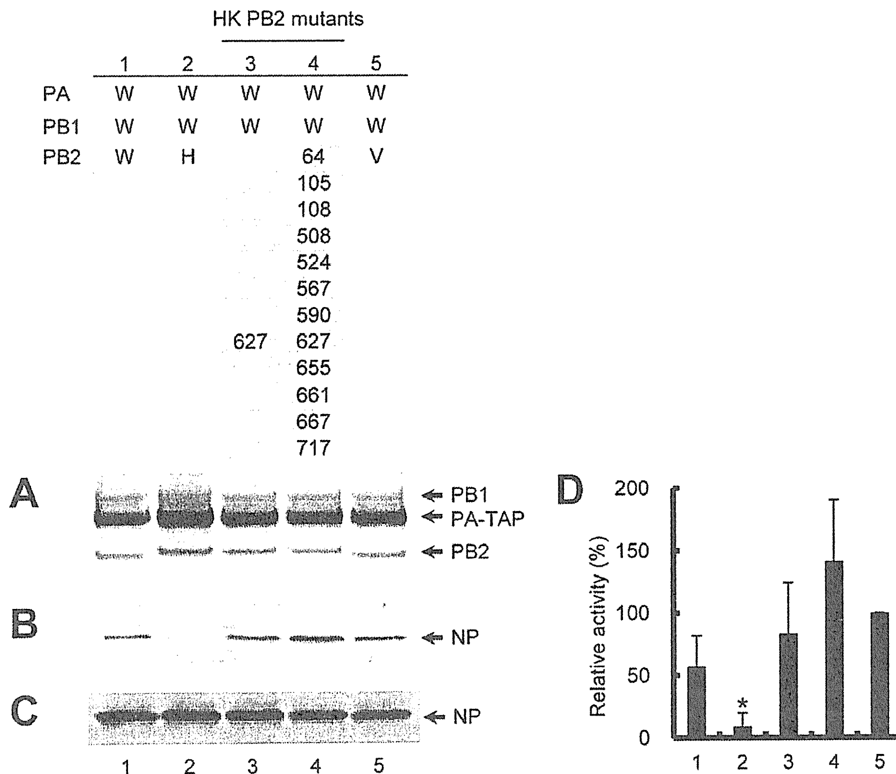

Figure 5. Accumulation of RNP reconstituted from hybrid polymerase. Hybrid RNP was reconstituted in a background of WSN (W) by replacing only PB2 subunit with the HK PB2 wild type (H) (lanes 2), HK PB2 mutants (lanes 3–4) and VN PB2 (V) (lanes 5). Reconstituted RNP in 293T cells was partially purified by using TAP-tagged PA (see materials and methods). The numbers in HK PB2 mutants indicate mutated positions in HK PB2. (**A and B**) Partially purified RNP analyzed by silver-stained 7.5% SDS-PAGE, and by western blotting using specific antibodies for NP, respectively. (**C**) Expression of NP in total cell lysate, analyzed by western blotting using specific antibodies for NP by 7.5% SDS-PAGE. The positions of PB1, PB2, PA-TAP and NP are shown on the right. (**D**) Quantification of results obtained in panel B by phosphorimaging. Data are expressed as percentages relative to VN PB2 (lane 5) (mean ± standard deviation; n = 3). * shows statistical significance at $P<0.01$ in a Student's t-test.
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modulating the function of assembled RNP. Position 108 may modulate the interaction with PB1 and preferentially affect transcriptional activity (Figure 2, lane 24), since it is close to the PB1 binding site (Figure 6A) [29,40]. Positions 508 and 524 are close to cap-binding sites [10,41] and the 627 domain (538–693) [42]. These positions, we speculate, may affect RNA binding, the function of 627 residue, or may modulate the interaction with host chaperone proteins, such as Hsp90, Hsp70 and CCT [43,44,45]. Importantly, an alignment of the PB2 sequences from typical human influenza viruses and avian influenza viruses currently circulating in poultry in some countries, with differing HA and NA subtypes, demonstrated that three positions 108, 508 and 524 were highly conserved among influenza A viruses (Figure 6B). This suggests that these residues are important for the function of RNP in influenza A viruses. In addition, an alignment of HK PB2 sequence with 60 human isolates of H5N1 demonstrated that Q508 and M524 were conserved only less than 2%. Perhaps, these residues might define specific features of HK PB2.

The E→K mutation at HK PB2 627 showed a significant reduction in vRNA promoter and cRNA promoter bindings in cross-linking experiments (Figure 4B). PB2 has been shown to bind both vRNA and cRNA promoters [16,40], but the precise binding site in PB2 has not yet been identified. Our findings suggest that the PB2 627 is involved in vRNA and cRNA promoter binding. A reduction of the promoter binding is clearly consistent with the reduction of replication initiation activity in vitro (Figure 4C). Structural studies of the C-terminal region of PB2 have shown that the K→E mutation at PB2 627 disrupts the positively charged surface of the protein without altering structure [42]. We propose that the mutation at HK PB2 627 alter the charge of the protein, thereby affecting the promoter binding and subsequent RNA synthesis.

A purified H5N1polymerase shows significantly higher polymerase activity in vitro when compared to human strain A/WSN/33 (H1N1) [14,46,47]. The polymerase activity of WSN was increased by the introduction of the PB2 or PA subunit of H5N1. These findings are consistent with our observations that the HK PB2 remarkably enhanced the promoter binding activity and replication initiation activity of WSN polymerase (Figure 4). However, the HK PB2 significantly reduced the polymerase activity in vivo in the RNP reconstitution assay. This different result between in vitro and in vivo is also in agreement with the previous reports [14,47]. We speculate that a significant reduction of the polymerase activity in vivo, in spite of its strong activity in vitro, could be explained by the poor accumulation of RNP.

Introduction of HK PA into SW and NT polymerases increased the synthesis of mRNA, cRNA and vRNA (Figure 1, lanes 20 and 36). It is also worth noting that HK PA obviously relieved the inhibitory effect of HK PB2 (Figure 1, lanes 22 and 38). This suggests a functional cooperation between PB2 and PA [48].

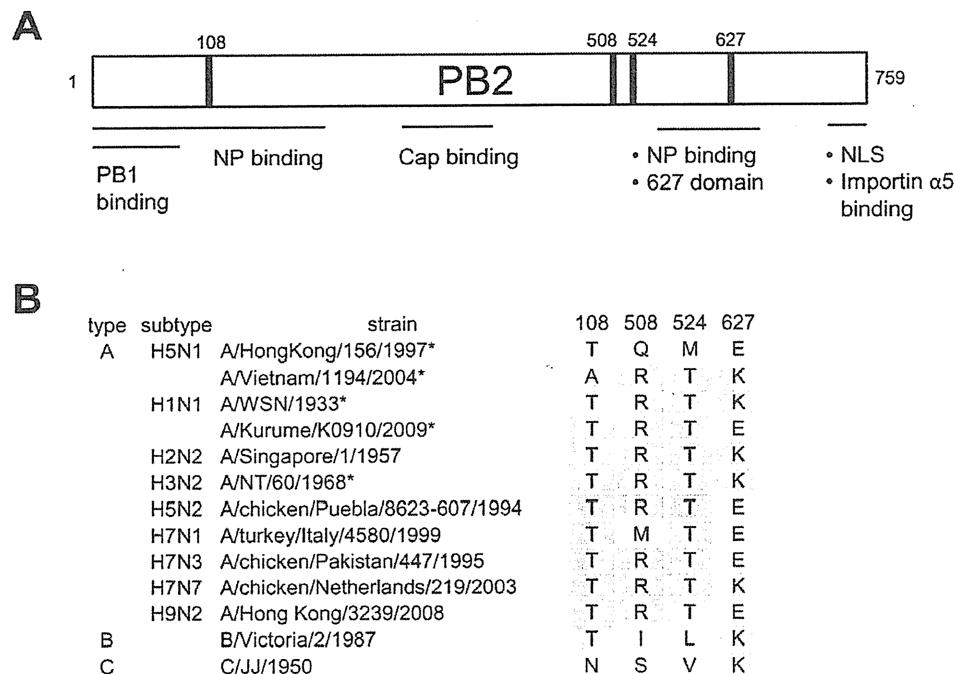

Figure 6. Alignment of PB2 subunit. (A) Functional map of PB2 subunit. (B) Alignment of amino acid residues in PB2 which are important for the accumulation of RNP. * shows influenza strains used in this study. Gray shading indicates high evolutionary conservation between influenza A, B and C virus sequences.
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These results are consistent with recent RNP reconstitution studies suggesting that PA plays a major role in increasing activities of hybrid polymerase [14,47,49]. However, such a tendency could not be observed in hybrid WSN polymerase, suggesting that the PA subunit cannot always overcome the restriction of genetic reassortment.

In summary, we have found that the PB2 subunit of influenza A/HongKong/156/1997 (H5N1) has a strong inhibitory effect on the RNP activity when introduced into the polymerase of other influenza strains. In addition, four residues at positions 108, 508, 524 and 627 of the PB2 subunit appear to be important determinants that are involved in the accumulation of functional RNP and in modulating the polymerase activity. These results may suggest a possible mechanism by which the generation of replicative reassortant virus of influenza is highly restricted.

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Author Contributions

Conceived and designed the experiments: KH YN NH HW. Performed the experiments: YN KH TK. Analyzed the data: YN KH TK. Contributed reagents/materials/analysis tools: YN KH TK. Wrote the paper: KH.

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ORIGINAL ARTICLE

Antimicrobial susceptibility and genetic characteristics of *Haemophilus influenzae* isolated from community-acquired respiratory tract infection patients in Shanghai City, China

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Abstract *Haemophilus influenzae* is an important causative pathogen of community-acquired respiratory infection in China. In this study we investigated 37 *H. influenzae* strains isolated from patients with community-acquired respiratory tract infections (CARTI) in Shanghai city between Dec 2008 and Apr 2009. *H. influenzae* clinical isolates were identified, and β-lactamase production tests were conducted and minimal inhibitory concentrations (MIC) were measured. Pulsed-field gel electrophoresis (PFGE) was introduced as an effective fingerprinting method. Two isolates (5.4%) were verified as serotype b strains, and 30 strains (81.1%) were nontypeable *H. influenzae*. Furthermore, 10 (27.0%) were β-lactamase-producing ampicillin-resistance (BLPAR) (TEM-1 type) strains, 11 (29.8%) were low-β-lactamase-nonproducing ampicillin-resistant *H. influenzae* (Low-BLNAR) strains, and the rest were β-lactamase-negative ampicillin-susceptible (BLNAS) strains. Minimum inhibitory concentrations (MIC_{90}; μg/ml) were 2 for ampicillin/sulbactam, 0.05 for cefotaxime, 16 for cefaclor, 2 for azithromycin, 0.12 for levofloxacin, and 4 for imipenem. Fingerprint typing by PFGE revealed 23 independent patterns for the isolates. Pattern A (defined in this study) was predominant in BLPAR strains, and a variety of other patterns were detected in Low-BLNAR and BLNAS strains. Although the incidence of ampicillin resistant *H. influenzae* is increasing in CARTI patients in China, current antimicrobial chemotherapy seems to be effective.

Keywords Antimicrobial susceptibility · *Haemophilus influenzae* · BLPAR and Low-BLNAR · PFGE

Abbreviations

ABPC/SBT	Ampicillin/sulbactam
AZM	Azithromycin
BLNAR	β-lactamase-nonproducing ampicillin-resistant *Haemophilus influenzae*
BLNAS	β-lactamase-nonproducing ampicillin-susceptible *Haemophilus influenza*
BLPAR	β-lactamase-producing ampicillin-resistant *Haemophilus influenzae*
CAP	Community-acquired pneumonia
CARTI	Community-acquired respiratory tract infections
CCL	Cefaclor
CLSI	Clinical and Laboratory Standards Institute
CTX	Cefotaxime
Hib	Serotype b *Haemophilus influenzae*
IPM	Imipenem

Some of the results obtained in this study were presented at the 59th annual meeting of the Japanese Society of chemotherapy, and all the results obtained in this study have been submitted to forthcoming 86th annual meeting of the Japanese Association for Infectious Diseases for an oral presentation in April 2012.

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Low-BLNAR	Low β-lactamase-nonproducing ampicillin-resistant *Haemophilus influenzae*
LVFX	Levofloxacin
MIC	Minimum inhibitory concentration
NTHi	Nontypeable *Haemophilus influenzae*
PBPs	Penicillin-binding proteins
PFGE	Pulsed-field gel electrophoresis

Introduction

Haemophilus influenzae is a pleomorphic Gram-negative coccobacillus which is regarded as an important community acquired pathogen. *H. influenzae* initially colonizes the nasopharynx, and naturally-acquired disease caused by *H. influenzae* seems to occur only in humans [1]. Six serotypes (a–f) have been identified on the basis of capsular polysaccharides. *H. influenzae* type b (Hib) seems to be the most important serotype involved in childhood pneumonia, meningitis, and bacteremia. However, Nontypeable *H. influenzae* (NTHi) is reported to cause otitis media, sinusitis in children, and is associated with community-acquired pneumonia (CAP) [2] and acute exacerbations of chronic bronchitis [3, 4]. Because ampicillin has long been used as the first drug of choice for treatment of *H. influenzae* infections, TEM-1 and ROB-1 type β-lactamase-positive ampicillin-resistant (BLPAR) strains are identified in almost all isolates with reduced susceptibility to ampicillin. Furthermore, β-lactamase-negative ampicillin-resistant (BLNAR) strains have recently been reported to have a mechanism of resistance that reduces the affinity of ampicillin for penicillin-binding proteins (PBPs) [5, 6]. The global prevalence of BLNAR strains remains low [7–9], but in some countries, for example Japan, BLNAR strains are encountered with increasing frequency [10–12]. In China, many studies have reported that *H. influenzae* was frequently discovered in CAP patients, and the proportion of ampicillin-resistant strains in isolates had increased steadily from 8.5 to 14.8% [13, 14] in adults and from 2.4–14.5% in children [15, 16]. Nevertheless, clinical details about antimicrobial-resistant strains, for example BLNAR and BLPAR, are still limited in China. Furthermore, *H. influenzae* isolates analyzed in most studies were provided by central hospitals affiliated with universities which usually only admit critical patients, and most of these had already received chemotherapy. The characteristics of the spread of *H. influenzae* in communities are still unclear. This study was conducted to address such issues.

Materials and methods

Study sites

Eleven hospitals cooperated in this study between December 2008 and April 2009. These facilities are community-level hospitals, with fewer than 200 beds, located in 11 independent districts of Shanghai city. During the study period, Outpatients diagnosed with community-acquired respiratory tract infection (CARTI) caused by *H. influenzae* were included in this study. Biological specimens were collected from all participants during a hospital visit (usually the first visit). Clinical specimens (including sputa and throat swabs) were sent to the clinical microbiology laboratory at Zhongshan hospital where the *H. influenzae* isolates were identified.

Bacterial strains

Gram-stained smears and cultures of specimens, obtained as recently as possible, were prepared to identify *H. influenzac* isolates. Isolates were inoculated on chocolate agar plates and cultured at 37°C overnight in 5% CO_2 for subculture. β-lactamase production was detected by means of a disc impregnated with nitrocefin (Becton–Dickinson, Sparks, MD, USA). Serotyping was performed by slide agglutination with antisera purchased from Difco Laboratories (Detroit, MI, USA).

Antimicrobial susceptibility test

Minimum inhibitory concentration (MIC) was determined by the broth-dilution method, in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [17]. The susceptibility of 37 *H. influenzae* isolates was tested against 6 antibiotics: ampicillin/sulbactam (ABPC/SBT), cefotaxime (CTX), cefaclor (CCL), azithromycin (AZM), levofloxacin (LVFX), and imipenem (IPM) (Oxoid, UK). *H. influenzae* ATCC 49247 was also tested as the reference strain.

Genetic identification of antimicrobial resistance-related genes

PCR was performed on 37 *H. influenzae* isolates to identify antimicrobial resistance-related genes, in accordance with the manufacture's manual (Wakunaga Pharmaceutical, Hiroshima, Japan), as described elsewhere [18, 19]. P6 primers were used to amplify the P6 gene which encodes the P6 membrane protein specific for *H. influenzae*, TEM-I primers to amplify part of the bla_{TEM-1} gene, PBP3-S primers to identify an Asn526 → Lys amino acid substitution in the *ftsI* gene, and PBP3-BLN primers to identify

an Asn526 → Lys and Ser385 → Thr amino acid substitution in the *ftsI* gene.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed on all the strains to determine genetic relatedness, as described elsewhere [20, 21]. The DNA was digested with *Sma* I (Takara Shuzo, Shiga, Japan). CHEF Mapper Pulsed Field Electrophoresis Systems (Bio-Rad Life Science Group, Hercules, CA, USA) were used for the electrophoresis, with a potential of 6 V/cm, switch times of 0.47 and 63 s, and a run-time of 20 h and 18 min. After staining with Ethidium bromide, interpretation of PFGE patterns was based on the criteria described by Tenover et al. [22].

Results

Characteristics of the strains

This study included 37 patients (male 18, female 19) diagnosed as CARTI (including 18 acute pharyngitis, 2 acute tonsillitis, 11 acute bronchitis, 2 acute pneumonia, and 4 exacerbation of bronchiectasis). The average age was 55.4 years (17–99). Only 6 patients with underlying diseases (chronic cardiac disease, hypertension, diabetes mellitus, and chronic pulmonary diseases) were recorded. None of the patients had received any antibiotic therapy before the specimens were obtained. A total of 37 *H. influenzae* isolates were isolated from these patients. Of these, 12 (32.4%) were obtained from sputum, and the other 25 (67.6%) were recovered from throat swabs. On the basis of the characteristics of resistant genes, 16 (43.2%) were β-lactamase-negative ABPC-susceptible (BLNAS) strains, 10 (27.0%) were TEM-1-type β-lactamase-positive ampicillin-resistant (BLPAR) strains, and 11 (29.8%) were Low-BLNAR. The BLNAR strain was not detected in this study (Table 1). A total of 3 different serotypes were verified in 7 (18.9%) encapsulated *H. influenzae* strains. Of these, 2 were serotype b (Hib), 3 were serotype c, and 2 were serotype d. All the Hib strains and 2 serotype c strains were BLPAR. The other encapsulated isolates were BLNAS strains. The remaining 30 (81.1%) were verified as nontypeable strains (NTHi), of which 11 were Low-BLNAR, and 6 were BLPAR strains (Table 1).

Antimicrobial susceptibility test

The respective MIC_{50} (MIC_{90}) values (μg/ml) against BLNAS, BLPAR, and Low-BLNAR strains were 0.5 (0.5), 1 (2), and 0.5 (1) for ABPC/SBT; 0.015 (0.03), 0.015 (0.03), and 0.03 (0.06) for CTX; 2 (4), 2 (4), and 8 (32) for CCL; 1 (2), 0.5 (1), and 1 (2) for AZM; 0.03 (0.12), 0.015 (0.12), and 0.015 (0.03) for LVFX; and 0.5 (1), 0.25 (1), and 1 (4) for IPM (Table 2). All the isolates were susceptible to CTX, AZM, LVFX, and IPM, and there were minor differences among the activity of each of these antibiotics against BLNAS, BLPAR, and Low-BLNAR strains. One BLPAR strain was resistant to ABPC/SBT, and 4 strains were resistant to CCL, all of which were Low-BLNAR strains.

Interpretation of PFGE

Molecular typing by PFGE revealed 23 independent patterns. Only 2 PFGE patterns were detected among BLPAR strains. In this group, 8 strains were pattern A; of these, 6 were nontypeable and the other 2 were serotypes b and c. The other 2 pattern B strains were serotypes b and c (Fig. 1). In the Low-BLNAR group, 4 strains were pattern A, and the other 7 strains had different PFGE patterns (C–I) (Fig. 2). Of the 14 patterns (J–W) detected in the BLNAS group, only 1 was verified as pattern A, and this was nontypeable. Additionally, 2 NTHi strains were pattern J (Fig. 3). A total of 13 (35.1%) strains of, predominantly, pattern A were detected in all groups, of which 2 were encapsulated strains (Hib: 1, serotype c: 1) and 11 were NTHi strains.

Table 1 Characteristics of strains and distribution of serotypes

Strain	Serotypes (%)				Sputum	Throat swab (%)
	b	c	d	Nontypeable		
BLNAS	0	1	2	13	5	11
BLPAR (TEM-1)	2	2	0	6	3	7
Low-BLNAR	0	0	0	11	4	7
BLNAR	0	0	0	0	0	0
Total	2 (5.4)	3 (8.1)	2 (5.4)	30 (81.1)	12 (32.4)	25 (67.6)

BLNAS β-lactamase-nonproducing ampicillin-susceptible *H. influenzae*, *BLPAR* β-lactamase-producing ampicillin-resistant *H. influenzae*, *Low-BLNAR* low β-lactamase-nonproducing ampicillin-resistant *H. influenzae*, *BLNAR* β-lactamase-nonproducing ampicillin-resistant *H. influenzae*

Table 2 Susceptibility of strains to selected antibiotics

Strain	MIC_{50} (MIC_{90}) / (range) (μg/ml)					
	ABPC/SBT	CTX	CCL	AZM	LVFX	IPM
BLNAS	0.5 (0.5) / (0.12–2)	0.015 (0.03) / (0.008–0.06)	2 (4) / (0.03–16)	1 (2) / (0.25–4)	0.03 (0.12) / (0.008–1)	0.5 (1) / (0.06–4)
BLPAR (TEM-1)	1 (2) / (0.25–4)	0.015 (0.03) / (0.008–0.06)	2 (4) / (2–4)	0.5 (1) / (0.12–4)	0.015 (0.12) / (0.008–0.25)	0.25 (1) / (0.12–1)
Low-BLNAR	0.5 (1) / (0.5–2)	0.03 (0.06) / (0.008–0.06)	8 (32) / (2–64)	1 (2) / (0.25–2)	0.015 (0.03) / (0.008–0.06)	1 (4) / (0.25–4)
Total	0.5 (2) / (0.12–4)	0.015 (0.03) / (0.008–0.06)	2 (16) / (0.03–64)	1 (2) / (0.12–4)	0.015 (0.12) / (0.008–1)	0.5 (4) / (0.06–4)

BLNAS β-lactamase-nonproducing ampicillin-susceptible *H. influenzae*, *BLPAR* β-lactamase-producing ampicillin-resistant *H. influenzae*, *Low-BLNAR* Low β-lactamase-nonproducing ampicillin-resistant *H. influenzae*; *ABPC/SBT* Ampicillin/sulbactam, *CTX* Cefotaxime, *CCL* Cefaclor, *AZM* Azithromycin, *LVFX* Levofloxacin, *IPM* Imipenem

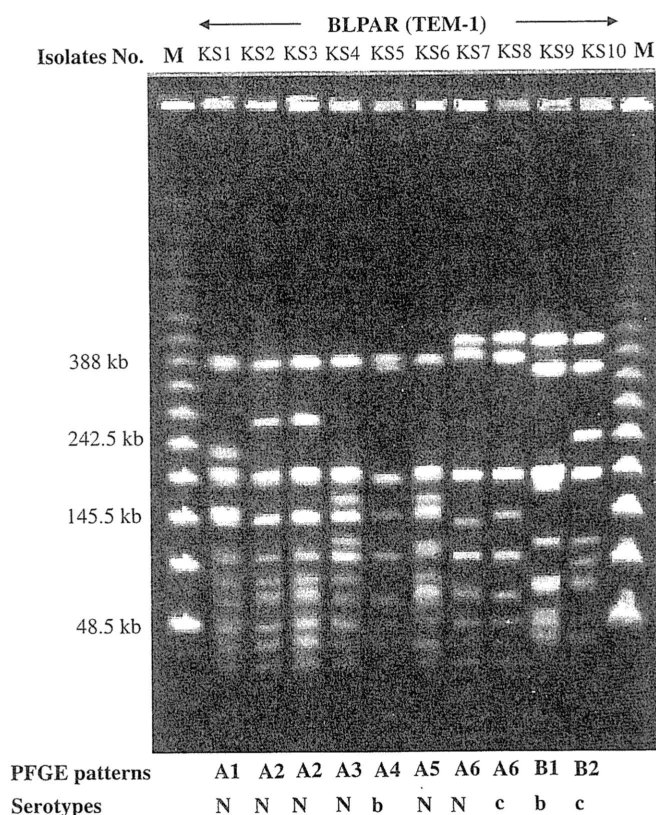

Fig. 1 PFGE results for BLPAR (TEM-1) strains. DNA fragments were digested by *Sma* I restriction endonuclease; *lane M* contains a molecular size marker. *N* nontypeable, *b* serotype b, *c* serotype c

Discussion

H. influenzae is an important causative pathogen, and surveillance of antimicrobial-resistant strains is of great concern. Reportedly, up to 30% of isolates have been regarded as β-lactamase-positive strains [23]. TEM-1 enzyme has been reported to be responsible in 90–95% of isolates, and ROB-1 enzyme in 5–10% [24, 25]. In Asian countries, *H. influenzae* is isolated from approximately 15.1% of CAP patients [26]. Farrell et al. [10] reported infrequent isolation of β-lactamase-positive *H. influenzae* strains—6.4, 19.9, and 8.0% in China, Hong Kong, and Japan compared with 67.9 and 52.6% in Taiwan and South Korea, respectively. Recently, Wang et al. [27] reported that BLPAR accounted for 14.3% of all strains isolated from pediatric outpatients in Beijing. In our study, 27% of isolates were classified as BLPAR strains, and all these were TEM-1 type. This result suggested the frequency of isolation of BLPAR might be increasing in urban areas of China. The prevalence of BLNAR strains was 9.3% in

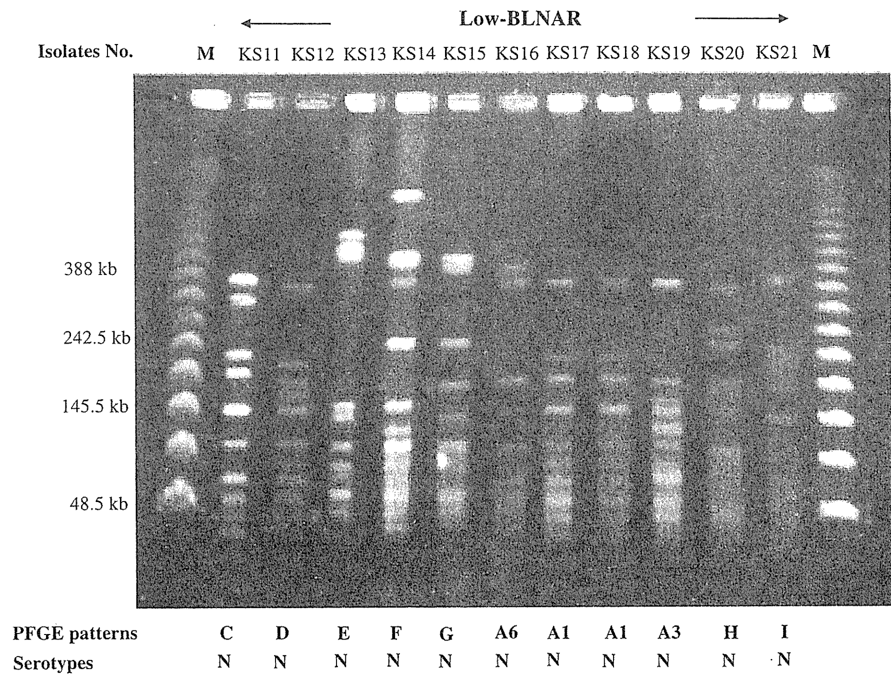

Fig. 2 PFGE results for Low-BLNAR strains. DNA fragments were digested by *Sma* I restriction endonuclease; *lane M* contains a molecular size marker. *N* nontypeable

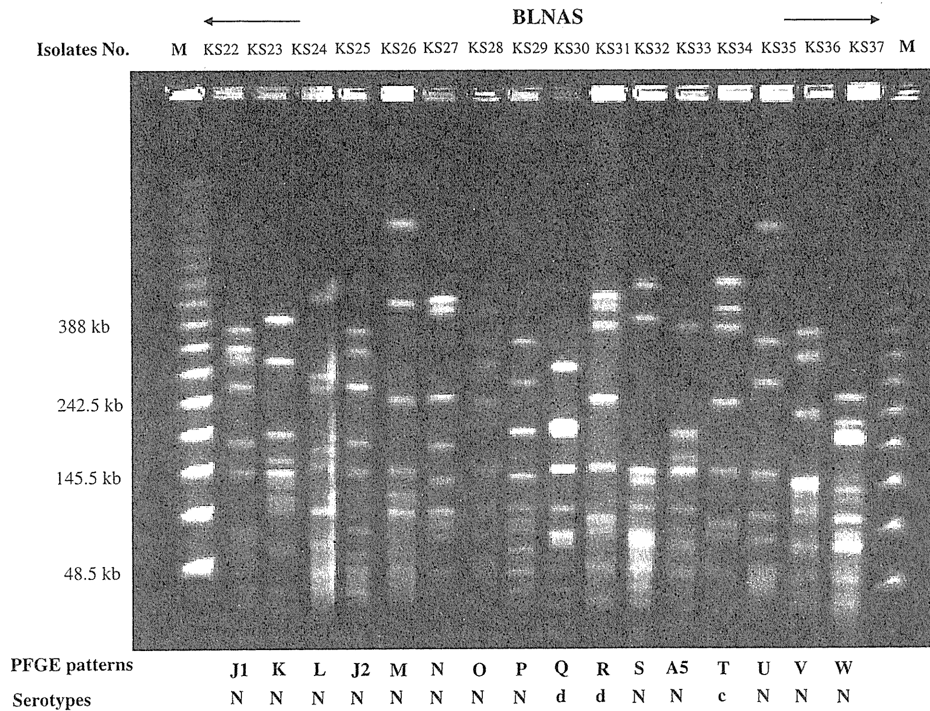

Fig. 3 PFGE results for BLNAS strains. DNA fragments were digested by *Sma* I restriction endonuclease; *lane M* contains a molecular size marker. *N* nontypeable, *c* serotype c, *d* serotype d

Spain between 1998 and 1999, 1.3% in France in 1999, and 2.4% in the USA between 2002 and 2003 [7, 28, 29]. Recently, Blasi et al. [30] reported that 5.2% of isolates were BLNAR strains worldwide; of these 97.5% were collected from Japan, but no such isolates were collected from China. In our study, 11 isolates were classified as Low-BLNAR, but no BLNAR strain was detected, which is consistent with the results of previous studies [15, 16, 27]. BLNAR strains are regarded as resistant to other β-lactam agents and cephalosporins. The characteristics of the antimicrobial resistance of these strains remain of great concern in clinical prescription. Although the prevalence of

BLNAR is still rare in China, monitoring on BLNAR seems necessary to develop more practicable chemotherapy guidelines.

β-Lactam antibiotics and new quinolones are usually prescribed as empiric antibiotic therapy for respiratory tract infections in China. For those suspected of having *Mycoplasma* or *Chlamydia* infections, macrolides such as erythromycin or azithromycin are recommended, and, for severe cases, intravenous penicillin, ampicillin, cefuroxime, or cefotaxime are suggested [31, 32]. It is well known that β-lactam/β-lactamase inhibitor combinations are usually highly active against *H. influenzae*. However, it should be noted that in this study we found one BLPAR strain with resistance to ABPC/SBT. The mechanism is currently unknown and we are working on it. It is also noteworthy that for the 2 cephalosporins tested, 4 strains (10.8%), all of which were Low-BLNAR strains, were completely resistant to CCL, and that CTX, a third-generation cephem, was much more active than CCL. Reportedly, 3% of strains were resistant to CCL in Shanghai and Beijing, and 1% in Guangzhou in 2002, and 4.2% in Beijing in 2004 [15, 27]. Compared with these results, the greater number of resistant isolates suggested reduced activity of CCL, and prescription of CCL should be recommended only for susceptible isolates according to MIC. Macrolides have also been widely used in China, and resistance to macrolides, especially of *Streptococcus pneumoniae*, has already been reported in China [33]. Recent studies have, however, reported high activity of AZM against *H. influenzae* [15, 16]. In this study, all the strains were susceptible to AZM, which is consistent with previous results. CTX, LVFX, and IPM were also highly active against all the strains. However, ABPC/SBT and CCL-resistant strains were found. These results indicate that more attention should be paid to the abuse of antibiotics in China.

PFGE is well known as one of the most sensitive fingerprint methods. In this study, BLPAR strains seemed to be concentrated in PFGE pattern A, and 4 Low-BLNAR strains (36.4%) were also pattern A; however, BLNAS strains had a variety of genetic patterns. These results suggested that ampicillin-resistant strains might be much more closely related genetically than are ampicillin-sensitive strains. Two BLPAR strains isolated from different hospitals were verified as Hib and were susceptible to all the antibiotics tested in this study except ampicillin. One Hib strain was PFGE pattern A, and the other was pattern B. These results provided evidence that at least 2 types of Hib strains were spreading horizontally in Shanghai, China. Compared with other developing Asian countries, in children aged less than 5 years the unadjusted incidence of Hib meningitis was low, estimated to be 0.98 per 100,000 child-years in China [34]. The Hib conjugate vaccine seems to be effective not only in the prevention of invasive infections but also for reduction of nasopharyngeal carriage in young children and the incidence of respiratory tract infections such as bronchitis, etc.

This was the first clinical prospective study of the distribution of *H. influenzae* in community hospitals in a metropolitan city in China. It was also a pilot study to track the continual evolution of antimicrobial resistance of *H. influenzae* and of genetic determinants. Although this study had many limitations, for example small sample size and limited study period, it should be considered as one part of an essential approach to interrupt the spiral of antimicrobial resistance of *H. influenzae*.

In conclusion, these results demonstrated that BLPAR and Low-BLNAR strains with different genetics are increasing in urban areas of China. Although current empirical chemotherapy for CARTI patients seems to be effective, more surveillance should be implemented to improve appropriate treatments.

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Conflict of interest We declare that none of the authors has financial arrangements with any company whose product is mentioned prominently in this manuscript nor with any company making a competing product.

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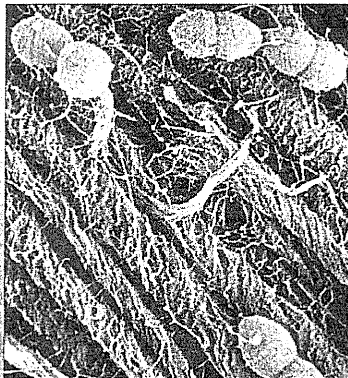

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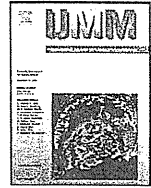

Fetuin A, a serum component, promotes growth and biofilm formation by *Aspergillus fumigatus*

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ABSTRACT

Aspergillus fumigatus is an all-important pathogenic fungus and is known for its angiotropism. When it invades human organs, *A. fumigatus* makes direct contact with blood and its components by causing inflammation and invading vascular structures. To learn the effect of its contact with blood on the development of infection, we examined the effect of serum on *A. fumigatus* growth. In Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, hyphal tip growth was accelerated, forming a thickened and well-networked biofilm associated with extracellular matrix, and fetuin A was identified as the active component in the serum that accelerates fungal growth leading to formation of a community. These results suggest that fetuin A is a novel accelerator of the growth of *A. fumigatus* and that it participates in the formation of thick biofilm.

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Introduction

Aspergillus fumigatus is a ubiquitous environmental mold and is one of the most prevalent pathogenic fungi aside from being a major causative pathogen of aspergillosis. Recently, it has been shown that *A. fumigatus* also forms biofilm (Mowat et al., 2007, 2008, 2009; Seidler et al., 2008; Loussert et al., 2010; Beauvais et al., 2007). These communities tend to be resistant to antifungal treatments (Rajendran et al., 2011; Mowat et al., 2008).

This fungus is known for its angiotropism, and during the development of aspergillosis, it makes direct contact with serum by growing into hemorrhaged infected tissues and by invading blood vessels (Bernard et al., 1997; Filler and Sheppard, 2006; Kamai et al., 2006). Even without overt hemorrhage, serum components are exuded from vessels into adjacent tissues (Signor et al., 2004). Serum is known to affect the growth of *A. fumigatus*. Several components were identified as effectors of the growth of *A. fumigatus* (Gifford et al., 2002; Rodrigues et al., 2005; Hissen et al., 2004; Wasylnka et al., 2005). Albumin, a major component of serum, accelerates fungal growth, and the addition of albumin to growth media decreases the susceptibility of *A. fumigatus* to amphotericin B and itraconazole (Gifford et al., 2002; Rodrigues et al., 2005). Iron, identified as another major component of serum, also accelerates growth (Hissen et al., 2004; Wasylnka et al., 2005) through interaction with serum albumin (Gifford et al., 2002). However, the effect of serum on *A. fumigatus* has not been fully elucidated.

In this study, we found that serum promoted the growth and the formation of biofilm by *A. fumigatus*. Furthermore, we identified a serum factor, fetuin A, that promoted the growth and the branching of hyphae, suggesting that fetuin A plays an important role for biofilm formation by *A. fumigatus*.

Materials and methods

Fungi used in this study and growth conditions

The *A. fumigatus* IFM49896 strain (Watanabe et al., 2004) was obtained from the culture collection of the Medical Mycology Research Center, Chiba University. Conidia were inoculated and were grown on potato dextrose agar (PDA: BD, Franklin Lakes, NJ, USA) slants at 25 °C for 2 weeks. From these slants, conidia were collected and suspended in 0.05% Tween-20 solution as described previously (Toyotome et al., 2008).

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Culture conditions for formation of biofilm

Conidia suspended in Dulbecco's modified Eagle's (DME) medium (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS: BD) were inoculated to a tissue culture dish at 3.75×10^4 of conidia per square centimeter of dish bottom, and cultured at 37 °C for 30 h in the presence of 5% CO_2. For comparison, conidia were inoculated to another tissue culture dish in DME without FBS and were used as control. To examine the effect of human serum, *A. fumigatus* was cultured for 48 h in DME containing 10% human serum. The effect of bovine serum albumin (BSA: Wako Pure Chemical Industries, Osaka, Japan) and fetuin A from fetal bovine serum (Merck Biosciences, Darmstadt, Germany) was investigated by adding each reagent to the DME medium at concentrations of 5 or 10 mg/ml and 2 mg/ml, respectively.

Measurement of the weight of the fungal community

A. fumigatus suspended in DME, DME with 10% FBS, DME with 10 mg/ml BSA, or DME with 2 mg/ml fetuin A was cultured at 37 °C in three 9-cm diameter dishes. After 30 h, the fungal community was recovered by filtering through Miracloth (Merck Biosciences), and the cells were weighed after lyophilization.

Staining with Calcofluor White M2R

Calcofluor White M2R (Fluorescent Brightener 28: CF, Sigma–Aldrich) was used for staining at a final concentration of 25 μM. Cells cultured for 30 h were then incubated with CF for 60 min at 37 °C. LSM5 Exciter (Carl Zeiss, Oberkochen, Germany)

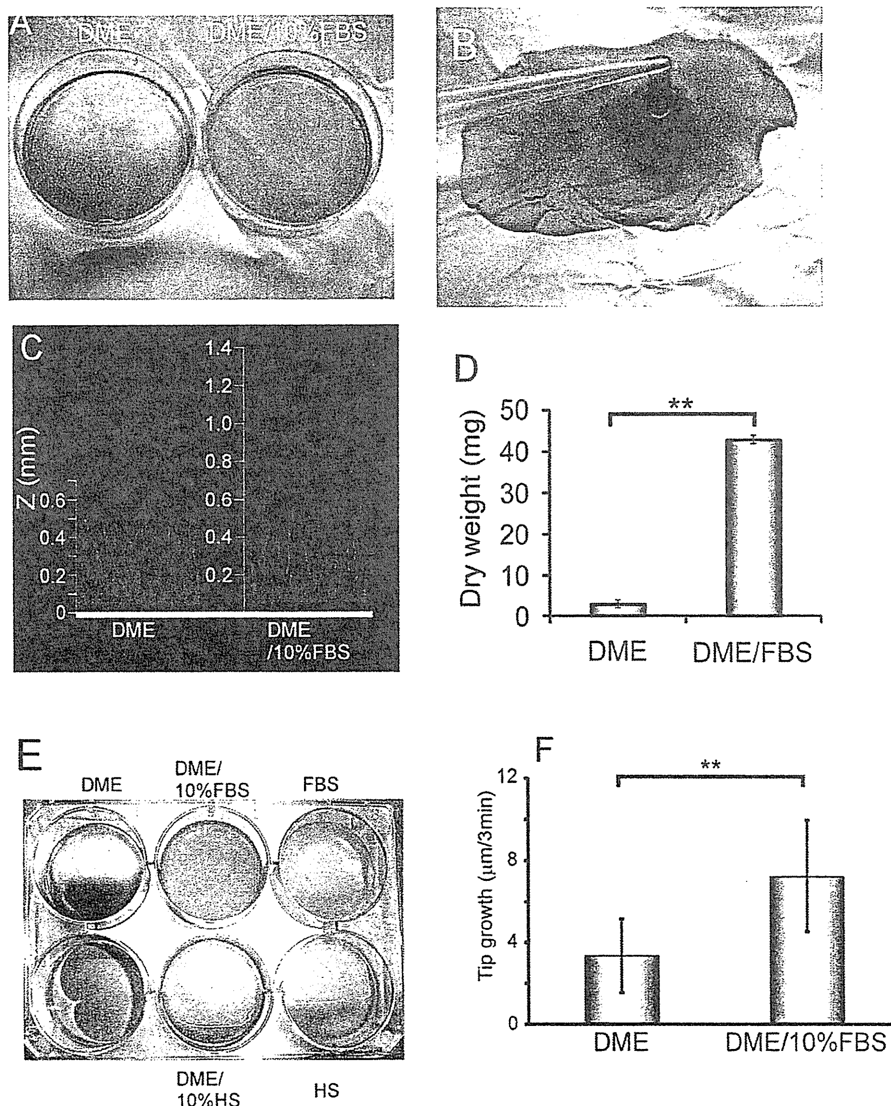

Fig. 1. (A) Appearance of a 30-h culture of *A. fumigatus* in DME (left) and in DME containing 10% FBS (right). The plate was placed on a sloped surface to visualize biofilm formation. The culture picked up from the dish had a slime-like appearance (B). (C) Side views of a Z-stack image of fungal cells cultured for 30 h in DME and DME/10% FBS. The cells were stained with Calcofluor White M2R. (D) Dry weight of the fungal community of *A. fumigatus* from a 9-cm diameter dish. Means and SDs were calculated from the results of 3 dishes from independent trials. *P*-values were calculated using Student's *t*-tests. Bars represent mean ± SD. $^{**}P < 0.01$. (E) Appearance of a 48-h culture of *A. fumigatus* in DME (upper left), DME containing 10% FBS (upper center), FBS alone (upper right), DME containing 10% human serum (lower center), and human serum alone (lower right). (F) Results of the BCT assay. *P*-values were calculated using Student's *t*-tests. Bars represent mean ± SD. $^{**}P < 0.01$.

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and ZEN 2008 software were used for constructing a side view of Z-stack images.

Staining with Fungiflora Y

Fungiflora Y (Thechnicon International, Inc., Tokyo, Japan), which specifically stains β-linked polysaccharides, was used for the staining of fungus balls and fungal cells with extracellular polysaccharides. The staining procedure was performed according to the manufacturer's instructions. An AXIO Imager.A1 equipped with AXIOCam MRc (Carl Zeiss) was used for taking the images. To quantify the frequency of branching, the distance from growth tip to the last branching point and the number of branches in a cluster were measured using a fluorescent microscope BZ-9000 (Keyence Co., Osaka, Japan).

Measurement of hyphal growth rate by monitoring hyphal tips

For measurement of the growth rate of hyphae, Bio-Cell Tracer (BCT) system (Hidan Co., Ltd., Chiba, Japan) was used (Ansheng et al., 1999). In each experimental condition, more than 17 hyphal tips were monitored, and the elongation of hyphae was recorded for calculation of growth rate.

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed as described previously (Yamaguchi et al., 2010). Briefly, fungal cells cultured in DME or DME/10% FBS on filter paper were fixed with 2.5% glutaraldehyde in phosphate buffer overnight at 4 °C. Fixed cells were post-fixed with 1% osmium tetroxide, dehydrated in ethanol, and substituted with iso-amyl acetate. After critical point drying, samples were coated with platinum–palladium and observed with an S-3400 scanning electron microscope (Hitachi High-Technologies Co., Tokyo, Japan) at 5 kV.

Fractionation of serum proteins

Serum proteins were concentrated 9-fold using Centriprep YM-30 (Millipore, Billerica, MA, USA), which has a 30,000-nominal molecular weight limit. The concentrated serum was diluted 9-fold with DME, and then cycles of concentration and dilution were repeated 3 times to eliminate low molecular weight components. During the procedure, components were cooled and placed on ice. The retentate and initial filtrate were used for the experiment as described below.

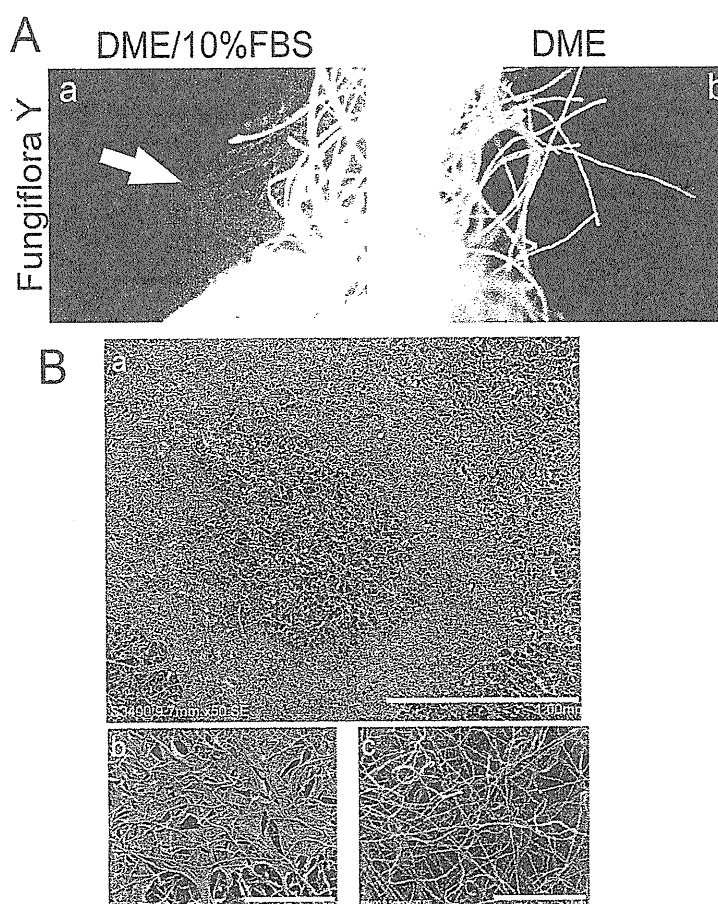

Fig. 2. (A) Staining of extracellular polysaccharides with Fungiflora Y. Panel a shows hyphae cultured in DME/10% FBS. Panel b shows hyphae cultured in DME. The white arrow indicates the extracellular polysaccharides stained with Fungiflora Y. (B) SEM images of *A. fumigatus* cultured in DME/10% FBS (panels a and b) or in DME (panel c). Scale bar: 1.0 mm (a), 100 μm (b and c).

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Isolation of glycoproteins

For the isolation of glycoproteins, we used Glycoprotein Isolation Kits with concanavalin A (ConA) resin and with wheat germ agglutinin (WGA) resin (Thermo Fisher Scientific Inc., Asheville, NC, USA). The procedure was performed according to the manufacturer's instructions. For elimination of sugar in the elution buffers, eluents were diluted 10-fold with DME and concentrated using Centriprep YM-30. Cycles of dilution and concentration were done as above, and the resultant solutions were used for the culture of *A. fumigatus*.

Mass spectrometry

The protein band that was stained with CBB was excised from the gel. After destaining and drying the excised pieces, proteins were digested by trypsin at 37 °C for 10–14 h. The digested peptides were extracted with acetonitrile solution and analyzed with a matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometer (4700 MALDI TOF/TOF Analyzer, Applied Biosystems Inc., Foster City, CA, USA). To identify proteins, we analyzed the mass spectrometer data using Mascot MS/MS Ions Search (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS, Matrix Science, London, UK).

β-Glucan assay

For the determination of β-glucan concentration in medium, BGStar A kit (Wako Pure Chemical Industries) was used. Measurement was performed according to the manufacturer's procedure.

Microtiter biofilm assay

Biofilm formation by *A. fumigatus* was assessed using 96-well (flat bottom) polystyrene (PST) microtiter dishes (NUNC, Roskilde, Denmark). Briefly, the concentration of conidia was initially adjusted to a turbidity of about 0.1 (OD_{490}). Conidia were then grown in 200 μl of DME, DME supplied with 10% FBS, and fetuin A (0.02 mg/ml, 0.2 mg/ml, and 2 mg/ml) at 37 °C for 30 h, 48 h, and 72 h. Biofilms were stained with 1% crystal violet at room temperature for 15 min. After thoroughly washing with water 3 times, the dye bound to the biofilms was extracted with 230 μl of 95% ethanol and was quantified by measuring absorbance at 595 nm using a microplate reader. All tests were examined in triplicate, and the average of each experiment was calculated. Biofilm architectures adherent to the wells were observed by optical microscopy at magnifications of 10× and 40×.

Pull-down assay

Pull-down assay was performed as follows. Resting conidia, swollen conidia from fungal cells cultured for 6 h (Toyotome et al., 2008), and hyphae were mixed with 500 μl of human serum. After 1 h, fungal cells were washed 5 times with TBS containing 0.1% Tween 20. The fungal cells were suspended in sample buffer for SDS-PAGE, after which they were boiled. The samples were developed using SDS-PAGE and subjected to Western blotting. Goat anti-human fetuin A antibody (R&D Systems, Inc., Minneapolis, MN) was used to detect human fetuin A.

Preparation of FITC-labeled fetuin A and examination of the association with A. fumigatus

The Fluorescein Labeling kit-NH2 (Dojindo, Kumamoto, Japan) was used to label FITC to fetuin A. *A. fumigatus* hyphae cultured with 10 mg/ml BSA on a coverslip were incubated with FITC-labeled fetuin A for 30 min at 37 °C in the presence of 5% CO_2. After the incubation, hyphae were washed 3 times with PBS, and then fixed with 4% paraformaldehyde.

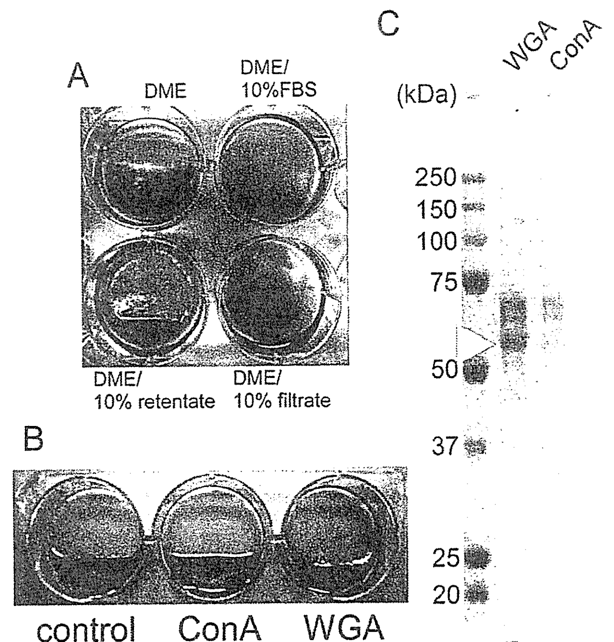

Fig. 3. (A) Growth of *A. fumigatus* in medium containing fractionated serum. (B) Growth of *A. fumigatus* in medium containing glycoproteins of serum purified by ConA or WGA resin for 126 h. The plates in A and B were placed on a sloped surface. (C) Glycoproteins purified by WGA or ConA were developed by SDS-PAGE. The arrowhead indicates the band excised for mass spectrometry analysis.

Staining with anti-human fetuin A antibody

Staining with anti-human fetuin A antibody was done on paraffin sections from aspergilloma patients using standard immunohistochemical protocols.

Statistics

Results of statistical analyses were shown as mean ± standard deviation (SD). Statistical significance was determined using either Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparisons test. *P*-values less than 0.05 were considered statistically significant. The statistical method used in each study is indicated in the figure legends.

Sera from healthy volunteers

Blood was collected from healthy volunteers based on policies of the ethics committee of the Medical Mycology Research Center, Chiba University. Informed consent was obtained from the donors.

Results

Serum promotes growth and biofilm formation of A. fumigatus

First, we examined the effect of FBS on the growth of *A. fumigatus*. After 30 h, fungal culture in DME medium containing 10% FBS, but not in DME only, formed a thick biofilm (Fig. 1A) that has a slime-like appearance (Fig. 1B). The fungal community formed in DME/10% FBS was thicker than that in DME medium alone, and the hyphae in the community were more tightly networked

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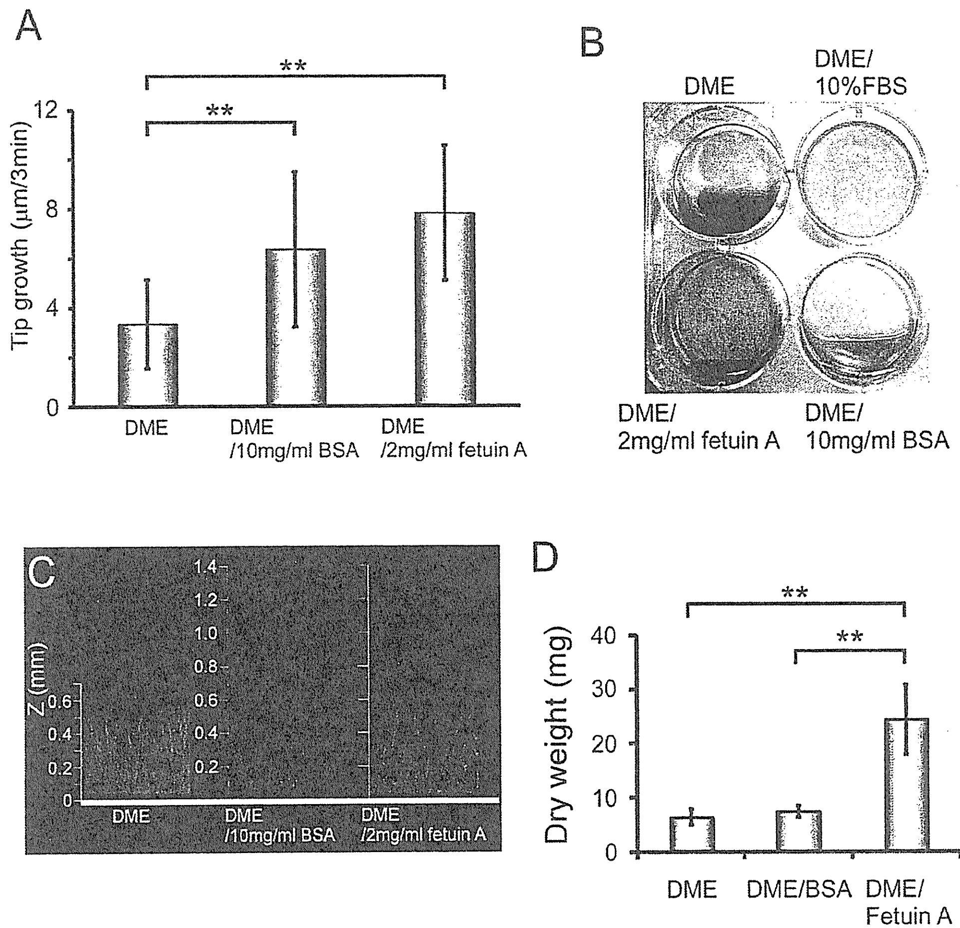

Fig. 4. Effect of fetuin A on the growth of *A. fumigatus*. (A) Results of the BCT assay. *P*-values were calculated using a one-way ANOVA followed by a Bonferroni's multiple comparisons test. Bars represent mean ± SD. $^{**}P < 0.01$. (B) Appearance of wells with cultured *A. fumigatus* in media for 48 h. The plate was put on a sloped surface. (C) Side views of Z-stack image of fungal cells cultured for 30 h in DME, DME/10 mg/ml BSA, and DME/2 mg/ml fetuin A. (D) Dry weight of the fungal community of *A. fumigatus* from a 9-cm diameter dish cultured for 30 h in DME and DME/10 mg/ml BSA. The mean and error bars indicating the SD were calculated from the results of 3 dishes from independent trials. *P*-values were calculated using one-way ANOVA followed by a Bonferroni's multiple comparisons test. Bars represent means ± SDs. $^{**}P < 0.01$.

(Fig. 1C). The dry weight of the fungal community cultured in FBS-containing medium was significantly increased (Fig. 1D). To determine whether or not the formation of the thick biofilm was specific to FBS, we investigated the growth of *A. fumigatus* in medium containing several sera from healthy adult volunteers. As seen in medium containing FBS, *A. fumigatus* formed a thick biofilm in human adult serum (Fig. 1E). These results proved that, in the presence of serum, *A. fumigatus* formed thick and large biofilm. Then, we observed the growth of each hypha under a microscope and analyzed the growth rate using BCT as described elsewhere (Ansheng et al., 1999). Monitoring of more than 17 hyphal tips in each condition revealed that hyphal growth in DME/10% FBS medium was significantly increased compared with those cultured in DME medium only (Fig. 1F). Taken together, these results show that serum accelerates the growth of *A. fumigatus*, supporting the formation of the thick biofilm.

Extracellular polysaccharides present in the biofilm of A. fumigatus formed in serum

To examine the presence of extracellular polysaccharides in the biofilm of *A. fumigatus*, we treated the fungal community with FungiFlora Y, which specifically stains β-linked polysaccharides. As shown in Fig. 2A, extracellular matrix around hyphae was clearly visualized by FungiFlora Y when cultured in DME/10% FBS. On the other hand, *A. fumigatus* hyphae cultured in DME alone were not associated with extracellular matrix. Moreover, observation of the fungal community under a scanning electron microscope revealed extracellular matrix around the hyphae cultured in DME/10% FBS (Fig. 2B). The matrix, which appeared to be mostly membranous, formed thin layers between hyphae (Fig. 2B-a and B-b) and inside the fungal community as well (data not shown). In contrast, cells cultured in DME alone formed only a trace of extracellular matrix (Fig. 2B-c), consistent with the results in Fig. 2A. These results indicate that, when cultured with serum, *A. fumigatus* forms a biofilm accompanied by extracellular polysaccharides.

Serum glycoproteins promote biofilm formation

To determine the component of serum proteins required for the formation of the biofilm, we fractionated serum components using a filter with a 30,000-nominal molecular weight limit and examined the growth of *A. fumigatus* in media containing either retentate or filtrate. *A. fumigatus* cultured with retentate, but not with filtrate, could form the thick and well-networked biofilm as seen in the fungal community cultured in FBS-containing medium (Fig. 3A). This result suggests that a serum component above 30 kDa induces the formation of the thick biofilm. Then, we sought to identify the active component in the serum fraction containing above-30-kDa proteins, focusing on glycoproteins, using a lectin

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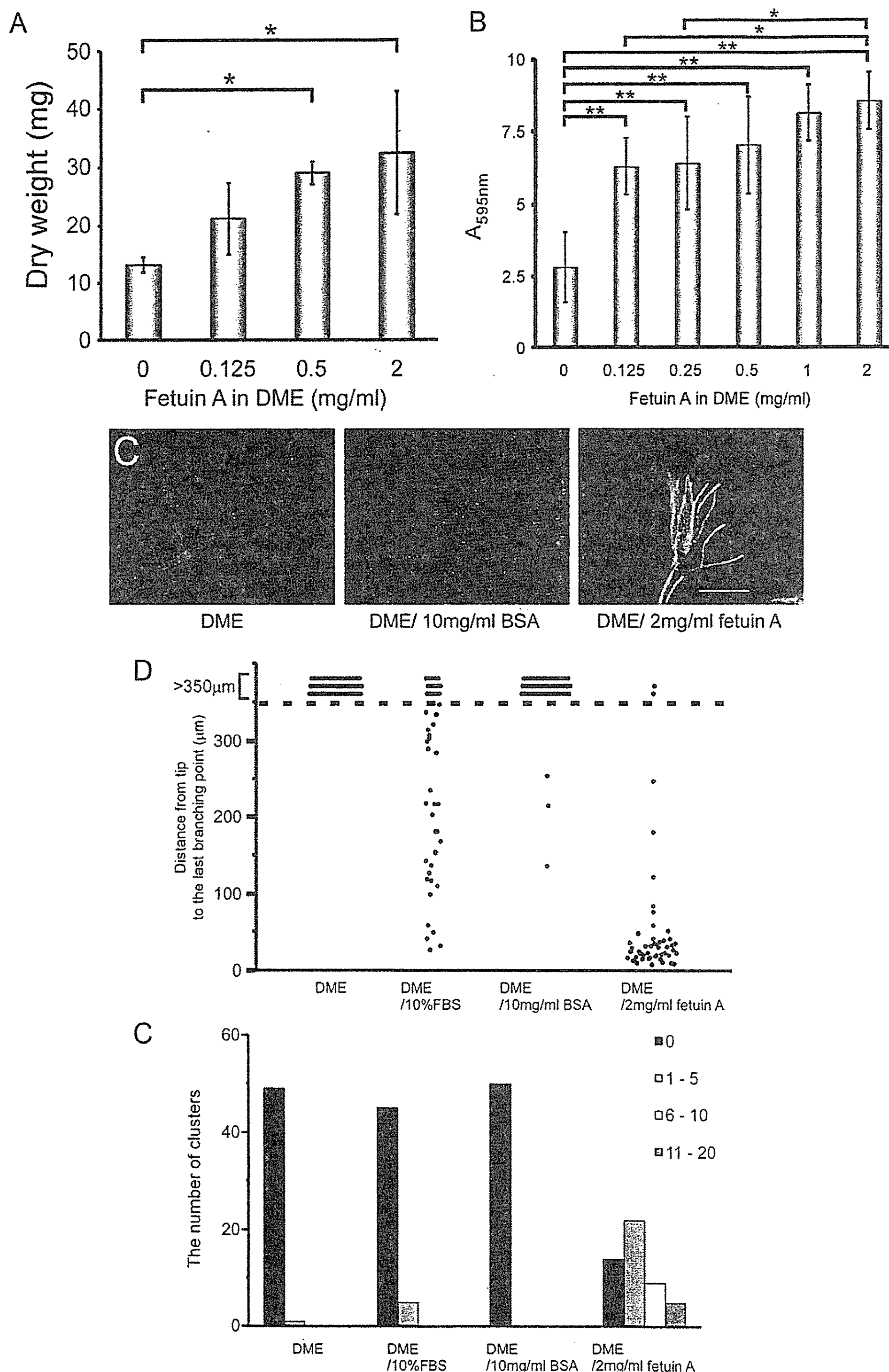

Fig. 5. (A) Dry weight of the fungal community of *A. fumigatus* cultured for 30 h in media containing various concentrations of fetuin A. (B) Biofilm formation by *A. fumigatus* cultured for 72 h in DME containing various concentrations of fetuin A. In *A* and *B*, the results were calculated from 3 independent experiments. $^{**}P<0.01$, $^{*}P<0.05$. (C) Images of *A. fumigatus* hyphae cultured in DME (left) and DME with 10 mg/ml BSA (middle) or 2 mg/ml fetuin A (right). Hyphae were stained with Fungiflora Y; scale bar indicates 50 μm. (D and E) The distances from growth tip to the last branching point (D) and the number of clusters within 50 μm from growth tip (E) cultured in DME, DME with 10% FBS, DME with 10 mg/ml BSA, or 2 mg/ml fetuin A. In each experiment, 50 growth tips were selected and measured. In *E*, black bars indicate the number of branches that did not have branching points within 50 μm from growth tip, light gray bars indicate clusters that have 1–5 branching point(s), white bars indicate 6–10 branching points, and dark gray bars indicate 11–20 branching points.

column kit. Using concanavalin A (ConA) or wheat germ agglutinin (WGA) resin, we excluded most of the albumin and recovered the glycoproteins from FBS. Then, we examined the growth of *A. fumigatus* cultured in medium containing the recovered proteins. No difference in appearance was observed between the growth of *A. fumigatus* in medium containing ConA resin-trapped proteins and in DME medium (Fig. 3B). On the other hand, *A. fumigatus* cultured in medium containing WGA resin-trapped proteins formed a thick

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fungal community, which was similar to the original serum fraction (Fig. 3B). As shown in Fig. 3C, the amount of an approximately 55-kDa protein in the proteins trapped by WGA was significantly higher than that in the ConA-trapped proteins.

Fetuin A, a serum glycoprotein promotes the growth and biofilm formation of A. fumigatus

We isolated the approximately 55-kDa protein band and analyzed it by mass spectrometry. The search result by MASCOT identified the protein as fetuin A. To confirm the effect of fetuin A, we examined its function in growth acceleration of *A. fumigatus* by adding fetuin A to the culture medium and compared its effect with that of BSA, which is known to promote fungal growth (Gifford et al., 2002; Rodrigues et al., 2005). Monitoring of hyphae using the BCT system showed that fetuin A as well as BSA significantly accelerated the growth of hyphae (Fig. 4A). As shown in Fig. 4B, fetuin A-containing medium induced a thick biofilm similar to that in the FBS-containing medium. On the other hand, *A. fumigatus* cultured in medium containing BSA failed to form a thick biofilm, and hyphae were rather scarce and not tightly networked as in the FBS-containing medium (Fig. 4B and C). The dry weight of the fungal community cultured in the fetuin A-containing medium was significantly higher than that cultured in DME or in the BSA-containing medium (Fig. 4D). As shown in Fig. 5A, even at a concentration of 0.5 mg/ml, which is comparable to the serum level in healthy individuals, fetuin significantly increased the dry weight. In a microtiter biofilm assay, biofilm formation by *A. fumigatus* in the fetuin A-containing medium was significantly higher than that in the DME medium after 72 h of culture (Fig. 5B). Furthermore, communities of microorganisms attached to the surface of the well in the fetuin A-containing medium could be clearly confirmed by both naked eye and microscopy (data not shown). Morphologically, hyphae cultured in fetuin A-containing medium were highly branched (Fig. 5C, 5D, and 5E). On the other hand, in medium containing 10 mg/ml BSA, the massive branching was not observed (Fig. 5C–E). Hyphae cultured in FBS-containing medium branched more frequently than hyphae cultured in DME or BSA-containing medium (Fig. 5D and E), suggesting that branching is important for the biofilm formation of *A. fumigatus*. Moreover, these results suggest that fetuin A supports the formation of a thick biofilm by *A. fumigatus* by inducing both hyphal elongation and massive branching.

Fetuin A directly binds to hyphae of A. fumigatus

To examine the association of fetuin A with *A. fumigatus*, we performed a pull-down assay. Hyphae of *A. fumigatus* were strongly associated with fetuin A in human serum (Fig. 6A). In addition, we analyzed the direct association using fluorescent-labeled fetuin A. As shown in Fig. 6B, FITC-labeled fetuin A was associated with the hyphae of *A. fumigatus*. These results suggest that fetuin A directly binds to hyphae of *A. fumigatus*.

Fungus ball includes β-linked polysaccharides and fetuin A

A fungus ball is a kind of biofilm formed in vivo (Loussert et al., 2010; Müller et al., 2011). To investigate the localization of β-linked polysaccharides and fetuin A, we stained lung sections from aspergilloma patients with FungiFlora Y or anti-human fetuin A antibody. As shown in Fig. 7A, β-linked polysaccharides were found in the space between hyphae in the fungus ball. Additionally, the fungus ball was strongly stained with anti-fetuin A antibody (Fig. 7B). These results suggest that a large amount of β-linked polysaccharides and fetuin A are included in fungus balls and are important for the formation of the ball.

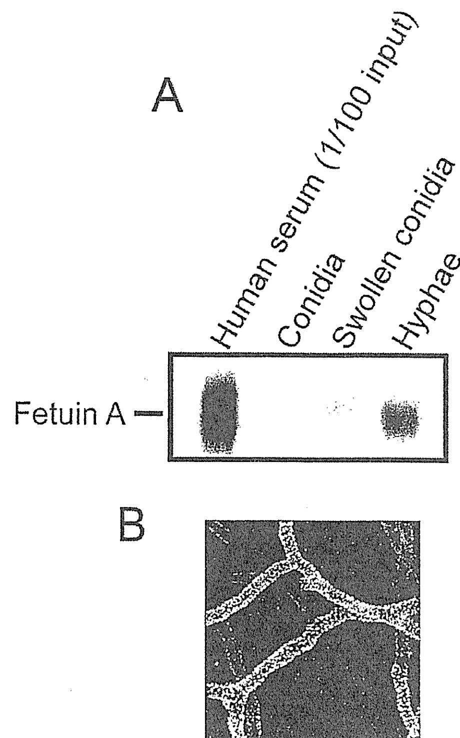

Fig. 6. The direct association of fetuin A with hyphae of *A. fumigatus*. (A) Fetuin A was pulled down with hyphae of *A. fumigatus*. (B) FITC-labeled fetuin A associated with hyphae of *A. fumigatus*.

Discussion

When *A. fumigatus* invades the human body in aspergillosis, the fungus makes direct contact with blood and tissue fluid in organs, and the interaction between blood or the tissue fluid and the fungus might affect the growth of *A. fumigatus*. In this study, we showed that in medium-containing serum, *A. fumigatus* growth was significantly accelerated, forming a thick biofilm associated with an extracellular matrix. Biofilm formation was observed in 2 other isolates of *A. fumigatus* (data not shown), suggesting that this effect is common among *A. fumigatus* isolates. Our finding suggests that serum is a promoter of growth and biofilm formation of *A. fumigatus*.

We showed that the thick fungal community contained extracellular matrix when cultured in serum. Seidler et al. (2008) reported that *A. fumigatus* biofilm was accompanied by extracellular-matrix formation between hyphae when co-cultured with bronchial epithelial cells. Beauvais et al. (2007) and Loussert et al. (2010) analyzed the extracellular matrix formed by *A. fumigatus* under various conditions and found polysaccharides including galactosaminogalactan and galactomannan, monosaccharides, melanin, and proteins as their components. As shown in Fig. 2A, the extracellular matrix contained β-linked polysaccharides. Additionally, the extracellular β-linked polysaccharides were found in fungus balls from aspergilloma patients (Fig. 7A). When *A. fumigatus* was cultured in FBS-containing medium, the soluble β-glucan concentration was about 2-fold higher than the concentration cultured in DME medium (64.9 ± 4.69 ng/ml vs. 35.3 ± 4.00 ng/ml). These data suggest that serum in the medium promoted the formation of biofilm-like substance by the fungus with abundant extracellular β-linked polysaccharides production including β-glucan.

After further analysis, we identified fetuin A, a serum glycoprotein containing sialic acids, as an accelerator of fungal growth

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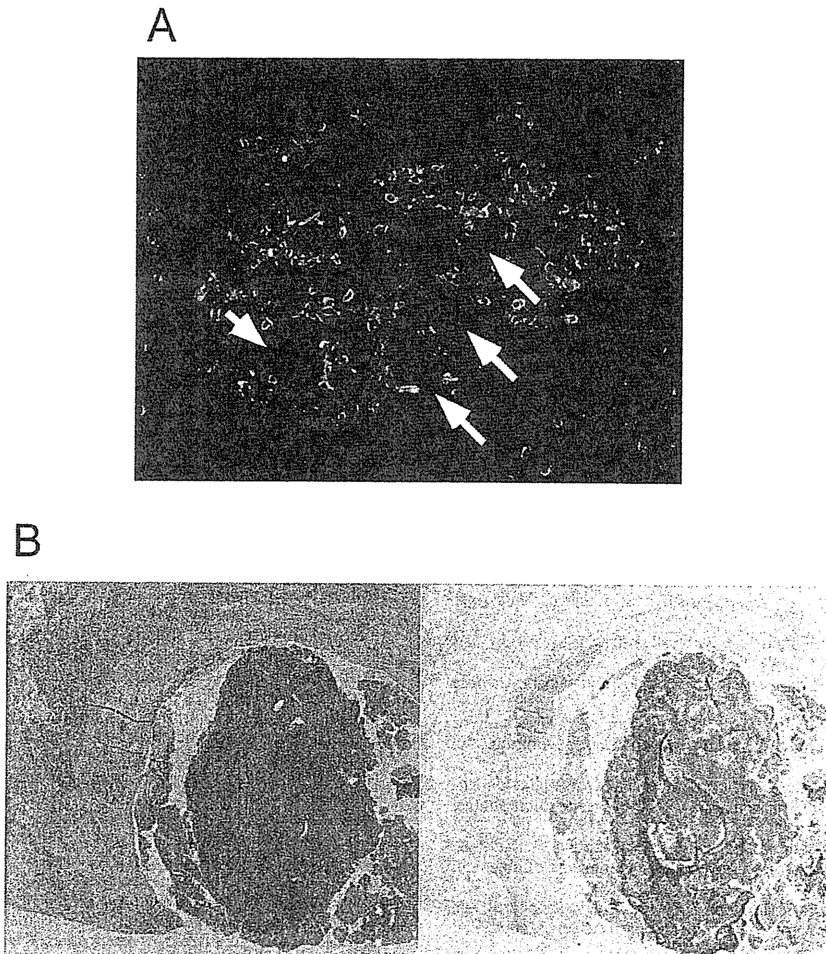

Fig. 7. (A) Staining fungus balls with FungiFlora Y. The arrows indicate a space between hyphae stained with FungiFlora Y. (B) Images of fungus balls and surrounding tissue stained with anti-human fetuin A antibody (left) and with Gomori's methenamine-silver nitrate, Grocott's variation (right).

and massive branching (Figs. 3–5). Fetuin A is known to have many functions (Jahnen-Dechent et al., 1997; Kundranda et al., 2005; Schäfer et al., 2003; Wang et al., 1998). Studies using fetuin A knock-out mice showed that fetuin A acts as an inhibitor of ectopic calcification (Schäfer et al., 2003) as well as a promoter of Lewis lung carcinoma tumorigenesis (Kundranda et al., 2005).

As described by Paisley et al. (2005), growth acceleration is important for virulence, suggesting that fetuin A is an important susceptibility factor. Interestingly, fetuin A was strongly accumulated in fungus balls (Fig. 7B) suggesting that fetuin A contributes to the growth promotion of *A. fumigatus*.

The novel function of fetuin A for *A. fumigatus*, namely, the induction of branching, contributes to the formation of the biofilm and fungus ball. The mechanism of the induction of branching, however, remains unknown. BSA is known as another fungal growth accelerator, but the addition of BSA alone did not induce the massive branching and the increase of dry weight observed with the addition of fetuin A. Since fetuin A, but not BSA, is highly glycosylated, the polysaccharide chains might be important for the induction of branching. Our data indicated that fetuin A is directly associated with *A. fumigatus* hyphae (Fig. 6). Recently, Warwas et al. (2010) reported that fetuin A was a good substrate of sialidase from *A. fumigatus*. Since the sialidase predicted an extracellular protein, fetuin A might interact with this enzyme. The association of fetuin A with *A. fumigatus* via polysaccharides might affect the gene expression and morphology of this fungus. Further experiments are warranted to clarify the interaction between *A. fumigatus* and fetuin A and the role of fetuin A in the induction of branching.

We showed that fetuin A at a concentration of more than 0.5 mg/ml in DME medium clearly promoted the formation of a thick fungal community. The concentration of fetuin A in FBS is about 20 mg/ml, and it decreases to 0.5 mg/ml in adult bovine serum (Brown et al., 1992). In adult humans, the serum level of fetuin A is about 0.6 mg/ml (Kundranda et al., 2005). The concentration of fetuin A that we used in our experiment was within the achievable range observed in fetal/adult bovine or human serum. As shown in Fig. 7B, the level of fetuin A in fungus balls is higher than that in the surrounding area, suggesting that fetuin A is accumulated in fungus balls and could readily reach concentration levels that are enough to induce the formation of biofilm of *A. fumigatus*.

In summary, we showed the effect of serum on growth and biofilm formation of *A. fumigatus*. And we identified a serum protein, fetuin A, which accelerated the growth of *A. fumigatus* and facilitated the formation of thick biofilm. The mechanisms of the formation of the biofilm by serum and the induction of branching by fetuin A, however, remain unclear. Elucidating these mechanisms will help us to understand the roles of *A. fumigatus* biofilm formation in the establishment of infection.