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# Characterization and Identification of Distinct *Mycobacterium massiliense* Extracellular Proteins from Those of *Mycobacterium abscessus*

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***Mycobacterium massiliense*** **is an emerging pathogen and very similar to** ***Mycobacterium abscessus*** **of rapidly growing mycobacteria in the phenotype and genotype. Pathogenic bacteria secrete a diversity of factors into extracellular medium which contribute to the bacterial pathogenicity. In the present study, we performed the comparative proteome analysis of culture filtrate proteins from a clinical isolate of** ***M. massiliense*** **and** ***M. abscessus*** **strains using two-dimensional gel electrophoresis and liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). Interestingly, 9 proteins of** ***M. massiliense*** **were distinctly expressed from those of** ***M. abscessus*****. Bioinformatic analysis of the identified proteins revealed that 3 unique proteins corresponded to serine/arginine rich protein, membrane protein from** ***Streptomyces coelicolor*****, and one hypothetical protein from** ***Corynebacterium efficiens*** **YS-314, respectively. Culture filtrate proteins from** ***M. massiliense*** **induced the release of pro-inflammatory cytokines from macrophages in a dose-dependent manner but not that from** ***M. abscessus*****. Taken together, the functional study on the identified proteins uniquely produced from** ***M. massiliense*** **may provide not only the clues for the different pathogensis, but also help develop the diagnostic tools for the differentiation between two mycobacterial species.**

***Keywords*****:** *M. massiliense*, extracellular proteins

A variety of bacterial proteins are secreted or exported into the surrounding milieu during growth that play important roles in cell-cell communication, the detoxification of harmful chemicals, and the killing of potential competitors (Tjalsma *et al.*, 2000). In particular, mycobacterial secretory proteins are considered to be candidate antigens for immune protection against mycobacterial diseases or reagents for serological diagnosis (Rosenkrands *et al.*, 2000; Andersen, 2001; Batoni *et al.*, 2002). Pathogenic bacteria, including mycobacteria, release proteins that perform critical functions in virulence. Analyses of mycobacterial secretory proteins from culture filtrates (CFs) may further our understanding of the virulence factors underlying the mycobacterial evasion of protective immunity in susceptible hosts and provide candidates for the development of new vaccines and diagnostic tests (Trajkovic *et al.*, 2004).

Rapidly growing mycobacteria (RGM) have been reported in various clinical conditions and are recognized as significant human pathogens. They include *Mycobacterium chelonae*, *M. abscessus*, *M. fortuitum*, and *M. smegmatis* (Brown-Elliott and Wallace, 2002). *M. abcessus*, which belongs to the *M. chelonae-M. abscessus* group of RGM, is the most frequently encountered type in case reports on RGM. *M. abscessus*, the causative organism in approximately 80% of cases of RGM-related pulmonary disease, causes a wide range of clinical diseases, including cutaneous disease, osteomyelitis, post-traumatic wound infections, and chronic lung disease (Wallace *et al.*, 1983; Griffith *et al.*, 1993; Sanguinetti *et al.*, 2001; Brown-Elliott and Wallace, 2002).

Recently, case reports on new species of RGM, including *M. massiliense*, have appeared (Bari *et al.*, 2005; Simmon *et al.*, 2007; Cardoso *et al.*, 2008; Kim *et al.*, 2008; Tortoli *et al.*, 2008). Using *hsp65* and *rpoB* genes, the isolates of *M. abscessus* described to date have been shown to be relatively heterogeneous (Springer *et al.*, 1995; Ringuet *et al.*, 1999; Adekambi *et al.*, 2003). On the basis of *rpoB* sequencing, *M. massiliense* and *M. bolletii* have been distinguished from the *M. abscessus* group (Adekambi *et al.*, 2004, 2006). The two species are very similar in their phenotypic and genotypic features and are indistinguishable by conventional species-identification techniques. Thus, *M. massiliense* may be misclassified as *M. abscessus* by traditional methods (Adekambi *et al.*, 2004). In Korea, there are several reports on pulmonary disease caused by *M. abscessus* infection have remarkably increased (Ryoo *et al.*, 2008; Jeon *et al.*, 2009; Lee *et al.*, 2010). More recent study reported that the identification of *M. massiliense* by comparative sequence analysis of *rpoB* and *hsp65* for the first time (Kim *et al.*, 2007). *M. massiliense* is a common cause of wound infections, abscesses, and pneumonia (Adekambi and Drancourt, 2004). It was reported that sepsis due to *M. massiliense*, which was isolated from the blood of a kidney transplant patient, played a role in that patient's sudden death (Tortoli *et al.*, 2008).

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We compared the extracellular proteome of *M. massiliense* with that of *M. abscessus* strains. Those proteins present in *M. massiliense* but absent in *M. abscessus* deserve special consideration as antigens for new vaccines or diagnostic reagents against *M. massiliense*-related disease.

## Materials and Methods

### Bacterial strains and culture conditions

A type strain ATCC 19977, two clinical strains of *M. abscessus* [clinical strain 1 (ACS-1), clinical strain 2 (ACS-2)] and *M. massiliense* clinical strain 1 (MCS-1) were used in this study. Theses strains were confirmed by comparative sequence analysis of *rpoB* and *hsp65* as previously described (Kim *et al.*, 2007). All strains were initially cultured in 7H9 broth supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC, Becton Dickinson, USA) for 1 week at 37°C. Single cell suspensions of each strain were prepared following homogenization and filtration as previously described. Ten-fold serial dilutions from seedlots of each strain were plated on 7H10 agar supplemented with 10% OADC (Becton Kickinson) and CFU counts for each strain were performed to quantify the number of organism per ml. Seedlots of each strain were then kept in small aliquots at -80°C until use.

### Culture filtrate antigen

All strains were cultivated in modified Watson-Reid (mWR, pH 6.0) broth media as previously described (Sung *et al.*, 2004). Briefly, bacterial cells grown in mWR were removed by centrifugation at 10,000×g for 10 min followed by filtration (0.2 μm pore-size of filter, USA). The filtrate was then concentrated roughly 100-fold using Centricon Plus-80 (5-kDa molecular weight cutoff; Amicon, USA) and dialyzed in 10 mM PBS (pH 7.4). The concentration of soluble CF proteins was determined by BCA Protein Assay kit (Pierce, USA).

### Two-dimensional polyacrylamide gel electrophoresis (2-DE)

The CF antigens from each strain were prepared using a 2-D Clean-Up kit (Amersham Biosciences, Sweden). Each sample was separated in the first dimension using 7- or 11-cm IPG strips with a pH range of 4 to 7 (Bio-Rad, USA). The samples were then focused as follows using a PROTEAN IEF Cell (Bio-Rad): 250 V for 30 min, from 250 to 4,000 V for 2 h, and 4,000 V for 20,000 Vh in the case of the 7-cm IPG strip and 250 V for 30 min from 250 to 8,000 V for 35,000 Vh in the case of the case of the 11-cm IPG strip. The IPG strips were equilibrated prior to running in the second dimension. Electrophoresis in the second dimension was performed as described by Laemmli using 10-20% pre-cast gels (Bio-Rad) (Laemmli, 1970). The gels were stained with 0.25% Coomassie brilliant blue R250 (Bio-Rad).

### Analysis of protein expression

The protein expression levels of the differentially displayed on the identified protein spots between *M. massiliense* clinical strain 1 (MCS-1) and *M. abscessus* clinical strain 2 (ACS-2) were analyzed using Prodigy SameSpots software (Nonlinear dynamic Ltd, UK). The same spots on the different proteome gels were calibrated by outlining the probable spots of MCS-1 across gel image to those of ACS-2. The gel of MCS-1 was then aligned to the gel of ACS-2 by placing more than 21 selected vectors which regard overlapped spots of 2 gels as vector. The backgrounds of the same spots were normalized and the fold changes in the identified proteins of MCS-1 were then quantified by a direct cross-gel comparison to the reference spots of ACS-2.

### Bone marrow-derived macrophages (BMDM)

BMDM were obtained from 6-8 weeks old C57BL/6 female mice. Briefly, bone marrow cells from femur and tibia were cultured in Dulbecco's modified eagle's medium (DMEM) (Hyclone, USA) containing 2 mM L-glutamine supplemented with 100 U/ml penicillin, 100 μg/ml Streptomycin, 10% fetal bovine serum, and 10% of culture supernatant from L-929 cells at 37°C in a humidified atmosphere of 5% $CO_2$. After 4-5 days, non-adherent cells were removed and differentiated macrophages were incubated in antibiotic-free DMEM until use.

### Cytokine measurement by enzyme-linked immunosorbent assay (ELISA)

Supernatants from the BMDM treated with culture filtrate antigens were collected at the time indicated, sterile-filtered, and then stored at -80°C until use. The levels of cytokines were determined by ELISA using a commercial kit (eBioscience, USA) according to the manufacturer's instructions.

## Results

### Comparison of the extracellular proteomes of *M. massiliense* and *M. abscessus*

To identify proteins that may be used in serological diagnoses or as candidate immunogenic antigens, the CF antigen expression patterns of strains of *M. massiliense* and *M. abscessus* were compared. Because most of the mycobacterial CF antigens had a pI of 4-7 by 2-D gel analysis, and given that many studies of the mycobacterial extracellular proteome have focused on acidic pI ranges, we performed isoelectric focusing using the CF antigens of *M. abscessus* and *M. massiliense* with IPG strips and a pH range of 4 to 7. Most of the protein spots in our pilot experiments occurred within a pH range of 4.5 to 6, with a large number of spots having a pI near 5; few proteins were observed in the surrounding pH regions. A similar protein expression pattern was observed regardless of the strain (Fig. 1). Three spots around 30 kDa were strongly expressed in all strains. Two-dimensional gel electrophoresis pattern of *M. abscessus* clinical strain 2 (ACS-2) and *M. massiliense* clinical strain 1 (MCS-1) were remarkably alike. Interestingly, ten proteins from MCS-1 were distinct from those of ACS-2.

### Identification of differentially expressed proteins in *M. massiliense*

The three spots expressed in all strains and ten spots expressed differentially between ACS-2 and MCS-1 were analyzed further and identified by LC-ESI-MS (Fig. 2). The ten identified proteins independently expressed in *M. massiliense* were analyzed by blasting against *Actinomyces* spp. Detailed information, including the protein name, protein description, calculated pI value, and predicted molecular mass, is presented in Table 1. Of these, methyltransferase (MitM) was identified in two spots with different pI values but nearly the same molecular weight.

To confirm these results, the precise intensities of the identified spots between ACS-2 and MCS-1 were analyzed using Prodigy SameSpots software (Fig. 3). The expression levels of the three spots around 30 kDa were similar; the spots from ACS-2 also exhibited little greater fold-change compared

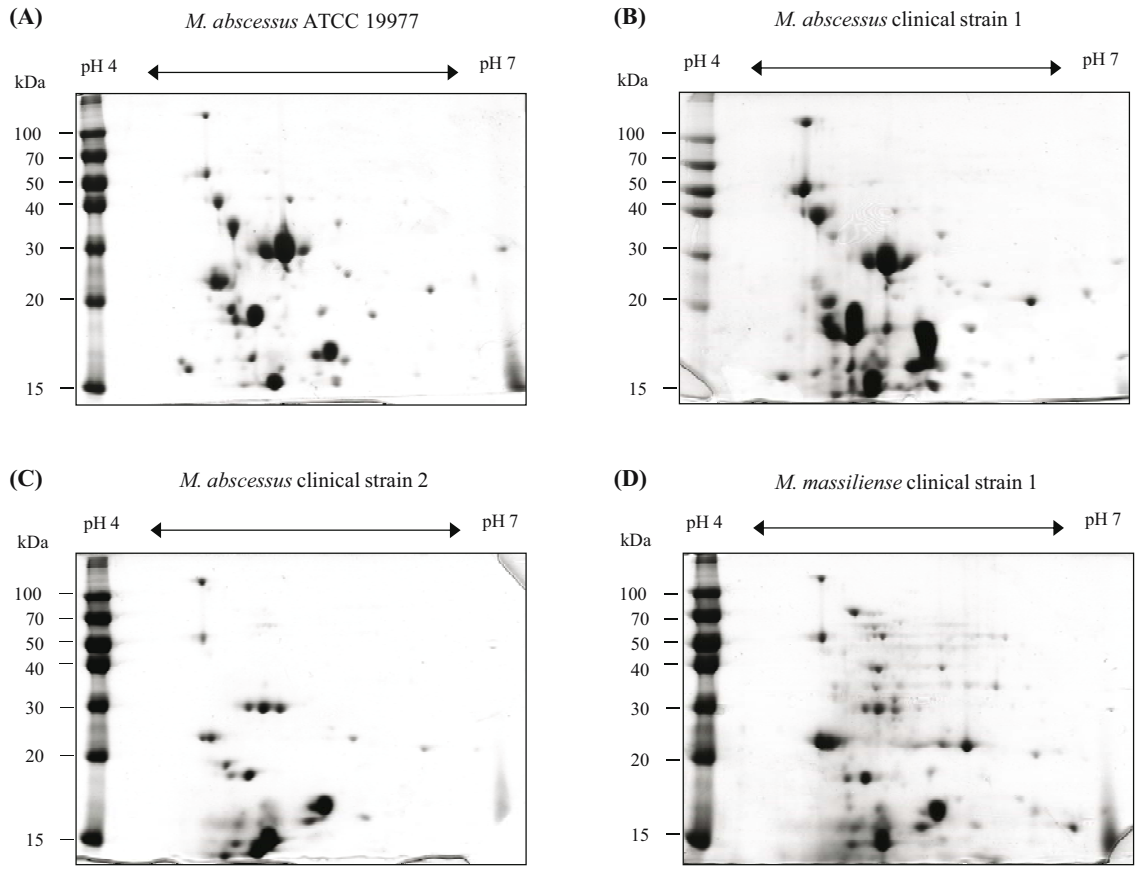


**Fig. 1.** 2-DE analysis of culture filtrates (CFs) from *M. abscessus* type strain ATCC 1997 (A), clinical strain 1 (B), clinical strain 2 (C), and *M. massiliense* clinical strain 1 (D). CFs were isolated from tubercle bacilli growing in modified Watson-Reid medium and concentrated. The concentrated CF proteins were then separated by isoelectric focusing using a 7-cm pH gradient strip (pH 4-7) in the first dimension and 12% SDS-PAGE in the second dimension. The proteins were stained with 0.25% Coomassie brilliant blue R250. The experiment was repeated three times to normalize different intensities between gels and obtained the same results.

to those from MCS-1 (1.4- to 1.7-fold change). However, the expression of the ten identified protein spots was clearly greater in MCS-1 than in ACS-2. Among the ten protein spots, a membrane protein corresponding to spot 9 exhibited the greatest fold-change (50,174-fold change) in expression as indicated by the spot intensity; in comparison, the other spots

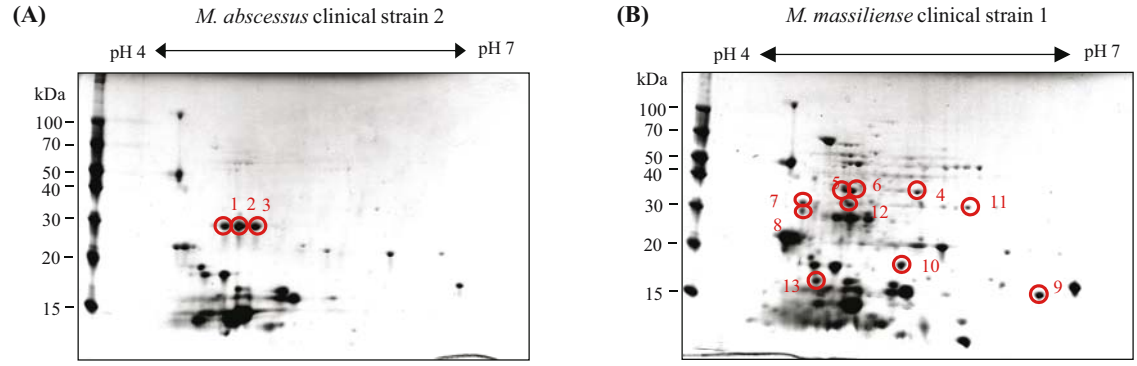


**Fig. 2.** 2-DE analysis of CFs from *M. abscessus* clinical strain 2 (A) and *M. massiliense* clinical strain 1 (B). To enlarge selected two gels in the figure1, the proteins were separated again by isoelectric focusing on 11-cm immobilized pH gradient strips (pH 4-7) in the first dimension and 10-20% gradient SDS-PAGE in the second dimension. The proteins were stained with 0.25% Coomassie brilliant blue R250, and differentially expressed 13 spots were identified by LC-ESI-MS. Identified spots are ringed. Their identities are listed in Table 3.

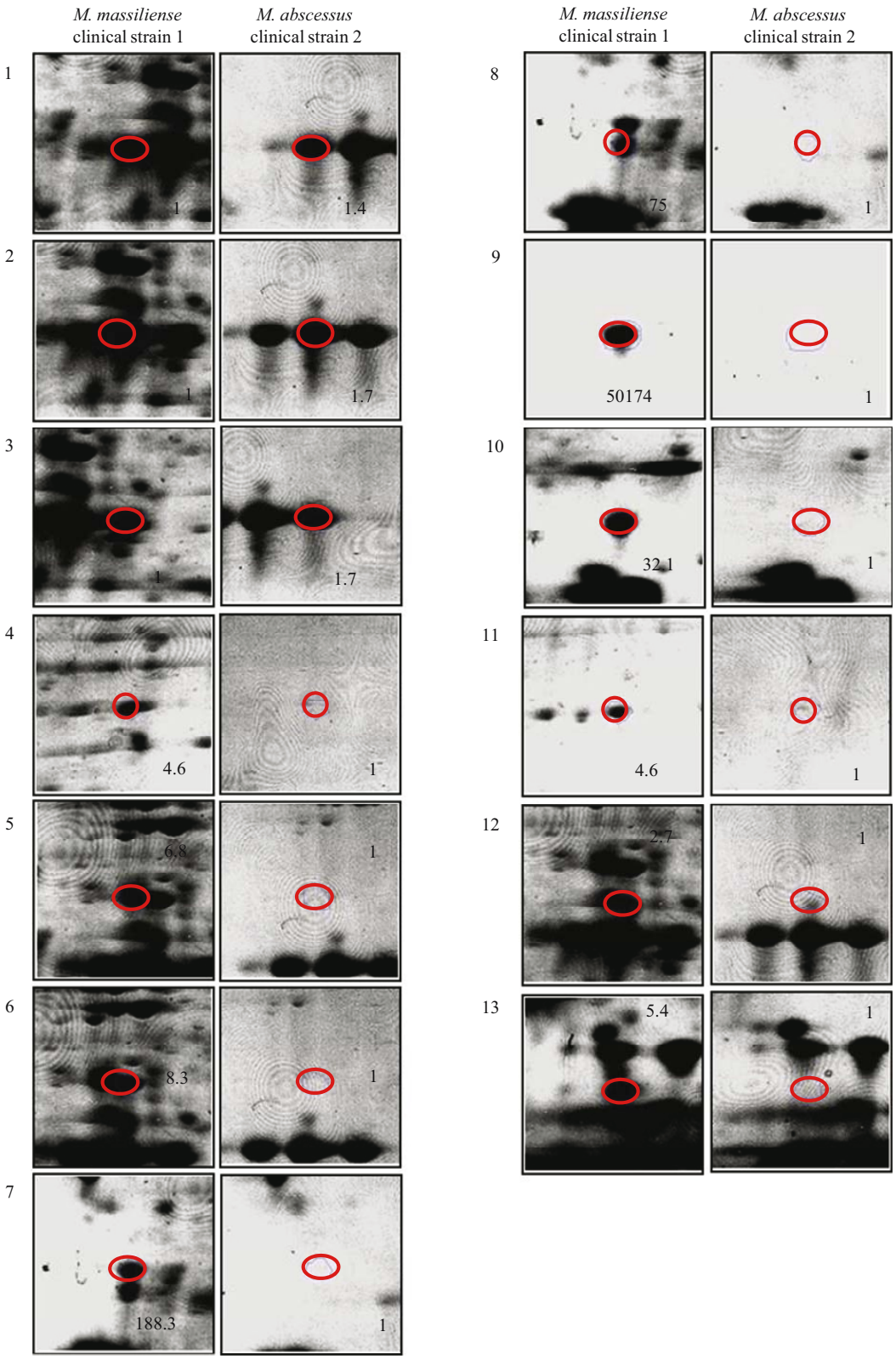


**Fig. 3.** Expression level of the identified proteins originated from *M. abscessus* clinical strain 2 (ACS-2) and *M. massiliense* clinical strain 1 (MCS-1). The identified spots between Strain ACS-2 and MCS-1 were analyzed using Prodigy SameSpots software (Nonlinear dynamic Ltd, UK). The backgrounds of the same spots were normalized to those in MCS-1 (box 1-3) or ACS-2 (box 4-13) and the fold changes of the identified proteins were then quantified by a direct cross-gel comparison to the reference spots. The fold changes of the proteins were expressed in the right portion.

**Table 1.** List of the best matched-proteins identified in *M. abscessus* clinical isolate 2 and *M. massiliense* clinical isolate 1

| Spot[a] | Identified protein[b] | *Actinomyces* spp.[b] | Isoelectric point (p*I*) | Molecular mass (kDa) |
|---|---|---|---|---|
| 1 | ABC-type sulfate transport system periplasmic component | *Actinobacillus pleuroneumoniae serovar* 1 str.4074 | 5.48 | 30.071 |
| 2 | CysNC | *Rhodococcus* spp. DS7 | 5.09 | 69.102 |
| 3 | GCN5-related N-acetyltransferase | *Kineococcus radiotolerans* SRS30216 | 5.50 | 30.520 |
| 4 | AfsR-like regulatory protein | *Streptomyces coelicolor* A3(2) | 5.49 | 118.799 |
| 5 | NysJ (PKS) | *Streptomyces noursei* | 5.07 | 562.670 |
| 6 | Ferrochelatase | *Symbiobacterium thermophilium* IAM 14863 | 5.89 | 36.452 |
| 7 | Modular polyketide synthase (PKS) | *Streptomyces avermitilis* MA-4680 | 4.94 | 806.333 |
| 8 | Putative response regulator | *Amycolatopsis mediterranei* | 5.59 | 25.293 |
| 9 | Membrane protein | *Streptomyces coelicolor* A3(2) | 10.75 | 13.300 |
| 10 | Serine/arginine rich protein | *Streptomyces coelicolor* A3(2) | 12.16 | 18.908 |
| 11,12 | MitM | *Streptomyces lavendulae* | 4.84 | 31.052 |
| 13 | Hypothetical protein | *Corynebacterium efficiens* YS-314 | 6.22 | 17.715 |

[a] Spot numbers indicate at Fig. 2.
[b] The nomenclature from the *Actinomyces* spp. genomes was used.

showed at least a 2.7-fold change.

## Bioinformatic analysis of distinct proteins between two mycobacterial species

To further characterize the differentially released proteins from *M. massiliense*, the identified proteins were examined for homology with proteins from *Mycobacteria* spp. (Table 2). As shown in Table 2, six of the 12 *Actinomyces* spp. proteins showed significant homology with proteins from *M. abscessus.* The proteins the best matching to those in *Actinomyces* spp. were further analyzed for the homology with those existing in *M. abscessus*. As a results, three proteins, a serine/arginine-rich protein, a membrane protein from *Streptomyces coelicolor*, and a hypothetical protein from *Corynebacterium efficiens* YS-

**Table 2.** Homology with *Mycobacteria* spp. and bioinformatic characterization of the identified proteins

| Spot[a] | Mycobacterium[b] | Protein | Identity (%) | Putative function[c] |
|---|---|---|---|---|
| 1 | *M. abscessus* | Sulfate ABC transporter, periplasmic protein | 45 | Bacterial periplasmic transport systems use membrane-bound complexes and substrate-bound membrane-associated, periplasmic binding proteins (PBPs), to transport a wide variety of substrates, such as, amino acids, peptides, sugars. |
| 2 | *M. abscessus* | CysN/CysC bifunctional enzyme | 48 | Bifunctional enzyme which catalyzes the first and second steps in the sulphate activation pathway; |
| 3 | *M. abscessus* | Putative acetyltransferase | 32 | Unknown |
| 4 | *M. tuberculosis* H37Rv | Transcriptional regulatory protein | 41 | Transcriptional regulation |
| 5 | *M. marinum M* | Type I modular polyketide synthase | 44 | Responsible for condensation steps 15 to 17 in the nystatin polyketide backbone synthesis (PKS) |
| 6 | *M. abscessus* | Ferrochelatase | 29 | Ferrochelatase (protoheme ferrolyase or HemH) is the terminal enzyme of the heme biosynthetic pathway. It catalyzes the insertion of ferrous iron into the protoporphyrin IX ring yielding protoheme. |
| 7 | *M. marinum M* | Type I modular polyketide synthase | 42 | Responsible for condensation steps 15 to 17 in the nystatin polyketide backbone synthesis (name=polyketide synthase) |
| 8 | *M. smegmatis str*. MC2 155 | DNA-binding response Regulator MtrA | 49 | ND[d] |
| 9 | *M. kansasii* ATCC 12478 | Hypothetical protein | 32 | ND |
| 10 | *M. avium* 104 | Cytochrome P450 monooxygenase | 33 | ND |
| 11,12 | *M. abscessus* | Putative methyltransferase | 36 | Methyltransferase |
| 13 | *M. abscessus* | Putative 2,3-dihydroxybiphenyl 1,2-dioxygenase or glyoxalase/ bleomycin resistance protein | 33 | Unknown |

[a] Spot numbers indicate at Fig. 2.
[b] The nomenclature from the *Mycobacterium* genomes was used.
[c] The list putative functions were obtained from BLAST.
[d] ND, Not determined

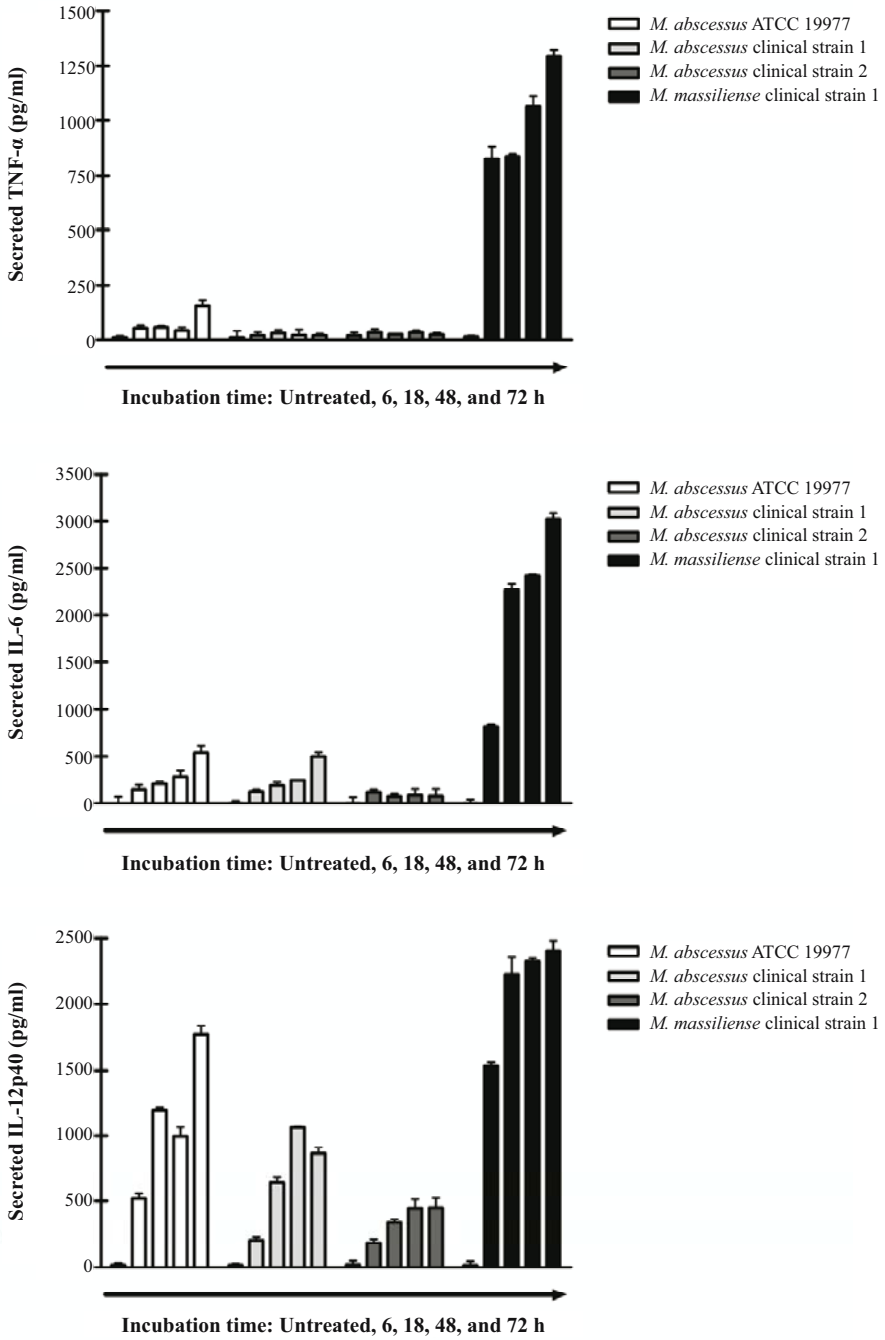


**Fig. 4.** TNF-α, IL-6, and IL-12p40 production in response to mycobacteria in BMDMs from mice. Differentiated BMDMs were challenged with *M. abscessus* ATCC 19977, two clinical strains or *M. massiliense* clinical strain 1 at an MOI of 1 for 4 h, washed off, and further incubated for indicated times. The supernatants were harvested after 18 h, 48 h, and 72 h to assess cytokines by ELISA.

314 has different names and functions. Thus, these three proteins were considered as unique proteins of *M. massiliense*. Although the protein corresponding to the spot 13 showed 33% homology to the putative 2,3-dihydroxybiphenyl 1,2-dioxygenase or glyoxalase/bleomycin resistance protein of *M. abscessus*, the function of that in *M. abscessus* has been predicted while the best-matched protein in *Actinomyces* spp. still remains as a hypothetical protein. In addition, the protein in *M. abscessus* contains glyoxylase superfamaily conserved domain whereas that in *Actinomyces* spp. does the LabA (a well conserved group of bacterial proteins with no defined function)-like peptide sequence. Thus, this protein was designated as a unique protein differentially expressed in *M. massiliense*.

## Cytokine production by macrophages in response to *M. massiliense* and *M. abscessus* CF antigens

To investigate the immunogenic capacity for macrophage

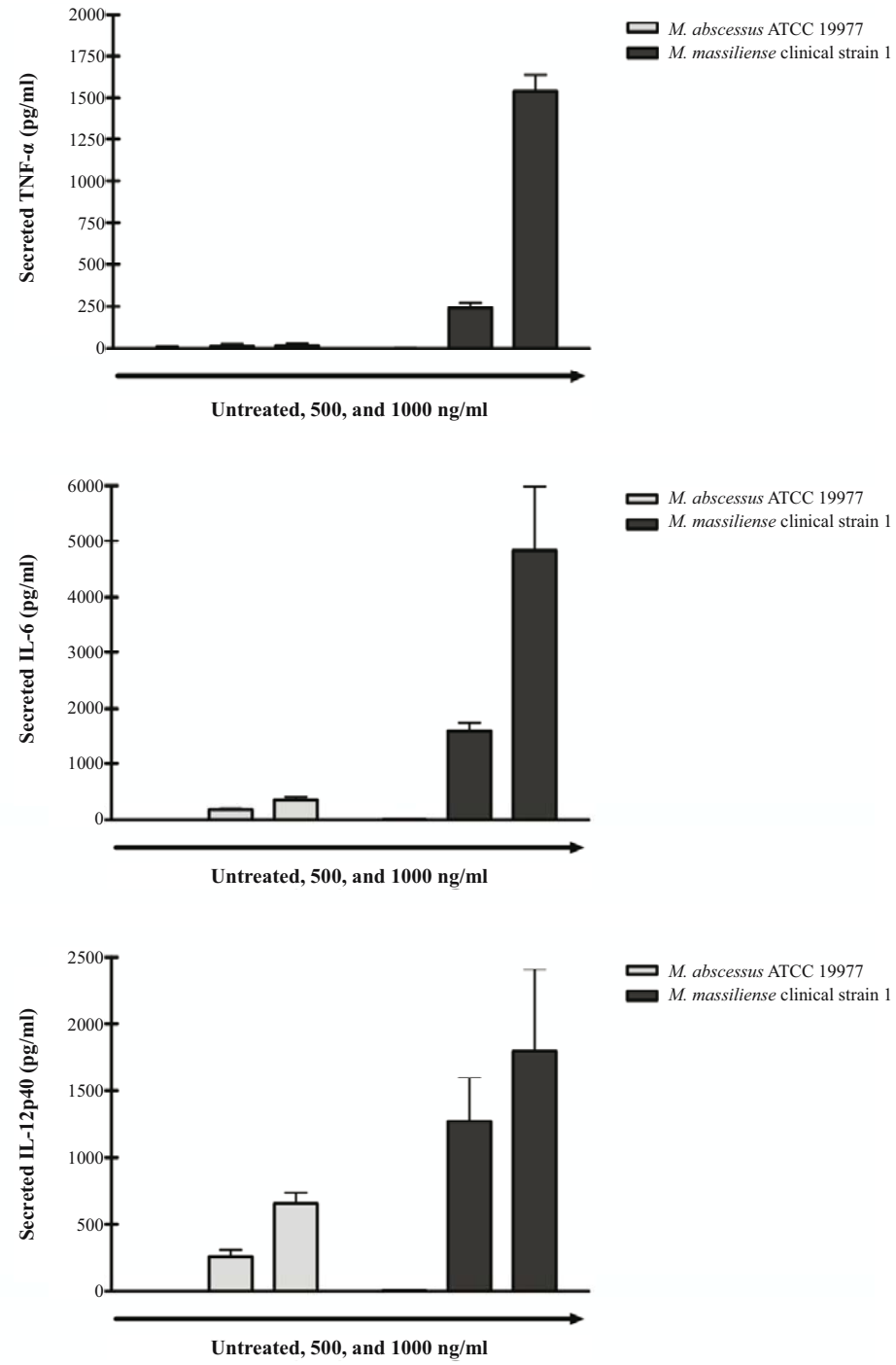


**Fig. 5.** TNF-α, IL-6, and IL-12p40 production in response to CFs in BMDMs from mice. BMDMs were treated with CFs from *M. abscessus* ATCC 19977, two clinical strains or *M. massiliense* clinical strain 1 with a concentration of 1,000 ng/ml at time courses. The supernatants were harvested after 6 h, 18 h, 48 h, and 72 h, followed by evaluation of cytokine production by ELISA.

activation by the treatment with CF proteins, we compared the stimulatory effects of CF proteins from *M. abscessus* and *M. massiliense* on bone marrow-derived macrophages (BMDMs) by measuring the released cytokines at various time points post-treatment. Interestingly, a large prolonged increase in the secretion of TNF-α, IL-6, and IL12p40 from BMDMs stimulated with CF antigens from MCS-1 was detected compared to that for the other strains (Fig. 4). Moreover, pro-inflammatory cytokine release by BMDMs in response to the CF antigens of MCS-1 was augmented in a dose-dependent manner, suggesting that *M. massiliense* MCS-1 secretes highly immunogenic proteins (Fig. 5).

## Discussion

In this report, we compared the expressed protein profile of *M. massiliense* with that of *M. abscessus*. The recent determination of the complete genomic sequence of *M. abscessus*, which encodes 4,920 proteins, will increase our understanding of the biology and pathology of the pathogen. The availability of the entire genomic sequence of *M. abscessus* makes it possible to study the proteome of the organism, and we analyzed CFs of the pathogen using 2-D electrophoresis. Recently, *M. massiliense* was classified as a distinct species from *M. abscessus*. Since then, it has been shown that the two species may have been misclassified because they are very closely related and cause a similar spectrum of human infections.

We selected two clinical strains and one type strain of *M. abscessus* and one clinical strain of *M. massiliense* for use in detecting differentially expressed proteins using our experimental system. Specifically, we examined differential protein expression between the CF antigens of *M. abscessus* clinical strain 2 (ACS-2) and *M. massiliense* clinical strain 1 (MCS-1). The protein spots were identified using *M. abscessus* and *Actinomyces* spp. because the genomic sequence of *M. massiliense* is unknown. Nine proteins (an AfsR-like regulatory protein, nystatin J [NysJ], ferrochelatase, a modular polyketide synthase [PKS], a putative response regulator, a membrane protein, a serine/arginine-rich protein, MitM, and one hypothetical protein) were expressed at higher levels in CFs of MCS-1 compared to those of ACS-2. The AfsR-like regulatory protein, NysJ, ferrochelatase, modular PKS, putative response regulator, and MitM were homologous with *M. abscessus*; however, these genes may be conserved in *Actinomyces* spp. Notably, three unique proteins were strongly expressed in *M. massiliense*. Bioinformatic analysis of the identified proteins revealed that the three unique proteins corresponded to a serine/arginine-rich protein, a membrane protein from *S. coelicolor*, and a hypothetical protein from *C. efficiens* YS-314. The Afs-R-like regulatory protein of *S. coelicolor* A3(2) controls secondary metabolic reactions. The serine/threonine kinase AfsK, which phosphorylates serine and threonine residues on AfsR, is part of a two-component regulatory system (Umeyama *et al.*, 2002). These systems supposedly sense nutritional conditions, including carbon and nitrogen availability, and environmental conditions, including temperature, osmolality, and stress (Umeyama *et al.*, 1999, 2002; Sharma *et al.*, 2004). Some of the stimuli sensed by these systems are transferred to pathway-specific activators leading to antibiotic production. In other words, phosphorylated AfsR awakens "sleeping" antibiotic-production genes by stimulating transcription.

Ferrochelatase, the terminal enzyme in heme biosynthesis, catalyzes the insertion of ferrous iron into protoporphyrin IX to form protoheme IX. The ferrochelatases isolated from various organisms are similar in terms of their catalytic properties, indicating that the major features of their reactions are conserved (Jones and Jones, 1969; Harbin and Dailey, 1985; Ponka, 1999). It was reported that mammalian ferrochelatase has an iron–sulfur cluster and that nitric oxide inhibits ferrochelatase activity in part by targeting its central iron–sulfur cluster (Savostin *et al.*, 1975).

NysJ is a type I modular PKS that polymerizes simple fatty acids into repeated units (modules), each of which is responsible for one condensation cycle in the synthesis of a polyketide chain. NysJ is required for condensation steps 15 to 17 in nystatin polyketide backbone synthesis (Brautaset *et al.*, 2000; Fjaervik and Zotchev, 2005).

Modular PKSs are enzyme complexes that produce polyketides (Khosla *et al.*, 1999; Jenke-Kodama *et al.*, 2005). Polyketides are natural products with diverse biological activities and pharmacological properties. Polyketide antibiotics, antifungals, cytostatics, antiparasitics, animal growth promoters, and anticholesterolemics are in commercial use (Castoe *et al.*, 2007; Ridley *et al.*, 2008). For example, nystatin has antifungal effects, whereas avermectin has antiparasitic effects, crythromycin has antibacterial effects, erythromycin and rapamycin have immunosuppressive effects, and daunorubicin has antitumor effects. In addition, mycolactones from the environmental human pathogen *M. ulcerans* are highly related macrocylic polyketides with immunosuppressive and cytotoxic properties (George *et al.*, 1999).

MitMs are enzymes that catalyze the transfer of a methyl group to DNA. Among them, MitM, which corresponded to spots 11 and 12 in this study, is an S-adenosylmethionine-dependent MitM that catalyzes key chemical modifications at defined positions in mycolic acids, which are major and specific components of the cell envelope in mycobacteria (Asselineau *et al.*, 2002; Alahari *et al.*, 2009).

Various cytokines have been shown to be secreted from $CD4^+$ T cells in response to a wide range of mycobacterial antigens or mycobacteria; in contrast, the number of reports related to *M. abscessus*- or *M. massiliense*-induced innate immune mechanisms is much smaller (Flynn and Chan, 2001). A recent study showed that a rough variant of *M. abscessus* strongly induced TNF-α secretion from murine macrophages (Irani and Maslow, 2005). TNF-α is essential for the formation and maintenance of granulomas as well as host immune responses as a pro-inflammatory cytokine. The initial interaction of mycobacteria with mononuclear phagocytes gives rise to a cytokine profile that is dominated by the pro-inflammatory molecules TNF-α, IL-6, and IL12p40, which activate macrophages (Keane *et al.*, 2001; Dimakou *et al.*, 2004). TNF-α- and IL-6-deficient mice infected with *M. tuberculosis* display increased bacterial loads and decreased survival time compared with control mice (Saunders *et al.*, 2000; Roach *et al.*, 2002). In this study, TNF-α, IL-6, and IL12p40 production in response to the CF antigens from MCS-1 was significantly greater than that from the other strains, which suggests that the differentially expressed CF proteins from *M. massiliense* play a crucial role in immunopathogenesis in murine models. Although one clinical strain of *M. massiliesne* might not be representative for this species, it would be more helpful for development of diagnostic methods or understanding of the pathogenesis if the immunogenic proteins are further studied in this strain due to its clinical relevance.

Recently, the complete genomic sequence of *M. abscessus*, which encodes 4,920 proteins, was revealed. This information has provided insight into the pathogenicity of this bacterium, and whole-genome analysis has both revealed the absence of mycobacterial virulence genes and established the genetic basis for the unique pathogenicity of *M. abscessus*.

Our results suggest that the three unique proteins that were

strongly expressed in *M. massiliense* play a role in protective immune responses and may help distinguish *M. massiliense* from *M. abscessus*. In addition, these proteins may be used as candidates for a future specific anti-*M. massiliense* vaccine. Therefore, further study of the three identified proteins, including the creation of recombinant proteins, should be undertaken.

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