAN63648, from *Myroides odoratus*), Uvs123 (AAP70377, from uncultured bacterium), and VIM-1 (CAB46686, from *P. aeruginosa*).

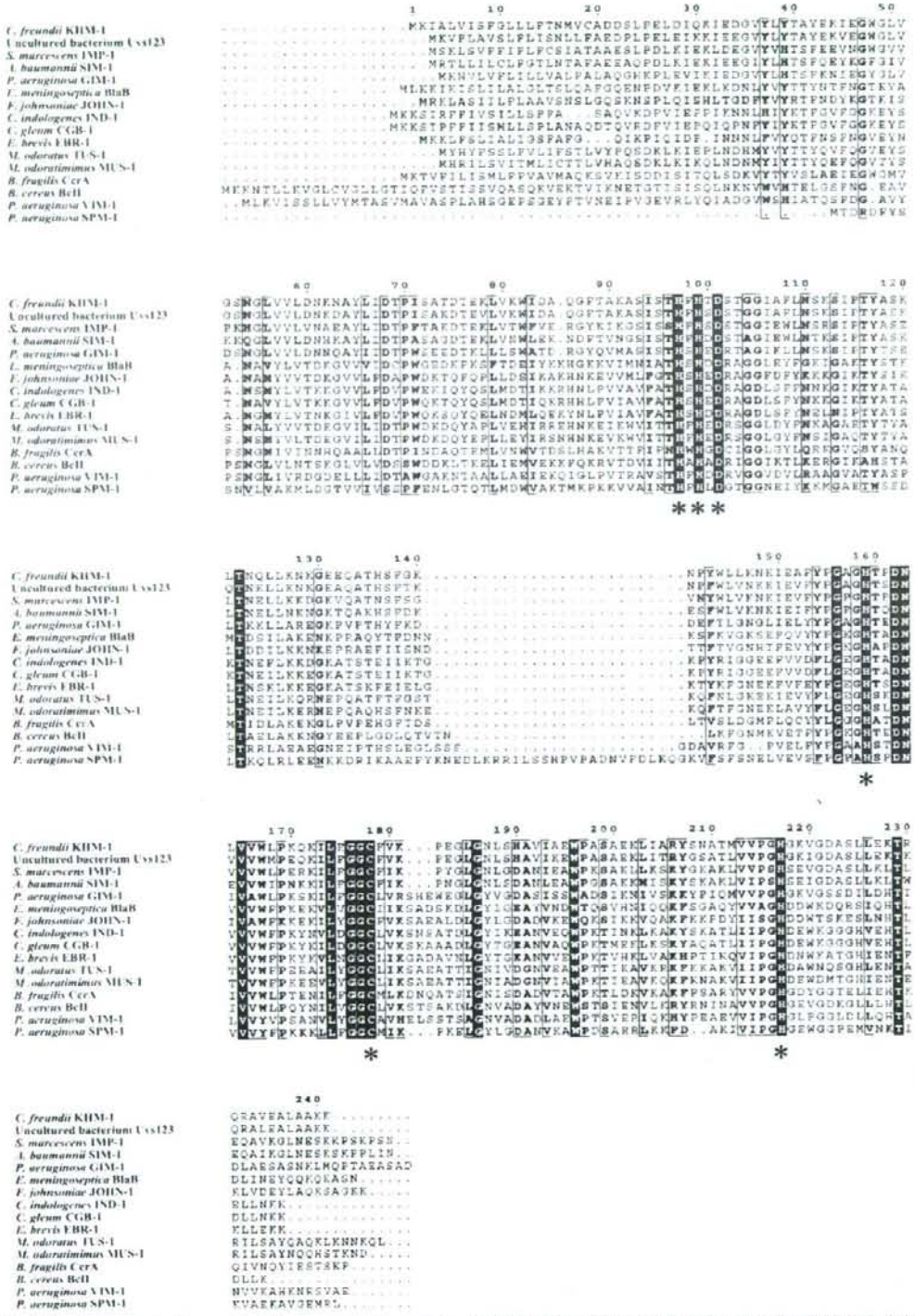

FIG. 2. Multiple-sequence alignments of the amino acid sequence of KHM-1 from *Citrobacter freundii* isolate KHM243 with those of other MBLs. The sequence sources are the same as those indicated in the legend to Fig. 1. Sequence comparison was performed by aligning the protein amino acid sequences by use of the ClustalW program (http://clustalw.ddbj.nig.ac.jp/top-e.html). The residues known to be involved in metal binding are indicated by asterisks. Identical residues are shaded.

TABLE 2. Kinetic parameters of β-lactamase KHM-1 with various substrates

Substrate	K_m (μM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Penicillin G	1,340 ± 56	23 ± 0.9	1.7×10^4
Ampicillin	978 ± 111	19 ± 2	1.9×10^4
Cephaloridine	4.4 ± 0.95	686 ± 12	1.6×10^8
Cefoxitin	81 ± 4	1,178 ± 164	1.4×10^7
Cefotaxime	13 ± 1.5	2,181 ± 208	1.7×10^8
Ceftazidime	8 ± 0.4	118 ± 3	1.5×10^7
Moxalactam	71 ± 8	2,794 ± 260	3.9×10^7
Aztreonam	—[b]	—	—
Meropenem	12 ± 3	0.4 ± 0.015	3.3×10^4
Imipenem	268 ± 53	15 ± 3	5.6×10^4

[a] The K_m and k_{cat} values represent the means of three independent experiments ± standard deviations.

[b] —, no hydrolysis was detected under conditions with a substrate concentration of up to 1 mM and an enzyme concentration of up to 840 nM.

der the experimental conditions adopted. This activity was inhibited by EDTA but was recovered by addition of Zn^{2+} (data not shown). The kinetic parameters, including K_m, k_{cat}, and the k_{cat}/K_m ratio, were determined for several different β-lactams (Table 2). Relatively higher values of the k_{cat}/K_m ratio ($>10^7$ $M^{-1} \cdot s^{-1}$), as a result of low values of K_m and high values of k_{cat}, were observed with the cephalosporins tested (cephaloridine, cefoxitin, cefotaxime, ceftazidime, and moxalactam); and lower values of the k_{cat}/K_m ratio ($<10^5$ $M^{-1} \cdot s^{-1}$) were observed with penicillin G, ampicillin, meropenem, and imipenem.

During 1997 and 1998, 104, 13, and 5 clinical isolates of *C. freundii*, *C. koseri*, and other *Citrobacter* spp., respectively, were collected in the hospital and were screened for imipenem resistance. Of these, four isolates of *C. freundii* showed reduced susceptibilities to imipenem (MICs, >8 μg/ml). However, bla_{KHM-1} was not detected in any of these isolates except the one from the patient infected with strain KHM243. A laboratory-based survey of other isolates of the family *Enterobacteriaceae* is in progress to detect bla_{KHM-1}.

Nucleotide sequence accession number. The nucleotide sequence data for bla_{KHM-1} and its flanking region from 774 bp upstream to 806 bp downstream reported here have been deposited in the EMBL/GenBank/DDBJ databases under accession number AB443628.

This study was supported in part by the Ministry of Health, Labor and Welfare of Japan (grants H18-Shinko-011, H19-Shinko-011, and H20-Shinko-011).

We thank M. Nakano (Jichi Medical School, Tochigi, Japan) for helpful comments on the manuscript.

REFERENCES

1. **Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto.** 2000. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38:**40–43.
2. **Bush, K.** 2001. New beta-lactamases in gram-negative bacteria: diversity and impact on the selection of antimicrobial therapy. Clin. Infect. Dis. **32:**1085–1089.
3. **Castanheira, M., M. A. Toleman, R. N. Jones, F. J. Schmidt, and T. R. Walsh.** 2004. Molecular characterization of a β-lactamase gene, bla_{GIM-1}, encoding a new subclass of metallo-β-lactamase. Antimicrob. Agents Chemother. **48:**4654–4661.
4. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Franceschini, N., B. Caravelli, J. D. Docquier, M. Galleni, J. M. Frère, G. Amicosante, and G. M. Rossolini.** 2000. Purification and biochemical characterization of the VIM-1 metallo-β-lactamase. Antimicrob. Agents Chemother. **44:**3003–3007.
6. **Gilmour, M. W., N. R. Thomson, M. Sanders, J. Parkhill, and D. E. Taylor.** 2004. The complete nucleotide sequence of the resistance plasmid R478: defining the backbone components of incompatibility group H conjugative plasmids through comparative genomics. Plasmid **52:**182–202.
7. **Inoue, M., J. Itoh, and S. Mitsuhashi.** 1983. pMS76, a plasmid capable of amplification by treatment with chloramphenicol. Plasmid **9:**86–97.
8. **Jacoby, G. A., and L. S. Munoz-Price.** 2005. The new β-lactamases. N. Engl. J. Med. **352:**380–391.
9. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
10. **Lauretti, L., M. L. Riccio, A. Mazzariol, G. Cornaglia, G. Amicosante, R. Fontana, and G. M. Rossolini.** 1999. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from a *Pseudomonas aeruginosa* clinical isolate. Antimicrob. Agents Chemother. **43:**1584–1590.
11. **Lee, K., J. H. Yum, D. Yong, H. M. Lee, H. D. Kim, J. D. Docquier, G. M. Rossolini, and Y. Chong.** 2005. Novel acquired metallo-β-lactamase gene, bla_{SIM-1}, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob. Agents Chemother. **49:**4485–4491.
12. **Levesque, C., L. Piche, C. Larose, and P. H. Roy.** 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. **39:**185–191.
13. **Liebert, C. A., R. M. Hall, and A. O. Summers.** 1999. Transposon Tn*21*, flagship of the floating genome. Microbiol. Mol. Biol. Rev. **63:**507–522.
14. **Makino, K., K. Oshima, K. Kurokawa, K. Yokoyama, T. Uda, K. Tagomori, Y. Iijima, M. Najima, M. Nakano, A. Yamashita, Y. Kubota, S. Kimura, T. Yasunaga, T. Honda, H. Shinagawa, M. Hattori, and T. Iida.** 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholera*. Lancet **361:**743–749.
15. **Nakamura, M., S. Sato, T. Ohya, A. Suzuki, and S. Ikeda.** 1985. Possible relationship of a 36-megadalton *Salmonella* Enteritidis plasmid to virulence in mice. Infect. Immun. **47:**831–833.
16. **Ochman, H., A. S. Gerber, and D. L. Hartl.** 1988. Genetic applications of an inverse polymerase chain reaction. Genetics **120:**621–623.
17. **Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato.** 1994. Molecular characterization of an enterobacterial metallo beta-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38:**71–78.
18. **Rasmussen, B. A., and K. Bush.** 1997. Carbapenem-hydrolyzing β-lactamases. Antimicrob. Agents Chemother. **41:**223–232.
19. **Sekiguchi, J., T. Asagi, T. Miyoshi-Akiyama, T. Fujino, I. Kobayashi, K. Morita, Y. Kikuchi, T. Kuratsuji, and T. Kirikae.** 2005. Multidrug-resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6')-Ie* gene cassette encoding a novel aminoglycoside acetyltransferase. Antimicrob. Agents Chemother. **49:**3734–3742.
20. **Taito, R. C., H. Rempel, R. L. Rodriguez, and C. I. Kado.** 1985. The aminoglycoside-resistance operon of the plasmid pSa: nucleotide sequence of the streptomycin-spectinomycin resistance gene. Gene **36:**97–104.
21. **Toleman, M. A., A. M. Simm, T. A. Murphy, A. C. Gales, D. J. Biedenbach, R. N. Jones, and T. R. Walsh.** 2002. Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY antimicrobial programme. J. Antimicrob. Chemother. **50:**673–679.
22. **Voget, S., C. Leggewie, A. Uesbeck, C. Raasch, K.-E. Jaeger, and W. R. Streit.** 2003. Prospecting for novel biocatalysts in a soil metagenome. Appl. Environ. Microbiol. **69:**6235–6242.
23. **Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann.** 2005. Metallo-β-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306–325.
24. **Young, D., J. M. Bell, B. Ritchie, R. Pratt, M. A. Toleman, and T. R. Walsh.** 2007. A novel sub-group methalo-β-lactamase (MBL), AIM-1 emerges in *Pseudomonas aeruginosa* (PSA) from Australia. abstr. C1-593, p. 75. Abstr. 47th Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washington, DC.