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Nasal Commensal *S. epidermidis* Bacteria Counteract Influenza Virus

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Running title: Nasal bacteria counteract influenza virus

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ABSTRACT

Several microbes including *Staphylococcus epidermidis* (*S. epidermidis*), a Gram-positive bacterium, are living inside the human nasal cavity as commensals. The role of these nasal commensals in host innate immunity is largely unknown. Bacterial interference in nasal microbiome may create an ecological competition between commensal bacteria and pathogenic species. We here demonstrate that culture supernatants of *S. epidermidis* significantly suppress the infectivity of various influenza viruses including San Diego swine-origin influenza (S-OIV) H1N1 virus (SD-H1N1-S-OIV). Via high-performance liquid chromatography (HPLC) together with mass spectrometry, a giant extracellular matrix-binding protein (Embp) has been identified as an active component that contributes to the anti-influenza effect of *S. epidermidis*. The anti-influenza activity was abrogated when Embp was mutated, validating that Embp is essential for *S. epidermidis* against viral infection. We also illustrate that both *S. epidermidis* bacterial particles and Embp can directly bind to SD-H1N1-S-OIV. Injection of a recombinant Embp fragment containing a fibronectin-binding domain into the embroyonated eggs increased the survival rate of influenza virus-infected chick embryos. These results suggest that the presence of *S. epidermidis* in the nasal cavity may serve as a defense mechanism against influenza virus infection.

IMPORTANCE

Owing to rapid antigenic drift, antigens for development of flu vaccines must be updated annually based on global influenza surveillance. Anti-viral drugs have limited efficacy if administered late in infection, and widespread use is likely to result in the emergence of resistant viral strains. Examination of how bacterial members of the nasal microbiome impact the infectivity of airborne pathogens may be key to controlling an influenza infection. The use of commensal bacteria to inhibit pathogens may have even greater potential than vaccine use, because these bacteria are natural competitors of pathogens and their action does not fully require host immune stimulation. We have identified Embp, a fibronectin-binding protein which can be shed from bacterial surface, as an active and essential component of *S. epidermidis* against influenza viruses. *S. epidermidis* bacteria are permanent inhabitants of nasal cavity in humans, birds and pigs. The use of *S. epidermidis* and/or its derived products as anti-influenza agents may not only prevent the viral invasion in humans but also control the interspecies transmission of influenza viruses.

Bacterial interference, or bacteriotherapy, in which commensal bacteria are used to prevent colonization of the host by pathogens, has been shown to be a promising modality for combating infections (1-7). The concept of bacterial interference was utilized in clinics in the treatment of a family with recurrent staphylococcal infections and for prevention of urinary tract infection (8). Therapeutic application of bacterial interference by active colonization using a “nonpathogenic” *Staphylococcus aureus* (*S. aureus*) strain, 502A, was successful in controlling outbreaks of *S. aureus* infections in the 1960s and for treatment of patients with recurrent furunculosis (9-11). More intriguingly, data from previous publication demonstrated that introduction of live *Staphylococcus epidermidis* (*S. epidermidis*) into patient volunteers who were *S. aureus* carriers significantly eliminates the *S. aureus* colonization (12). The findings indicate that it is safe to inoculate *S. epidermidis* into human nasal cavities as an interfering agent. It has been shown that *S. epidermidis* comprises 90%-100% of staphylococci from the human nasal cavity (13, 14). *S. epidermidis* is also a commensal bacterium in turkeys and chickens (4-6). It has been reported that *S. epidermidis* isolates from turkeys were used as interfering agents to help control *S. aureus* infection (5-7). Field administration of interfering *S. epidermidis* into 200,000 turkeys in 21 flocks decreased the *S. aureus* colonization and increased the survival rate of *S. aureus*-infected turkeys (5). Prior injection of interfering *S. epidermidis* into embroyonated eggs led to a reduction in the lethality on challenge with influenza virus (A_2J 305) (15).

Influenza is a highly contagious infection of the upper respiratory tract of humans. The upper respiratory tract is usually the initial site of the invasion of

influenza virus. An effective defense against influenza virus at the initial infection will curb the systemic spread of virus. Vaccines have historically been the mainstay of infection control. However, influenza vaccines have to be updated annually due to seasonal antigenic drifts (16). Neuraminidase inhibitors, especially oseltamavir (Tamiflu), remain the primary treatment for various influenza viruses, but they have limited efficacy if administered late in infection, and widespread use is likely to result in the emergence of resistant viral strains (17). Thus, there is an unmet need to develop novel anti-viral agents with a lower tendency to develop resistant viruses.

Here we demonstrate that *S. epidermidis* significantly suppresses the infectivity of various influenza viruses via a giant extracellular matrix-binding protein (Embp). This work provides a potential therapeutic for influenza virus infection because the use of nasal commensal bacteria as anti-viral agents has three unique characteristics: (a) Unlike antibiotics, commensal bacteria are indigenous to humans, which may have a lower risk of causing serious complications or generating resistant viruses; (b) *S. epidermidis* bacteria are also native inhabitants of birds (5-7, 18, 19) and pigs (19, 20) and have been used as interfering agents for prevention of pathogen colonization in livestock (5-7). Thus, *S. epidermidis* may benefit both humans and other animals and help control interspecies transmission of influenza viruses; and (c) many endogenous anti-viral proteins such as interferon (IFN) have been identified (21). However, they are normally activated many hours after infection. Unlike these proteins, commensal bacteria in the human nasal cavity may provide instant effects on prevention of influenza viral invasion.

RESULTS

Inhibition of influenza virus-induced erythrocyte hemagglutination by the culture supernatants of *S. epidermidis.*

The inhibition of influenza virus by the culture supernatants of *S. epidermidis* was examined via the hemagglutination activity inhibition assay (HIA) by adding guinea pig erythrocytes to the mixture of various dilutions of influenza virus [influenza A virus (H1N1) A/Denver/1/57] in the presence of culture supernatants of *S. epidermidis* [American Type Culture Collection (ATCC)1457 and ATCC12228)]. The culture supernatants of both non-pathogenic strains (ATCC1457 and ATCC12228) of *S. epidermidis* completely block erythrocyte hemagglutination (Fig. 1A, Lanes 1 and 2), revealing an anti-viral property of *S. epidermidis*. In contrast, the bacterial growth medium [tryptic soy broth (TSB) medium] alone exhibited no inhibitory activity on hemagglutination (Fig. 1A, Lane 3), indicating that secreted factors of *S. epidermidis* in the culture supernatants rather than nutrients in TSB exert the anti-viral effect. To examine if *S. epidermidis* has a broad-spectrum anti-viral effect, guinea pig erythrocytes were mixed with various subtypes of influenza virus in the presence of culture supernatants of *S. epidermidis* (ATCC1457 and ATCC12228) or TSB. The bacteria supernatants significantly abrogate erythrocyte hemagglutination caused by influenza A virus (H3N2) A/Aichi/2/68 (Fig. 1B) and influenza B virus B/Brigit (Fig. 1C), suggesting that bacterial supernatants have inhibitory activities against influenza A and B.

It has been known that hemagglutination of erythrocytes is a common property of *S. epidermidis* strains (22), which is related to adherence and biofilm formation and

may be essential for the pathogenesis of biomaterial-associated infections caused by *S. epidermidis*. Interruption of the icaADBC operon essential for polysaccharide intercellular adhesin (PIA) synthesis by Tn917 insertions led to a hemagglutination-negative phenotype. Furthermore, an immunoglobulin G (IgG) fraction of antiserum to PIA greatly reduced erythrocyte hemagglutination. In addition, purified PIA led to a dramatic decrease of hemagglutination titers; however, it did not mediate hemagglutination by itself. These results refer PIA as the hemagglutinin of *S. epidermidis* or at least as its major functional component. Recently, it has been reported that *S. epidermidis* can produce poly-N-acetyl glucosamine cell surface polysaccharides to initiate erythrocyte hemagglutination (23). To examine if *S. epidermidis* secreted factors themselves have hemagglutination activities, which may compete the ability of virus to induce hemagglutination, we incubated erythrocytes with the culture supernatants of *S. epidermidis* (ATCC1457; Fig. 1D, Lane 1 or ATCC12228; Fig. 1D, Lane 2) alone in the absence of influenza virus. As shown in Fig. 1D, no hemagglutination activity was observed, indicating that secreted factors of *S. epidermidis* themselves do not have hemagglutination activities. The result is in agreement with the fact that *S. epidermidis* (ATCC12228) is a PIA (*icaADBC* operon)-deficient strain 0 (24, 25).

The secreted factor of *S. epidermidis* reduces swine (H1N1) virus-induced hemagglutination and is a papain-sensitive protein.

To examine if *S. epidermidis* exhibits an anti-viral property against influenza A swine (H1N1) virus, guinea pig erythrocytes were mixed with two clinically isolated

influenza H1N1 viruses [influenza A non-swine virus (H1N1) San Diego/51/08 (Fig. 2A) and influenza A swine virus (H1N1) San Diego/01/09, a San Diego H1N1 swine origin influenza A (IAV) virus (S-OIV) (SD/H1N1-S-OIV) (Fig. 2B) in the presence of culture supernatants of *S. epidermidis* (ATCC12228) or TSB. The culture supernatants of *S. epidermidis* significantly block both non-swine and swine virus-induced hemagglutination. To determine if proteins in the secreted factors of *S. epidermidis* confer the inhibitory effect against virus-induced hemagglutination, culture supernatants were filtered with a 10-KDa cutoff column, or digested with papain prior to the conduction of HIA (Fig. 3). In consistence with Fig. 1A, culture supernatants of *S. epidermidis* (ATCC12228) considerably reduced the erythrocyte hemagglutination induced by influenza A virus (H1N1) A/Denver/1/57. The inhibitory activity was completely abolished once bacterial supernatants were filtered with a 10 KDa cutoff column and digested with papain. The result suggests that proteins are active components in culture supernatants that contribute to anti-viral activity of secreted bacterial factors.

Mass spectrometric identification of a giant fibronectin-binding protein (Embp) as an active protein in *S. epidermidis* that confers inhibition of virus-induced hemagglutination.

To determine which protein of *S. epidermidis* contributes to the anti-viral effect, the culture supernatant of *S. epidermidis* (ATCC12228) was separated by reverse phase high-performance liquid chromatography (HPLC) using a C18 column. Twelve major fractions (Fig. 4A) were obtained in 20 min run time. 100 µl of each fraction was

collected for HIA as described in Fig. 1. The proteins eluted of fraction 12 exhibited the best potency in the reduction of SD/H1N1-S-OIV-induced hemagglutination (Fig. 4B). No inhibition of virus-induced hemagglutination was found in other fractions (data not shown). Proteins in fraction 12 were digested with trypsin and then subjected to a nano liquid chromatography linear trap quadrupole mass spectrometry (Nano-LC-LTQ MS/MS) analysis. A protein (Embp; 1 MDa; accession number: Q8CP76) was identified. Two internal peptides (SINAYNKAIQSLETQITSAKDN and QQVAEIIAQANKLNNEMGTLKT) of Embp were sequenced. The internal peptide (SINAYNKAIQSLETQITSAKDN; 3359-3380 amino acid residues) of Embp is presented in Fig. 4C.

Embp is essential for the anti-viral activity of *S. epidermidis.*

An Embp-mutant *S. epidermidis* strain was used to determine if Embp is an essential protein in *S. epidermidis* for inhibition of influenza virus infection. To this end we used *S. epidermidis* strains 1585, 1585v, and mutant M135 (26). *S. epidermidis* 1585 is a wild type strain that produces no Embp after growth in TSB. In contrast, *S. epidermidis* 1585v is a variant derived from strain 1585 in which, by a chromosomal re-arragenent, a 460 KDa isoform of Embp is over-expressed even in TSB, while mutant M135 is isogenic mutant of 1585v in which expression of Embp is interrupted by insertion of transposon Tn917. The culture supernatants (25 μl) of *S. epidermidis* 1585, 1585v, and M135 were added guinea pig erythrocytes in the presence of SD-H1N1-S-OIV [32 hemaggutinting unit (HAU)] for HIA as described in Fig. 1. The supernatant of *S. epidermidis* 1585 with no Embp expression did not affect virus-

induced hemagglutination. The supernatant of *S. epidermidis* 1585v with Embp expression significantly blocked virus-induced hemagglutination. However, the blockage of hemagglutination did not occur when the supernatant of *S. epidermidis* M135 was used (data not shown). To confirm this observation in human nasal cells, the culture supernatants (100 µl) of *S. epidermidis* 1585, 1585v, and M135 were added into the culture of nasal RPMI-2650 (Fig. 5A) or lung (A549) (Fig. 5B) epithelial cells in the presence of SD-H1N1-S-OIV. Consistent with results from the HIA, the supernatant of *S. epidermidis* 1585v, but not 1585 or TSB medium alone, dramatically reduced the virus-induced cell death. However, the use of supernatants from *S. epidermidis* M135 impeded completely the bacterial capability of diminishing virus-induced cell death, suggesting that Embp is essential for the activity of *S. epidermidis* against viral infection.

Although HIA (Figs. 1-3) indicates that the culture supernatants of *S. epidermidis* exert anti-viral activity, it cannot be used to measure viral colonization and invasion at the early stage of infection. Thus, the infection of a recombinant influenza virus carrying a green fluorescent protein (GFP) reporter gene in the NS segment (NS1-GFP-virus) (27) in nasal RPMI-2650 epithelial cells was examined. To investigate if *S. epidermidis* can block the early entry of viruses, RPMI-2650 cells were infected with NS1-GFP-virus in the presence of culture supernatants (100 µl) of *S. epidermidis* 1585, 1585v, and M135. As shown in Figure 5C, NS1-GFP-viruses replicate in RPMI-2650 cells in the presence of TSB medium or culture supernatant of *S. epidermidis* 1585 or M135. The replication was dramatically impeded when cells were incubated

with the supernatant of *S. epidermidis* 1585v, suggesting that Embp plays a key role in limiting the early infection of influenza virus in nasal cells.

The interaction of *S. epidermidis* and influenza virus.

To determine if *S. epidermidis* can directly bind to influenza virus, *S. epidermidis* (ATCC12228) (10^6 CFU) was incubated with SD-H1N1-S-OIV (32 HAU) in 1 ml virus growth medium for 24 h. Transmission electron microscopy demonstrates virus attaches to the surface of *S. epidermidis* (Fig. 6B and C). To examine if Embp has the ability to bind SD-H1N1-S-OIV, a recombinant protein (rEmbp6599) containing a domain (amino acids 6599-7340) of Embp was coated on the enzyme-linked immunosorbent assay (ELISA) plates and reacted with SD-H1N1-S-OIV. The rEmbp6599 contains both seven Found In Various Architectures (FIVAR) (a fibronectin-binding domain) and eight protein G-related albumin-binding (GA) domains. As shown in Fig. 5E, SD-H1N1-S-OIV attached to rEmbp6599-coated ELISA plates in a dose-dependent manner. Our results have demonstrated that culture supernatants of *S. epidermidis* significantly suppress the hemagglutination induced various influenza viruses including SD-H1N1-S-OIV (Figs. 1 and 2). Here, we illustrated that both *S. epidermidis* bacterial particles and Embp can directly bind to SD-H1N1-S-OIV (Fig. 6). Embp without a LPXTG motif attaches to bacterial surface non-covalently. The non-covalent attachment allows Embp to be release/shed from bacterial surface (26). These results indicate that *S. epidermidis* may act as a natural guardian in human nasal cavity, filtering influenza viruses from nostrils by binding viruses to surface and released Embp.

Embp decreases the mortality rate of influenza virus-infected chick embryos.

The embryonated eggs were used as infection model of influenza virus to determine if Embp can affect the mortality rate of virus-infected chick embryo. The 11-day-old embryonated eggs were injected with 100 µl rEmbp6599 (0.6 µg) following by injection of 50 egg infective dose 50% ($50EID_{50}$) EID_{50} of influenza virus [A/Puerto Rico/8/34 (H1N1). Eggs injected with recombinant immunodominant surface antigen B (r-isaB) of *S. epidermidis* following by injection of influenza virus served as a control. Like Embp, isaB lacks an LPXTG motif and is both secreted and cell-surface associated (28). The protein is expressed in both *S. epidermidis* and *S. aureus*. *S. epidermidis* isaB shares approximately 33% amino acid similarity to *S. aureus* isaB. As shown in Figure 7, approximately 50% chick embryos injected with r-isaB and influenza virus died whereas only 20% embryos injected with rEmbp6599 and influenza virus did not survival one day post-infection. All chick embryos injected with r-isaB and influenza virus die on day 5 post-infection. Approximately 30% chick embryos injected with rEmbp6599 and influenza virus survived up to day 8 post-infection. The result reveals that injection of rEmbp6599 of *S. epidermidis* enhances the survival rate of influenza virus-infected chick embryos.

DISCUSSION

At least two possibilities may explain human susceptibility to influenza virus infection in the presence of commensal *S. epidermidis* in the nasal cavity. First, the amount of *S. epidermidis* and/or Embp in the nasal cavity may be too low to confer full

protection. Second, the pre-existing naturally occurring antibody (NAb) to Embp may have been produced in hosts, neutralizing the anti-viral activity of Embp. It has been reported that NAb (IgG (29) and IgA (30)) to *S. epidermidis* has been produced in humans. It is worth measuring the titer and neutralizing activity of NAb to Embp. *S. epidermidis* is the dominant commensal bacterium (approximately 10% of all bacterial in nasal cavity) found in the human nasal cavity (1). A recent publication indicates that *S. epidermidis* colonizes in the nasal cavity of 96% (58/60) of healthy people (12). Another study found that *S. epidermidis* has a 100% colonization rate in the nares of 156 healthy volunteers (31). The Embp (Q8CP76) of *S. epidemidis* (ATCC12228) shares approximately 32% identity with Embp (Q931R6) of *S. aureus* (Mu50). The anti-influenza virus activity of *S. aureus* Embp is undetermined.

The full-length Embp (1 MDa) harbors FIVAR and GA domains (26, 32). The rEmbp2588 (amino acids 2588-3187) solely containing FIVAR domains exhibited no biofilm-inducing activity. In contract, rEmbp6599 (amino acids 6599-7340) containing both FIVAR and GA domains induced biofilm formation (33). Previously, it has been shown that a 32-KDa recombinant protein Embp32 within the FIVAR/GA domains could prevent *S. epidermidis,* but not *S. aureus* to bind fibronectin (32). Our results demonstrated that the culture supernatant of *S. epidermidis* 1585v, which contains N-terminal half truncated Embp, significantly reduced viral infectivity (Fig. 4). Thus we envision that the C-terminal half, which includes FIVAR, GA and DUF1542 (amino acids 9445-9831), is responsible for blocking influenza virus infection. The rEmbp2588 has a capability to bind fibronectin without inducing biofilm formation (26). If the rEmbp2588 has the anti-influenza virus activity, the possibility of biofilm formation

induced by Embp will be avoided when Embp is used as an anti-viral agent. It has been shown that Embp binds to fibronectin type III 12-14 domains, and suggest that fibronectin domain III12 is a major binding site (26). The alpha5beta1 integrin primarily binds in the RGD-containing domain (Type III 7-10) of fibronectin (34). Thus, Embp and integrin bind different domains of fibronectin. Fibronectin is a glycoprotein and contains both α2-3 and α2-6-inked sialic acids. Previous findings demonstrated that fibronectin directly interacts with envelope glycoproteins of influenza virus (35, 36). The binding was abrogated when fibronectin was pre-treated with neuraminidase, suggesting that the sialic acids of fibronectin are responsible for the affinity.

Although it is unclear how Embp binds to viruses, one possibility is mediated by fibronectin. Embp does not possess a LPXTG motif (26), suggesting a non-covalent attachment to the cell surface, which also explains why we can identify this protein in bacteria-free supernatants. In our laboratory, influenza viruses are isolated from Madin-Darby canine kidney (MDCK) cells and stored in the supernatants of cell culture. The cell supernatants contain cell medium and fetal bovine serum which is a source of exogenous fibronectin. As shown in Fig. 4, fibronectin in the viral stocks may act as a bridge, connecting *S. epidermidis* Embp together with viruses. However, cell medium containing exogenous fibronectin did not have capability to inhibit the virus-induced hemagglutination (Figs. 1-3) and viral colonization (Fig. 5), suggesting that fibronectin alone is not sufficient for inhibition of the infectivity of influenza virus. Our results have demonstrated that the anti-influenza nature of *S. epidermidis* was markedly restrained when Embp was mutated, verifying that Embp is indispensable for *S. epidermidis* against viral infection (Fig. 5). Our findings have also illustrated that

both *S. epidermidis* bacterial particles and Embp can directly bind to influenza viruses (Fig. 6). The non-covalent attachment allows Embp to be release/shed from bacterial surface. Based on these results, we suggest that *S. epidermidis* in host nasal cavity acts as a natural particle, filtering influenza viruses from host nostrils by binding viruses to Embp located on bacterial surface and released into extracellular milieu. Thus, *S. epidermidis* in the nasal microbiome may function as a front line defender against early steps of influenza virus infection.

Although it is undetermined whether Embp can bind influenza virus in the absence of fibronectin, the Embp domains responsible for viral binding (neutralization) and infectivity reduction (viral internalization) may be different. The viral neutralization of rEmbp6599 alone may be insufficient to confer complete protective immunity *in ovo* (Fig. 7). Thus, full protection may require a full-length or larger recombinant Embp that can both bind virus and reduce infectivity. It has been known that fibronectin is present in the chick embryo (37). However, chicken and mammal fibronectins have difference in glycosylation (38). If fibronectin contributes extensively to the anti-influenza virus activity of Embp, the distinction of fibronectins in chickens and humans may provide an explanation for partial protection of Embp-injected chick embryos from virus infection. Augmentation therapy using purified α1-antitrypsin (A1AT) such as human plasma-pooled Prolastin® has been used clinically for treatment of chronic obstructive pulmonary disease (COPD) (39). The use of recombinant Embp as a preventive or therapeutic anti-viral agent may have its own set of challenges because *1)* Embp may be immunogenic to induce antibody production and *2)* Embp proteins may not remain stable over long periods of time in the nasal passage. Enhancing the stability or

decreasing the immunogenicity of Embp by PEGylation (40, 41) may be needed before Embp is used as augmentation therapy for limiting influenza virus infection *in vivo*.

Conclusions.

Here we demonstrate that Embp of *S. epidermidis*, a nasal commensal bacterium, exhibits the anti-influenza virus activity. Unlike antibiotics, products from commensal bacteria are indigenous molecules in humans, which may have a lower risk of causing serious complications or generating virus resistance. Significance of this study includes *1)* highlighting the concept that inappropriate use of antibiotics may eliminate human commensals, making it more difficult to fight influenza; *2)* validating the function of human commensals as endogenous viral inhibitors opens a new area of research on the nasal microbiome (1, 42) against influenza virus infection. Anti-viral agents targeting an initial site (nasal cavity) of infection will prevent the systemic spread of viral infections; *3)* the use of Embp as augmentation therapy may complement the inability of current anti-viral drugs to fight unknown virus strains; and *4) S. epidermidis* bacteria are also commensals in nasal cavities of birds (5-7, 18, 19) and pigs (19, 20). The bacteria and/or their derived products may be used as interfering agents (5-7) in animal farms (e.g. chicken fields) using aerosol sprays in large-scale operations. The operation may efficiently control the interspecies transmission of influenza virus from animals to humans.

MATERIALS AND METHODS

Ethics statement.

Experiments of using embryonated eggs were performed at University of California, San Diego (UCSD). The UCSD ethics committee specifically approved this study under an approved Institutional Animal Care and Use Committee (IACUC) protocol (no. S10058).

Virus growth.

Two clinically isolated influenza H1N1 viruses (SD/H1N1-S-OIV and influenza A non-swine virus (H1N1) San Diego/51/08), NS1-GFP-virus and various subtypes of influenza virus obtained from ATCC were grown in virus growth medium containing Dulbecco's modified Eagle's medium (DMEM) with glutamine, 0.2% bovine serum albumin (BSA), 25 mM Hepes and 2 μg/ml TPCK-trypsin (Sigma Chemical Co., St. Louis, Mo.) and propagated in the monolayers of MDCK cells (43).

HIA.

HIA was performed in v-bottom 96-well microtiter plates (Costar, Cambridge, MA) at room temperature using 0.5% guinea pig erythrocytes (Lonza, Walkersville, Inc. MD). Hemagglutination titers indicated by the blue lines in Figs. 1-4 were determined by titration of virus samples in PBS followed by addition of an equivalent volume of guinea pig erythrocytes. The 50 μl of 0.5% guinea pig erythrocytes in phosphate buffered saline (PBS) were mixed with or without 25 μl of each dilution of influenza virus or 25 μl culture supernatants (10 μg/ml) of *S. epidermidis* as indicated in figure legends at room temperature for 40 min. Erythrocytes and virus mixed with bacterial

growth medium (TSB) served as a control. In one experiment, the culture supernatants of *S. epidermidis* were filtered with a MacroSep 10 KDa OMEGA cutoff column (Pall Gelman, Dreieich, Germany) or digested with papain (10 mg/ml) for 4 h before adding into the mixture of erythrocytes and virus. The data presented are representative of three separate experiments with similar results.

Protein identification via HPLC in conjunction with NanoLC-LTQ MS/MS analysis.

Proteins in the culture supernatants of *S. epidermidis* (ATCC12228) were separated by reverse phase HPLC using a LUNA C18 5 µm column, 250 x 4.6 mm (Phenomenex, Torrance, CA). A gradient mobile phase consists of a buffer A [(HPLC grade water plus 0.1% trifluoroacetic acid (TFA)] and a buffer B (acetonitrile plus 0.1% TFA). Flow rate was set on 1.0 ml/min and proteins were detected at 230 nm. Twelve fractions were collected after HPLC separation. The anti-viral activity of eluted proteins (100 µl) in each fraction was tested by HIA using SD/H1N1-S-OIV. The eluted proteins in fraction 12 with the most potent inhibition of virus-induced hemagglutination were digested with trypsin and subjected to Nano-LC-LTQ MS/MS (Thermo Scientific, San Jose, CA) (44). The automated Nano-LC-LTQ MS/MS setup consisted of an Eksigent Nano 2D LC system, a switch valve, a C18 trap column (Agilent, Santa Clara, CA), and a capillary reversed phased column (10 cm in length, 75 mm internal diameter) packed with 5 mm, C18 AQUASIL resin with an integral spray tip (Picofrit, 15 mm tip, New Objective, Woburn, MA). A reversed phase LC directly coupled to a LTQ ion trap mass spectrometer (Thermo Scientific, San Jose,

CA) was run using a linear gradient elution from buffer A (H_2O plus 0.1% formic acid) to 50% buffer A plus 50% buffer B (acetonitrile plus 0.1% formic acid) in 100 min. The instruments were operated in the data dependent mode. Data on the four strongest ions above an intensity of 26105 were collected with dynamic exclusion enabled and the collision energy set at 35%. Large-scale MS/MS spectra were extracted using default value by BioworksH 3.2. Charge state deconvolution and deisotoping were not performed. All MS/MS spectra were analyzed using in-house SorcererTM 2 system with SEQUEST (v.27, rev. 11) as the search program for protein identification. SEQUEST was set up to search the target-decoy ipi.MOUSE.v3.14 database containing protein sequences in both forward and reverse orientations (68627 entries in each orientation) using trypsin as the digestion enzyme with the allowance of up to five missed cleavages. The false positive rates were roughly determined by doubling the ratio between the number of decoy hits and the total number of hits. SEQUEST was searched with a fragment ion mass tolerance of 0.5 Da and a parent ion tolerance of 1.0 Da.

Cell viability and virus entry.

Human nasal epithelial cells (RPMI-2650; ATCC CCL-30) were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) at 37°C and 5%CO_2. Human lung epithelial cells (A549; ATCC CCL-185) were grown in Roswell Park Memorial Institute (RPMI) media containing 10% fetal bovine serum (FBS) at 37°C and 5%CO_2. Cells (10^7) were incubated with the culture supernatants (100 µl) of wild-type or M135 *S. epidermidis* in the presence of a 1/100 dilution of

SD/H1N1-S-OIV (32 HAU) or TSB medium overnight. Cell viability was quantified by an acid phosphatase (ACP) assay (45). Virus entry was determined by incubation of RPMI-2650 cells (10^7 cells) with NS1-GFP-virus (10^7 PFU) in the presence of TSB medium or culture supernatant of *S. epidermidis* 1585, 1585v or M135 (26) overnight in virus growth medium containing 2μg/ml TPCK-trypsin for 24 h at 37°C and 5%CO_2. The fluorescence of GFP within cells stained with a blue-fluorescent Hoechst 33342 nuclear dye was viewed with a Leica TCS SP2 confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL).

Expression of r-Embp and r-isaB.

The construction of *Escherichia coli* (*E. coli*) BL21AI over-expressing Embp6599 (amino acids 6599-7340) by using a pDEST17 expression vector (Invitrogen, Karlsruhe, Germany) was previously described (26). The expression of Embp6599 was induced by 0.2% L-arabinose for 4 h. The r-Embp6599 was affinity purified using a HiTrap chelating HP column (GE Healthcare Biosciences, Pittsburgh, PA). Purified r-Embp6599 was detected by a 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie Blue (Figure S1A). A vector encoding isaB (gi|57636443) was constructed by inserting PCR products of isaB into the p*Ecoli*-Nterm 6xHN vector (Clontech Laboratories, Inc., Mountain View, CA) at the SalI and HindIII restriction sites. Specific primers including the sense (5'-AATAGTCGACATGAAAAGGTTTGCAAAAGCATTTG-3') and anti-sense primers (5'-CAGAATTCTTATGACAATGTAGCACTTGTGACATACC-3') were designed to clone isaB from the *S. epidermidis* (ATCC12228). The expression of isaB from the *E. coli*

vectors was detected in the absence or presence of 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Fig. S1B). The r-isaB was obtained via In-Fusion Ready TALON Express Bacterial Expression and Purification kit (Clontech Laboratories, Inc., Mountain View, CA) and purified via a standard nickel resin purification protocol (Qiagen, Valencia, CA).

Transmission electron microscopy.

S. epidermidis (ATCC12228) (10^6 CFU) was incubated with SD-H1N1-S-OIV (32 HAU) in 1 ml virus growth medium for 24 h. After centrifugation of the mixture of bacteria and viruses at 5,000 g for 10 min, the pellets were fixed in Karnovsky's fixative (4% paraformaldehyde, 2.5% glutaraldehyde, 5 mM CaCl2 in 0.1M Na cacodylate buffer, pH 7.4) overnight at 4°C, followed by 1%OsO4 in 0.1M Na cacodylate buffer, pH 7.4, *en bloc* staining with 4% uranyl acetate in 50% ethanol, and subsequent dehydration using a graded series of ethanol solutions followed by propylene oxide and infiltration with epoxy resin (Scipoxy 812, Energy Beam Sciences, Agawam, MA). After polymerization at 65°C overnight, thin sections were cut and stained with uranyl acetate (4% uranyl acetate in 50% ethanol) followed by bismuth subnitrate. Sections were observed at an accelerating voltage of 60 kV using a Zeiss EM10C electron microscope (Carl Zeiss, Thornwood, NY).

Enzyme-linked immunosorbent assay (ELISA).

In order to investigate rEmbp6599 binding to influenza virus, 96-well ELISA polystyrene plates (Corning Life Sciences, Lowell, MA) were used. The rEmbp6599 or

BSA (0 - 6 μg/ml) was coated on ELISA plates and incubated with a 1/200 dilution of SD/H1N1-S-OIV (32 HAU) for 2 h followed by addition of a polyclonal antibody (IgG; 1:1000) to SD-H1N1-S-OIV for 1 h. The polyclonal antibody was generated in rabbits by immunization with the human influenza A (H1N1) 2009 monovalent vaccine (CSL Biotherapies Inc., King of Prussia, PA). Secondary horseradish peroxidase (HRP)-labeled donkey anti-rabbit antibodies were then added. The 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (BioRad Labs, Hercules, CA) was then added and the reaction was stopped using 1 N sulfuric acid (H_2SO_4). The optical density (OD) was measured on an ELISA plate reader at 450 nm wavelength.

Infections of influenza virus in the embryonated eggs.

The 10-day-old specific pathogen-free (SPF) chicken embryonated eggs were obtained from a local producer (McIntyre Poultry & Fertile Eggs, Lakeside, CA). Eggs were incubated at 37°C and 50 to 60% relative humidity in a specialized incubator Incubator (RX1, Lyon Technologies, Chula Vista, CA) for one day before infection of influenza virus. After the shells of 11-day-old embryonated eggs were wiped with a povidone-iodine prep pad (Medline Industries, Inc., Mundelein, IL), they were perforated at the blunt end and on the side using a hand drill. After perforation of the shell membrane, eggs were injected with 100 μl rEmbp6599 or r-isaB (0.6 μg) following by injection of $50EID_{50}$ of influenza virus [A/Puerto Rico/8/34 (H1N1) (ATCC)] (100 μl) via the allantoic route using a sterile 1-ml syringe. The holes were then sealed using an ethylene vinyl acetate glue gun. Survival was monitored for up to 8 days by daily candling. The EID50 was calculated according to the method

previously published (46). 31 eggs per group were used for three independent experiments. Survival data were plotted as Kaplan-Meyer curves and were analyzed statistically by a log rank test using Graph Pad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

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FIG 1 The inhibitory effect of culture supernatants of *S. epidermidis* on influenza virus-induced erythrocyte hemagglutination. (A, B, C) HIA [45] was performed by mixing 50 µl of 0.5% guinea pig erythrocytes in PBS with 25 µl of each dilution (as indicated) virus and 25 µl culture supernatants (10 µg/ml) of *S. epidermidis* [ATCC1457; Lane 1 and ATCC12228; Lane 2 in Panels A, B, and C) at room temperature for 40 min. Erythrocytes and virus mixed with bacterial growth medium (TSB) served as a control (Lane 3 in Panels A, B and C). Influenza A virus (H1N1) A/Denver/1/57 (A, ATCC; H1N1; 4 HAU), influenza A virus (H3N2) A/Aichi/2/68 (B, ATCC, H3N2; 8 HAU) and influenza B virus B/Brigit (C, ATCC, FluB; 16 HUA) were used for this assay. (D) The mixture of erythrocytes (50 µl) and culture supernatants (50 µl; 10 µg/ml) of *S. epidermidis* (ATCC1457 or ATCC12228) alone in the absence of virus (No virus) was prepared to determine if supernatants themselves have hemagglutination activities. (E) The 50 µl erythrocytes mixed with 50 µl PBS served as a positive reaction of hemagglutination. The HAU of each virus, the amount of virus needed for agglutinating erythrocytes, was determined by mixing 50 µl of each dilution of virus solution with 50 µl of 0.5% guinea pig erythrocytes suspended in PBS. The mixture was incubated at room temperature for 40 min before observation of erythrocyte aggregation.

FIG 2 The culture supernatants of *S. epidermidis* inhibit the erythrocyte hemagglutination induced by both non-swine and swine H1N1 viruses. HIA was performed as described in Fig 1. The 50 µl of 0.5% guinea pig erythrocytes (RBC) in PBS were mixed with 25 µl of each dilution (as indicated) of virus in the presence of 25 µl culture supernatants (Bacterial Sup.; 10 µg/ml) of *S. epidermidis* (ATCC12228) or bacterial growth medium (Growth Med. TSB) at room temperature for 40 min. (A) Influenza A non-swine virus (H1N1) San Diego/51/08 (32 HAU) and (B) SD/H1N1-S-OIV (32 HAU) were used for this assay.

FIG 3 The active component that contributes to the anti-viral activity of culture supernatants of *S. epidermidis* is a papain-sensitive protein with a molecular weight lower than 10 KDa. (A) HIA was performed as described in Fig. 1. The 50 µl of 0.5% guinea pig erythrocytes (RBC) in PBS were mixed with 25 µl of each dilution (2 to 128 dilutions) of virus in the presence of 25 µl culture supernatants (Bacterial Sup.; 10 µg/ml) of *S. epidermidis* (ATCC12228) or bacterial growth medium (Growth Med. TSB) at room temperature for 40 min. Influenza A virus (H1N1) A/Denver/1/57 (ATCC; H1N1; 32HAU) was used for this assay. (B) Before adding into the mixture of erythrocytes and virus, the culture supernatants were filtered with a MacroSep 10 KDa OMEGA cutoff column (Filtered) or digested with papain (10 mg/ml) for 4 h (Papain).

FIG 4 HPLC separation in combination with mass spectrometry for Embp identification. Proteins in the culture supernatants of *S. epidermidis* (ATCC12228) were separated by reverse phase HPLC using a LUNA C18 5 μm column. (A) Twelve fractions were collected after HPLC separation. The anti-viral activity of eluted proteins (100 μl) in each fraction was tested by HIA as described in Figure 2 using SD/H1N1-S-OIV. (B) Erythrocyte hemagglutination (Lane 1) was inhibited by SD/H1N1-S-OIV. (4 HAU) (Lane 2). The eluted proteins in fraction 12 (Lane 5), not fractions 7, 8 (Lanes 3, 4) and other fractions (data not shown), exerted a potent inhibition of SD/H1N1-S-OIV-induced hemagglutination. (C) Tryptic digests of proteins in fraction 12 of HLPC separation were subjected to Nano-LC-LTQ MS/MS. A sequenced peptide (SINAYNKAIQSLETQITSAKDN) is presented and assigned as an internal peptide of Embp (Q8CP76). The m/z value of each “y” and “b” ion in collision-induced dissociation (CID) spectra was indicated.

FIG 5 Mutation of Embp results in a loss of antiviral activity of *S. epidermidis* against influenza virus. (A, B) To test the essential role of Embp in viral infectivity, the culture supernatants (100 µl) of wild-type or M135 (Embp-mutant) *S. epidermidis* plus a 1/100 dilution of SD/H1N1-S-OIV (32 HAU) was added into nasal (RPMI-2650) (A) or lung (A549) (B) epithelial cells (10^7 cells) overnight. The incubation of cells with TSB medium plus virus serves as a control (C). Cell viability was determined by an ACP assay (45). The wild-type [1585 (Embp negative), 1585v (Embp positive)] and M135 (Embp-mutant) were used for this experiment. ***P < 0.001 (1585 vs. 1585v; n=3) by Student's *t*-test. (C) The RPMI-2650 cells (10^7 cells) were incubated with NS1-GFP-virus (10^7 PFU) in the presence of TSB medium (C) or culture supernatant of *S. epidermidis* 1585, 1585v or M135 overnight in virus growth medium (44). The replication of NS1-GFP-viruses (arrows) and fluorescence were viewed with a Leica TCS SP2 confocal microscope. Bar = 25 µm.

FIG 6 Influenza virus binds to *S. epidermidis* and rEmbp6599. *S. epidermidis* (ATCC1228; 10^6 CFU) was incubated without (A) or with (B, C) SD-H1N1-S-OIV (32 HAU) in 1 ml virus growth medium for 24 h. The transmission electron microscopy reveals that SD-H1N1-S-OIV (arrows) adheres to the surface of *S. epidermidis* [low (B) and high (C) magnification)]. ELISA was used to determine the ability of rEmbp6599 to bind to SD-H1N1-S-OIV (D). rEmbp6599 (a solid line) or BSA (a dashed line) (0 - 6 μg/ml) was coated on ELISA plates and incubated with a 1/200 dilution of SD/H1N1-S-OIV (32 HAU) for 2 h followed by addition of a purified antibody (IgG; 1:1000) to SD-H1N1-S-OIV for 1 h. The binding of rEmbp6599 to virus was detected by reading OD 450 nm. The data represents mean ± standard error (SE) (n=3, P< 0.05**; P< 0.001***; by Student's *t*-test, vs. BSA control). Bars (A, B) = 500 nm; Bar (C) = 150 nm.

FIG 7 Embp increases the survival rate of embryonated eggs infected with influenza virus**.** Eggs were injected with 0.6 μg/100 μl rEmbp6599 (a solid line) or r-isaB (a dashed line) following by infecting with 50EID_{50} of influenza virus [A/Puerto Rico/8/34 (H1N1) (ATCC)] on developmental day 11. Survival was monitored daily for 8 days, and the results are expressed as Kaplan-Meyer curves. The log rank test indicated that there were significant differences between eggs injected with viruses plus rEmbp6599 or r-isaB ($P < 0.0002$).

FIG 1

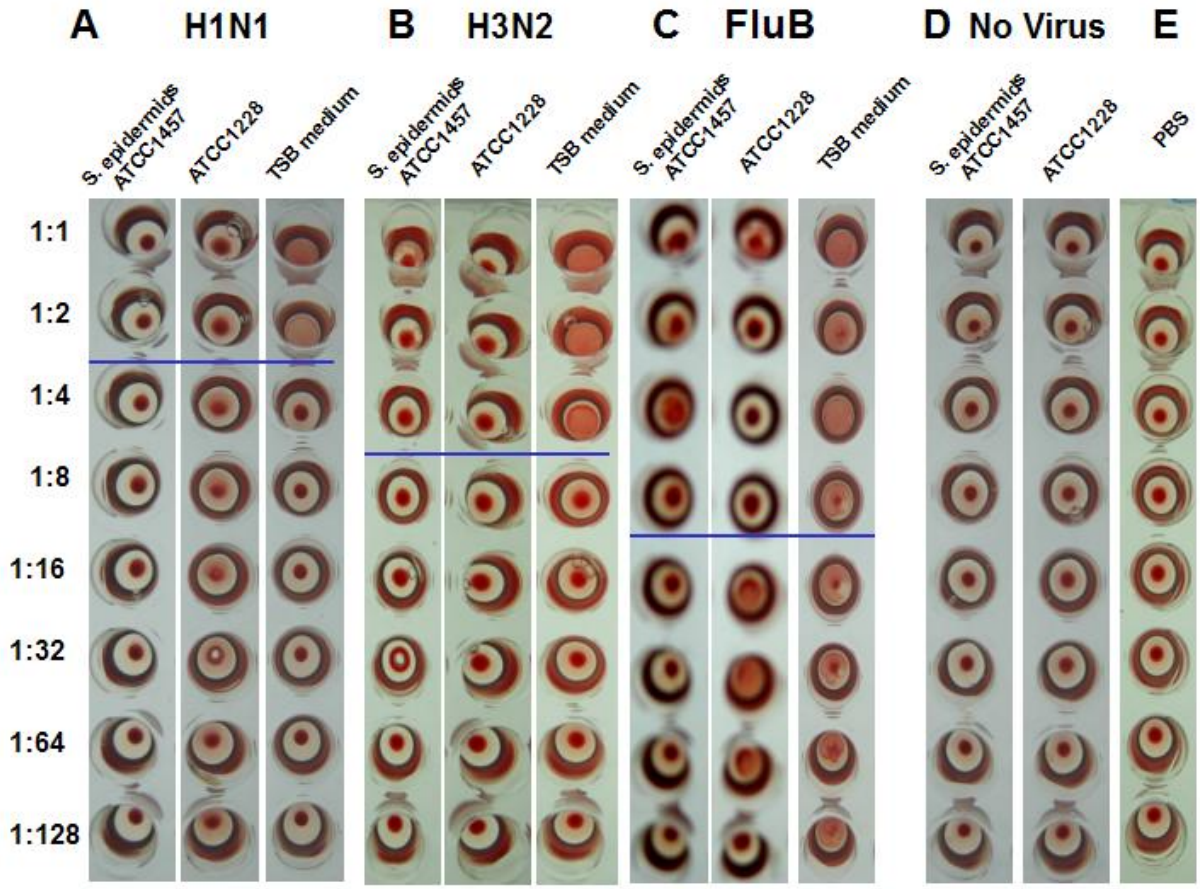

A
H1N1
B
H3N2
C
FluB
D
No Virus
E
S. epidermidis ATCC1457
ATCC1228
TSB medium
S. epidermidis ATCC1457
ATCC1228
TSB medium
S. epidermidis ATCC1457
ATCC1228
TSB medium
S. epidermidis ATCC1457
ATCC1228
PBS
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128

FIG 2

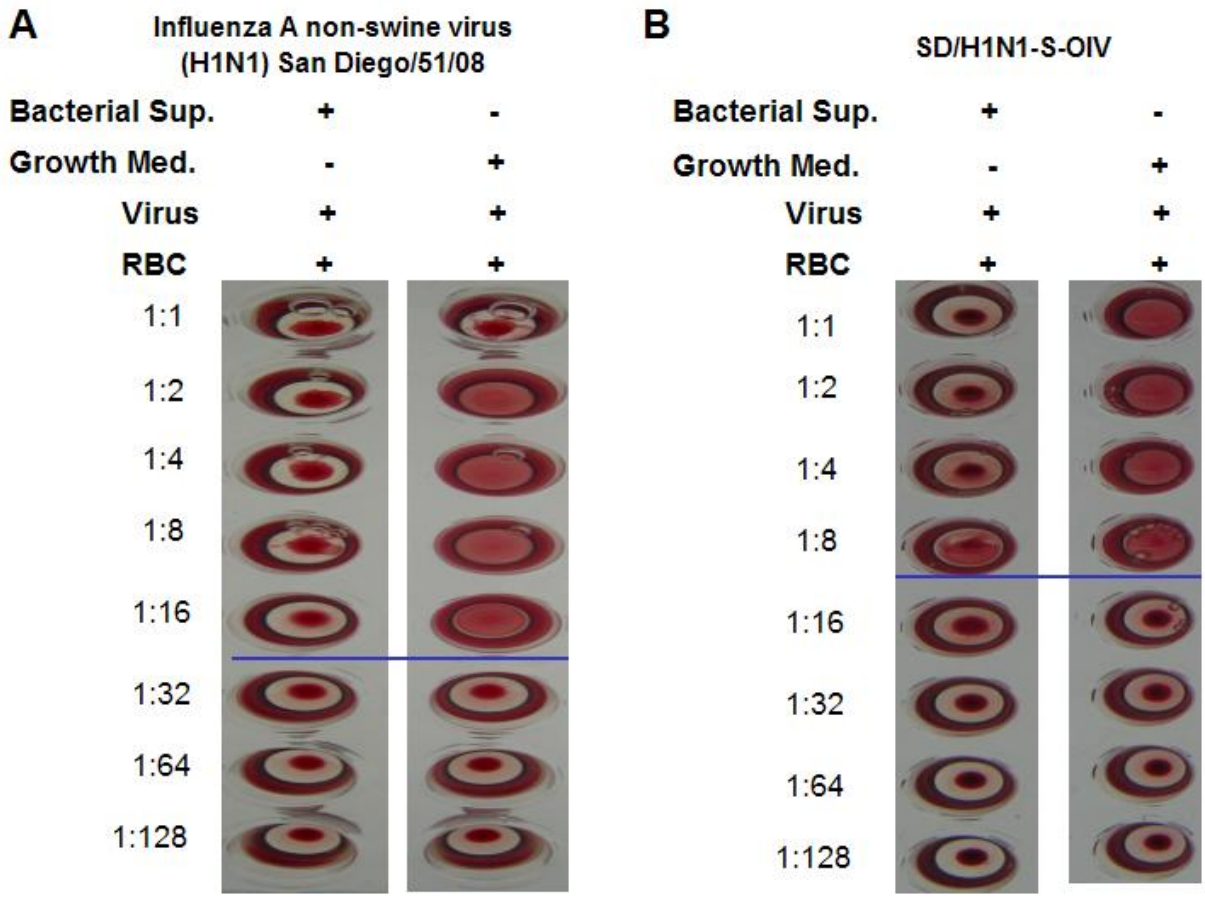

A
Influenza A non-swine virus (H1N1) San Diego/51/08
Bacterial Sup. + -
Growth Med. - +
Virus + +
RBC + +
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
B
SD/H1N1-S-OIV
Bacterial Sup. + -
Growth Med. - +
Virus + +
RBC + +
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128

FIG 3

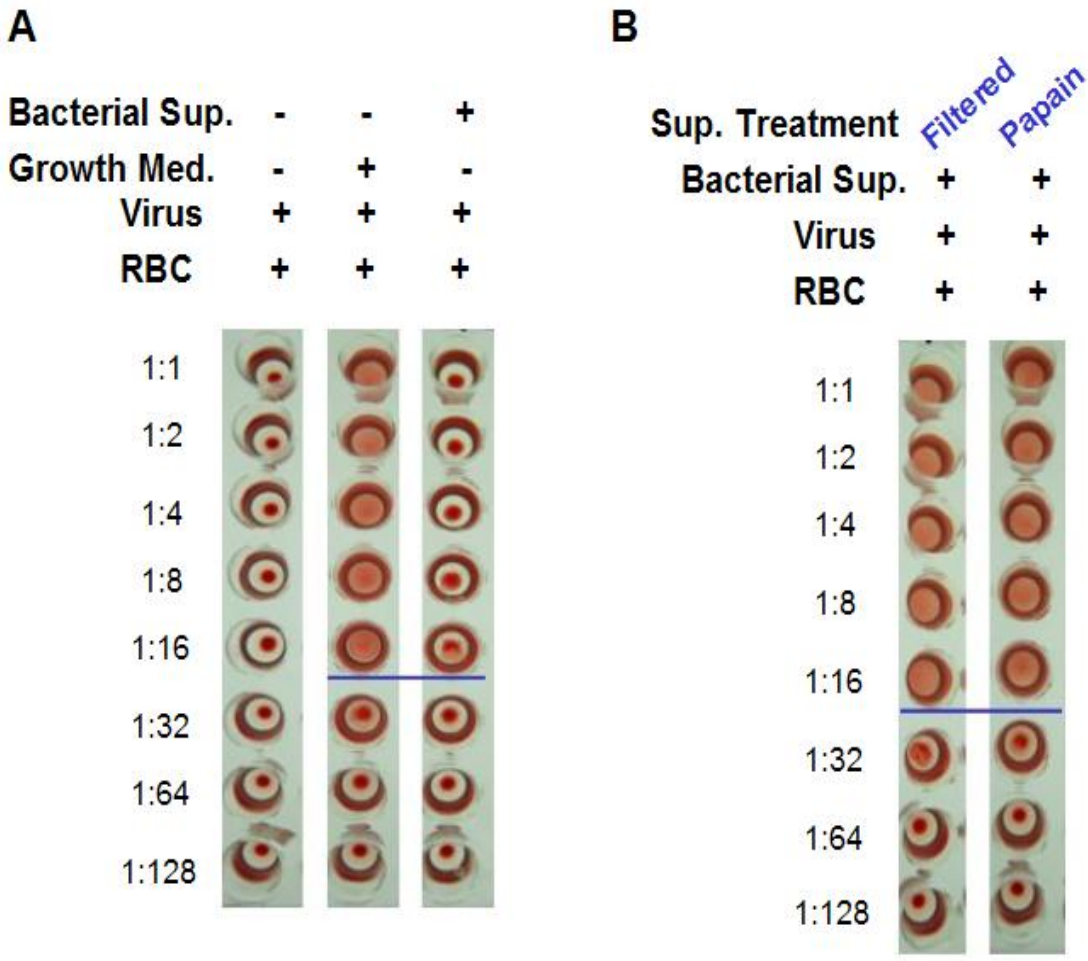

A
Bacterial Sup. - - +
Growth Med. - + -
Virus + + +
RBC + + +
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
B
Sup. Treatment Filtered Papain
Bacterial Sup. + +
Virus + +
RBC + +
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128

FIG 4

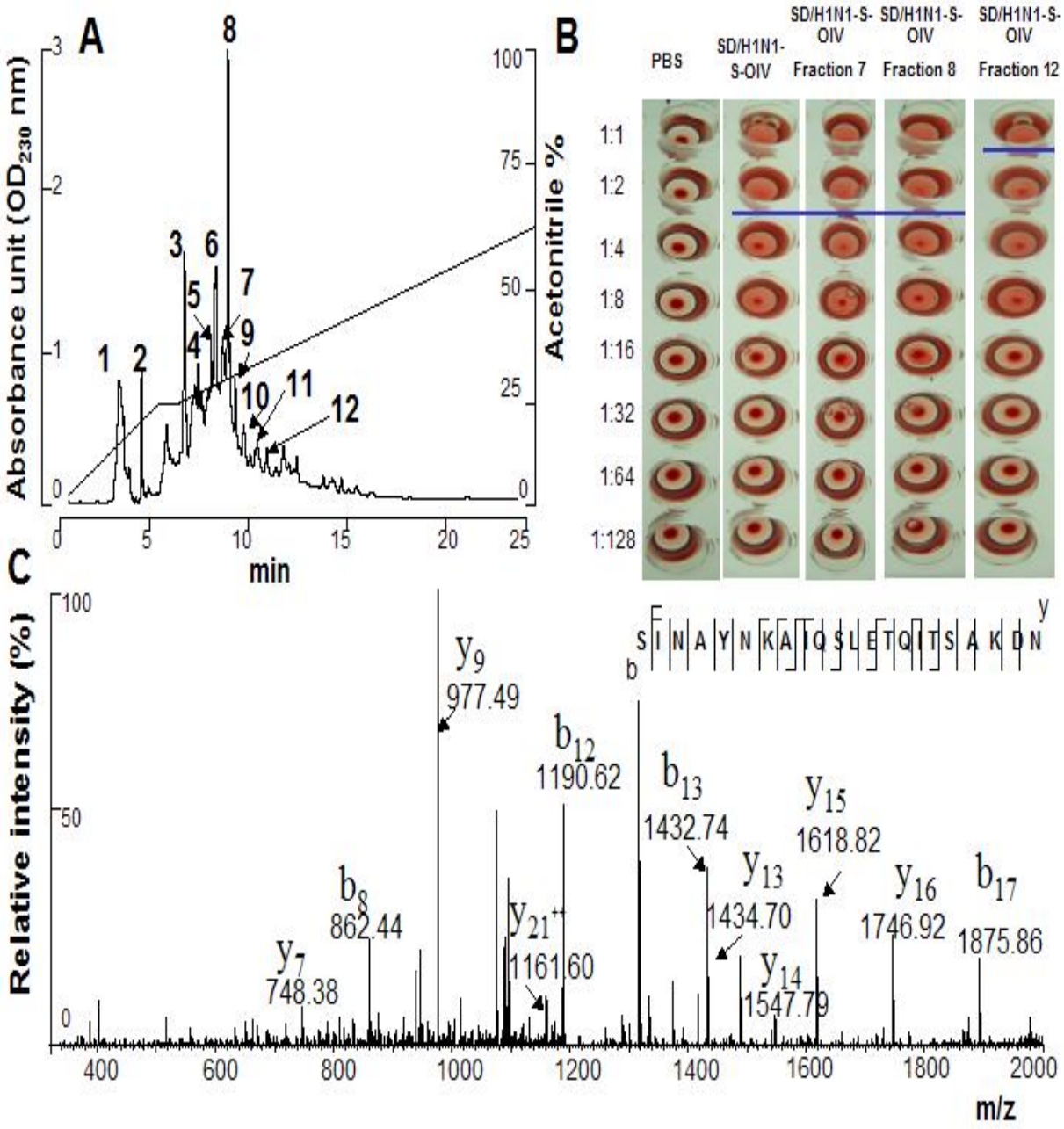

A
Absorbance unit (OD230 nm)
Acetonitrile %
min
B
PBS
SD/H1N1-S-OIV
SD/H1N1-S-OIV Fraction 7
SD/H1N1-S-OIV Fraction 8
SD/H1N1-S-OIV Fraction 12
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
C
Relative intensity (%)
m/z
y9 977.49
b12 1190.62
b13 1432.74
y15 1618.82
y13 1434.70
y16 1746.92
b17 1875.86
b8 862.44
y21++ 1161.60
y7 748.38
y14 1547.79

FIG 5

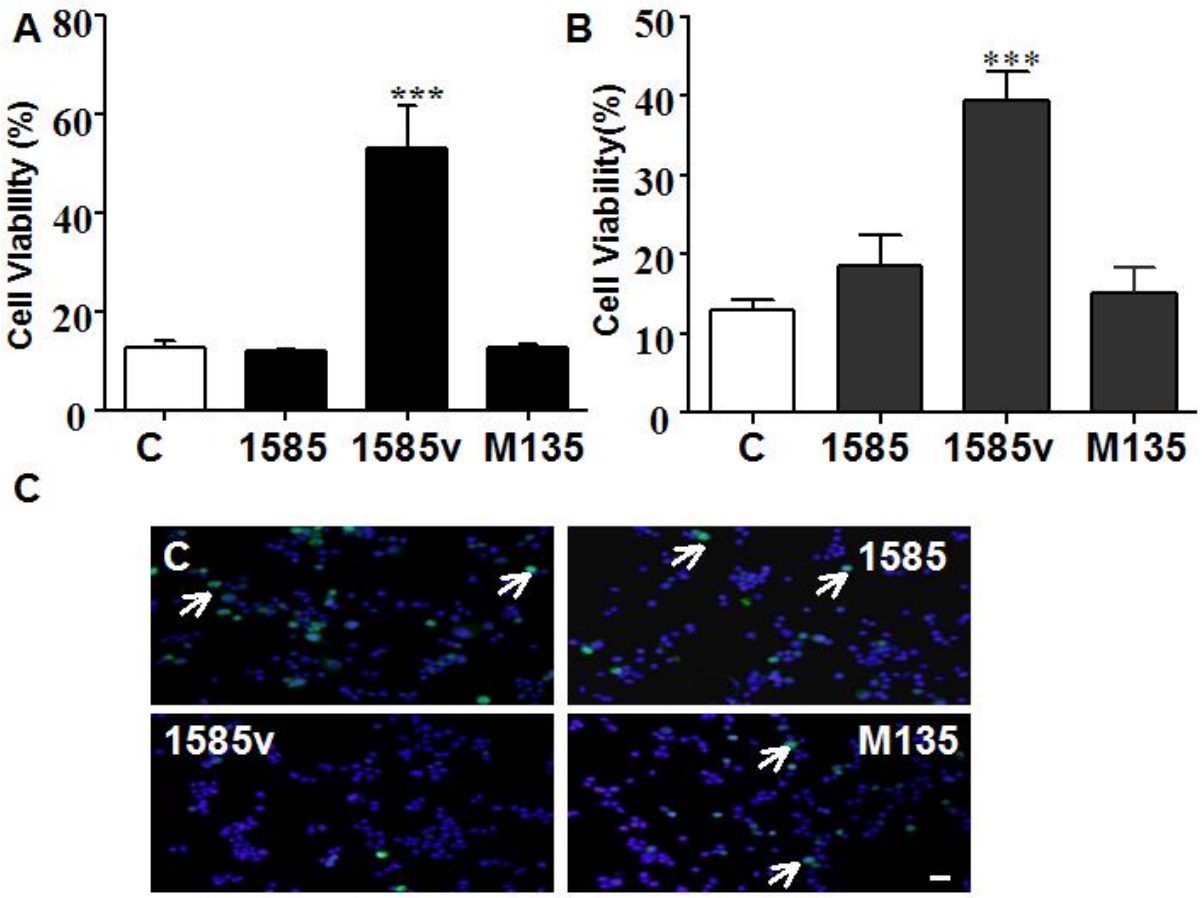

A
Cell Viability (%)
80
60
40
20
0
***
C
1585
1585v
M135
B
Cell Viability(%)
50
40
30
20
10
0
***
C
1585
1585v
M135
C
C
1585
1585v
M135

FIG 6

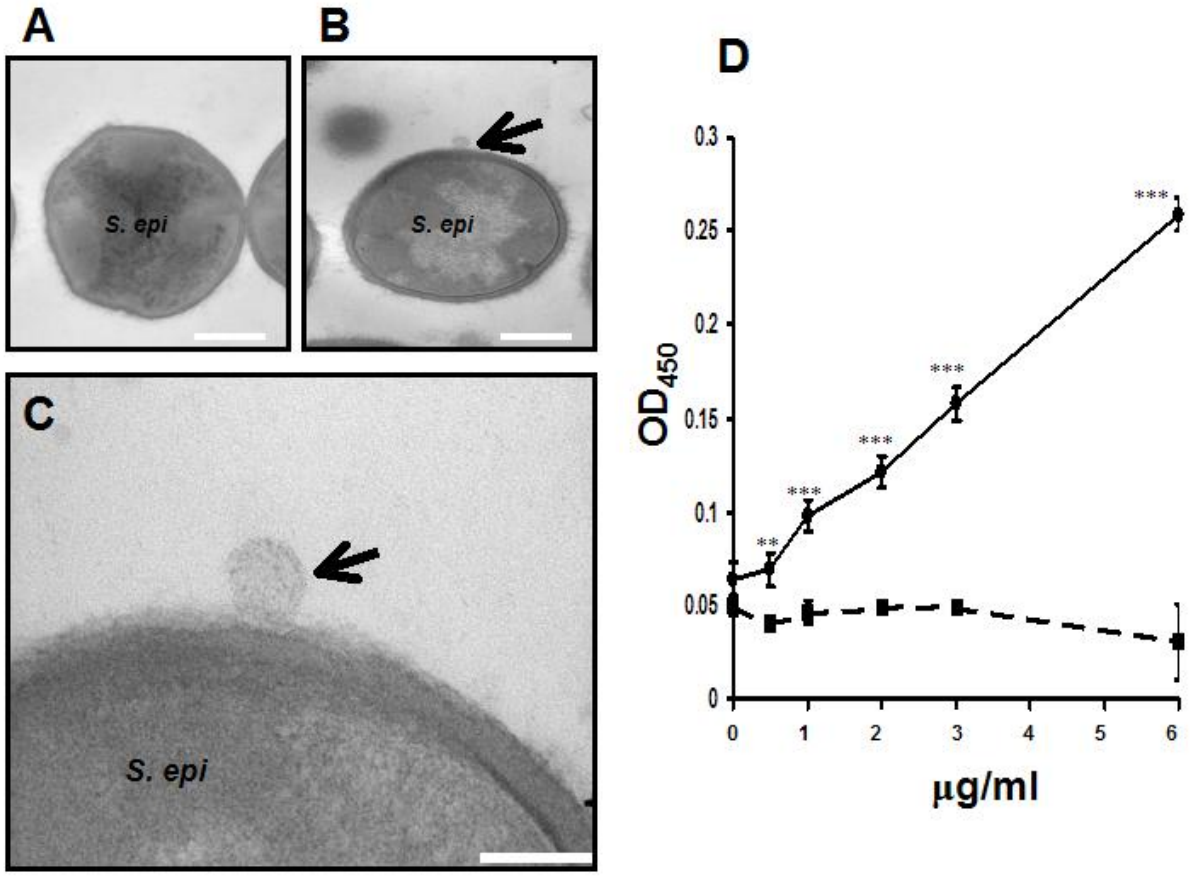
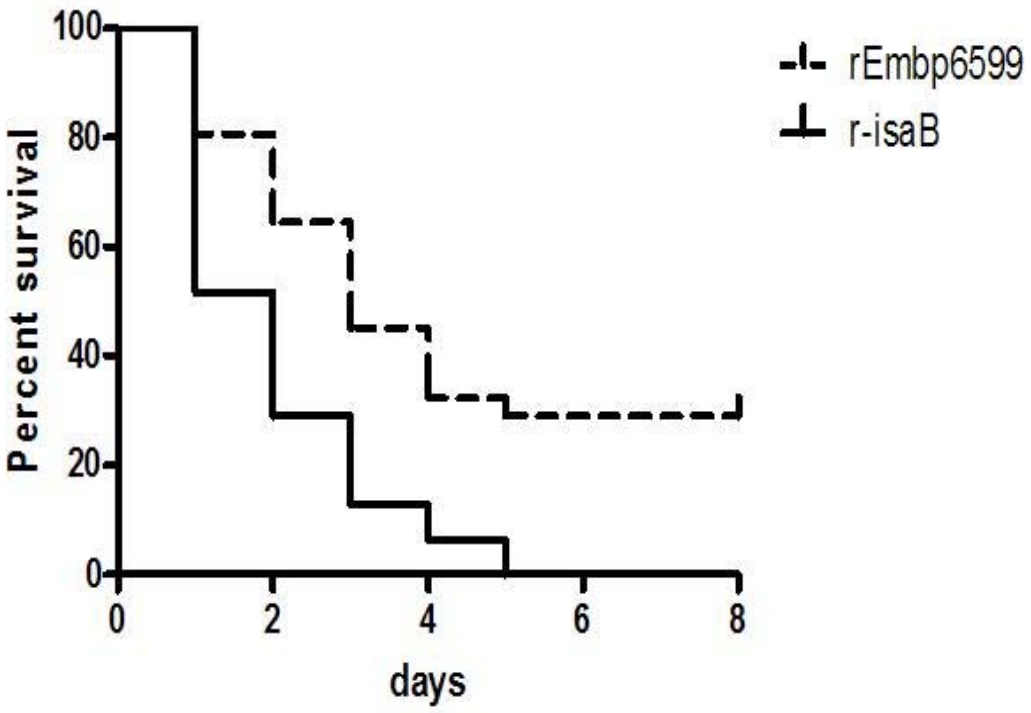


FIG 7


100
80
60
40
20
0
Percent survival
0
2
4
6
8
days
rEmbp6599
r-isaB

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