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RESEARCH ARTICLE OPEN ACCESS

Invasion of Intestinal Cells by *Staphylococcus aureus* is Mediated by Pyruvate Formate Lyase (Pfl) Protein

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Abstract

***Staphylococcus aureus* is a known enterotoxin-producing foodborne pathogen; however, the invasion mechanism of the bacterium into intestinal cells remains unclear. The aim of this study was to determine whether *S. aureus* can invade Caco-2 cells, and to elucidate the gene responsible for this invasion. Caco-2 cells were infected with *S. aureus* strains NCCP10862, KACC13236, KACC10768 and KACC11596, and their invasion efficiencies were evaluated. Proteins found in the invasive and noninvasive *S. aureus* strains were labelled with isobaric tags for relative and absolute quantification (iTRAQ), and the gene encoding the protein responsible for *S. aureus* invasion was deleted using a temperature-sensitive plasmid, pIMAY. The Caco-2 cell invasion efficiencies of the wild type and mutant *S. aureus* were then compared. Among the *S. aureus* strains, only NCCP10862 and KACC10768 were able to invade Caco-2 cells, and these strains had a higher level of pyruvate formate lyase (Pfl) protein expression than that of the noninvasive strains. Therefore, a *pflB*-deletion mutant of KACC10768 was prepared, which revealed a 60% decrease in invasion efficiency when compared to the wild type. These results indicate that certain *S. aureus* strains can invade intestinal cells, and the protein encoded by the *pfl* gene is involved in this invasion.**

Keywords: *Staphylococcus aureus*, invasion, pyruvate formate lyase, foodborne pathogen.

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INTRODUCTION

Staphylococcus aureus, a gram-positive and facultative anaerobic bacterium, is a foodborne pathogen that is a global health threat[1]. *S. aureus* is generally found on human skin, and when food is exposed to *S. aureus*-laden skin, it can result in food contamination and disease. *S. aureus* can grow and multiply up to 10^6 CFU/g under optimal growth conditions[2, 3], and produces endotoxins in the food[4,5]. Staphylococcal foodborne illness is caused by consuming food contaminated with *S. aureus* enterotoxins (SEs), and symptoms of the illness include vomiting and diarrhea[6]. SEs (SEA-SEE, SEG-SEJ, and SER-SET) are heat stable and resistant to low pH. SEA is the most common enterotoxin recovered from foodborne illness outbreaks followed by SED and SEB[7,8].

Therefore, this bacterium is considered as an enterotoxigenic rather than an invasive infection-causing bacterium. The possibility of *S. aureus* intestinal colonization and its implications on host health were previously reported by Gries *et al*.[9], wherein they suggested that the cecal mucus layer facilitates the intestinal colonization of methicillin-resistant *S. aureus*. Furthermore, Vriens *et al.*[10] reported the presence and interaction of *S. aureus* in the rectum of patients in surgical intensive care and liver transplant units. However, the mechanism underlying the intestinal invasion of *S. aureus* remains unclear.

Therefore, this study aimed to determine whether *S. aureus* can invade human intestinal cells, and to elucidate gene for the invasion.

MATERIALS AND METHODS

Inoculum preparation

Bacterial strains were purchased from National Culture Collection for Pathogens (NCCP; Seoul, Korea) and Korean Agricultural Culture Collection (KACC). Four *S. aureus* strains, NCCP10862, KACC13236, KACC10768, and KACC11596, were cultured on mannitol salt agar (MSA; Becton, Dickinson and Company, Sparks, MD, USA) and then inoculated into tryptic soy broth (TSB; Becton, Dickinson and Company). They were incubated at 35°C for 24 h. Hundred microliters of the culture was sub-cultured into fresh TSB, followed by incubation at 35°C for 24 h. The subcultures were centrifuged at 1912 xg and incubated at 4°C for 15 min. Thereafter, the cell pellets were washed twice with phosphate buffered saline (PBS; pH 7.4; 0.2 g KH_2PO_4, 1.5 g $Na_2HPO_4 \cdot 7H_2O$, 8.0 g NaCl, and 0.2 g KCl in 1 L of distilled water), and eventually suspended in 10 mL of PBS. The suspensions to be inoculated were diluted with PBS to OD_{600} = 0.01.

Intestinal cell invasion assay

The intestinal cell line, Caco-2, was purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured for 72 h at 37°C and 5% CO_2 in Eagle's minimum essential medium (MEM; Gibco, Penrose, Auckland, New Zealand), supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin (PS; Gibco), until the Caco-2 cells formed a monolayer, which was then detached by trypsin. Thereafter, the cells were washed twice with Dulbecco's phosphate buffered saline (DPBS; Welgene, Daegu, Korea), and suspended in MEM supplemented with 20% FBS. For the invasion assay, diluted Caco-2 cells, at a final concentration of 5.0 x 10^4 cells mL^{-1}, were placed in 24-well plates and incubated at 37°C and 5% CO_2 for 48 h. One milliliter of each *S. aureus* culture was inoculated into the plate and incubated for 2 h at 37°C and CO_2 to allow *S. aureus* invasion into Caco-2 cells. Aliquot of the inoculum was properly diluted in BPW and 100 µL of the diluents was spread-plated on TSA plates. Colonies grown on TSA plates were counted after 24 h of incubation, and the bacterial cell counts were used as initial bacterial cell counts. Planktonic *S. aureus* cells in the supernatant were discarded, and the attached *S. aureus* cells were treated with gentamicin (50µg mL^{-1}) or PBS for 2 h. The Caco-2 cell membrane was then lysed using 0.1% TritonX-100 for 20 min. About 100-µL aliquots of the suspensions were spread-plated on TSA, and incubated at 37°C for 24 h. The colonies were manually counted to enumerate the invasive *S. aureus*. Invasion efficiency (%) was calculated as follows: (invaded bacterial cell counts/initial bacterial cell counts) x 100.

Isobaric tags for relative and absolute quantification (iTRAQ) screening for invasion-related protein

To identify the protein responsible for *S. aureus* invasion of Caco-2 cells, bacterial cells harvested from 1–1.5 mL of *S. aureus* culture were lysed by suspension in lysis buffer (iNtRON, Seongnam, Korea) and lysostaphin (0.5 mg mL^{-1};

Sigma Aldrich, St. Louis, MO, USA), and incubation at 37°C for 2 h. The lysates were then treated with SMART bacterial solution (iNtRON), and centrifuged (8,000 xg and 5 min). Proteins in the supernatants were stored at -80°C until used. The proteins were precipitated using acetone at -20°C. After acetone was then removed by vaporization, the precipitated proteins were resuspended in urea buffer (50 mmol l^{-1} Tris pH 8.3, 3 M urea), and digested by trypsin (1:10) at 37°C for 16 h. The digested proteins were labeled with the iTRAQ reagent suspended in isopropanol (Global standard; GS-113, 10862-116, 13236-117, 10768-119, and 11596-121) for 1 h at 25°C, and then, completely dried in a SpeedVac centrifuge. The labeled samples were fractionated using cation exchange chromatography on Agilent 1100 system (Agilent Technologies, Santa Clara, CA, USA), and analyzed using LC-MS/MS with a hybrid quadrupole-TOF LC-MS/MS spectrometer (Applied Biosystems, Foster City, CA, USA). Peptides and proteins were then identified and quantified using the ProteinPilot Software 2.0.1 (Applied Biosystems), and differences in protein expression among the *S. aureus* strains were analyzed.

Gene mutation

iTRAQ data revealed that the invasive *S. aureus* had a higher level of pyruvate formate lyase expression (Pfl) than that of the noninvasive strain. Thus, a *pflB* deletion mutant was constructed as described by Lee *et al.*[11]. Table 1 lists the bacterial strains, primers, and plasmids used in this study.

Table 1. Bacterial strains, plasmids, and primers used in this study

Strains	Description	Reference
E. coli DC10B	High efficiency cloning strain	Monk *et al.*[12]
Plasmid	*E. coli* DH10B(*dcm*-)	
pIMAY	*E. coli-S. aureus* shuttle vector, temperature- sensitive	Monk *et al.*[12]
pIMAY-$pflB_{trunc}$	pIMAY ligated with truncated *pflB*	This study
Primer	Sequence (5'→3')	
pflB-F(PstI)	GGA ACA AGT ACT GCA GTT ATC GAA	This study
pflB-R(SalI)	TGT TAA CTG TCG ACA TTC TTC TGG	This study
pflB'-F	TAA GCC GTC CAG CAG AAA	This study
pflB'-R	CCG TAT ACA ACG TTT GAA G	This study

Construction of pIMAY-$pflB_{trunc}$

Chromosomal DNA of wild type invasive strain was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), and was used as template for following PCR amplification. The *pflB* gene was amplified using pflB-F (PstI) and pflB-R (SalI) primers with PCR conditions as follow; 95°C-5 min, 35 cycles of 95°C-30 sec, 43°C-30 sec and 72°C-1 min, and 72°C-5 min. After the amplified *pflB* was purified with QIAquick gel extraction kit (Qiagen), the gene was digested with *Eco*RV (Elpisbio, Daegen, Korea), which targets two sites (900 bp distance) of the *pflB* gene. The digested *pflB* fragments were self-ligated with DNA ligation kit (Takara, Shiga, Japan) to form truncated *pflB*. The truncated *pflB* and pIMAY that was a shuttle vector were then digested by restriction enzymes, *Pst*I and *Sal*I, and the two DNA fragments were ligated by the DNA ligation kit to produce pIMAY-$pflB_{trunc}$.

Transformation and cloning in *E. coli* DC10B

pIMAY-$pflB_{trunc}$ was transformed into high-efficiency cloning strain, *E. coli* DC10B as described by Monk *et al.*[12]. Briefly, competent *E. coli* DC10B cells were prepared using a chemical method[13], and stored in -80°C deep freezer until used. The competent cells were mixed with pIMAY-$pflB_{trunc}$ in microcentrifuge tube on ice, and the transformation was induced by heat-shock at 42°C for 30 sec. The transformants were spread-plated on LB agar containing 15 µg mL^{-1} of chloramphenicol (Cm). The plates were then incubated at 37°C for 24 h to obtain *E. coli* DC10B containing pIMAY- $pflB_{trunc}$.

Electroporation and allelic exchange

pIMAY-$pflB_{trunc}$ was extracted from the *E. coli* DC10B with DNA Plasmid SV kit (GeneAll, Seoul, Korea) according to the manufacturer's protocol.

pIMAY-$pflB_{trunc}$ was precipitated using ethanol, and the plasmid DNA was then transformed into *S. aureus* by electroporation. To induce single-crossover integration, the *S. aureus* strain containing pIMAY-$pflB_{trunc}$ was grown at 37°C for 24 h in LB broth containing Cm (15 μg mL^{-1}). The culture was streaked on LB agar + Cm, and incubated at 37°C for 24 h. One *S. aureus* colony from the agar was then cultured in LB broth without Cm at 28°C, followed by streaking on LB agar containing 1 μg mL^{-1} of anhydrotetracycline (ATc) to induce excitation of remained plasmid fragment. Eventually, the colony was dually streaked on LB agar containing ATc and Cm. The object grown only on LB agar+ATc not on LB agar+Cm was considered as a mutant *S. aureus* strain which contained truncated *pflB* instead of original *pflB*[11]. Existence of $pflB_{trunc}$ on *S. aureus* genome was eventually confirmed by DNA sequencing and PCR analysis using pflB'-F and pflB'-R primers, which were created from sequences lying outside the truncated *pflB* (Cosmogenetech, Seoul, Korea). DNA sequencing was analyzed using BigDye™ Terminator v3.1 Cycle Sequencing kit and 373 DNA Analyzer (Applied Biosystems). The lengths of *pflB* in wild- (2,250 bp) and mutant-type (1,350 bp) strains were compared.

Statistical analysis

The experiments were repeated twice, and two samples were used in each replication (*n* = 4). Because the replication had random effects on a dependent variable (*S. aureus* invasion efficiency), a mixed procedure of SAS® (Version 9.2, SAS Institute, Cary, NC, USA) was used to analyze the results, and the least squares means of the invasion efficiency among *S. aureus* strains were then compared, using pairwise *t*-test at α = 0.05.

RESULTS AND DISCUSSION

The invasion efficiencies of the *S. aureus* strains were compared. Among the aforementioned strains, *S. aureus* strains NCCP10862 and KACC10768 showed Caco-2 cell invasion (36.3%–38.4%), but the other strains showed no cellular invasion (Fig. 1). These two strains were clinical isolates, and *S. aureus* KACC10768 was identified as a methicillin-susceptible strain. These strain-variation was also shown in other studies. Blevins *et al.*[14] showed that regulatory roles of *sarA* and *agr*, which control virulence factors in *S. aureus*, were strain-dependent. Belkum and Melles[15] also presented the strain variation of *S. aureus* strains in pathogenicity. To elucidate the strain variation of *S. aureus* in Caco-2 cell invasion, the proteins profiles of *S. aureus* strains NCCP10862 and KACC10768 were screened using iTRAQ. The results indicated that Pfl protein expression was upregulated in the invasive *S. aureus* strains, followed by an increase in the levels of elongation factor Tu and phosphopyruvate hydratase in the strains (Table 2). The elongation factor Tu and phosphopyruvate hydratase genes were not subjected to mutation because they are essential for *S. aureus* survival. Thus, only *pflB* gene was subjected to the mutation.

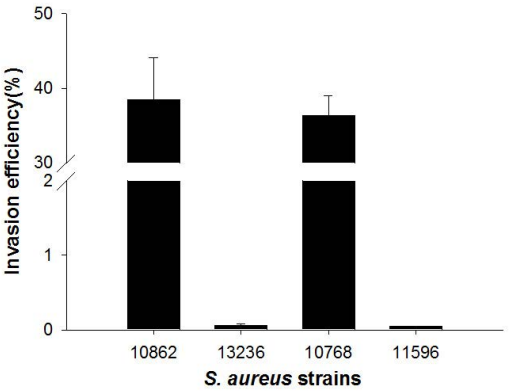

Fig. 1. Comparison of Caco-2 cell invasion efficiencies for *Staphylococcus aureus* strains NCCP10862, KACC13236, KACC10768, and KACC11596.

Table 2. Relative protein quantification of *Staphylococcus aureus* strains. 113-Global Standard (GS), 116: *S. aureus* NCCP10862, 117: *S. aureus* KACC13236, 119: *S. aureus* KACC10768, 121: *S. aureus* KACC11596

Name	116:113	117:113	119:113121:113
Elongation factor Tu	0.4105	0.8765	5.20070.7146
Phosphopyruvate hydratase	0.8292	0.7503	2.75680.7573

To elucidate the role of Pfl in *S. aureus* invasion, the *pflB* mutant of *S. aureus* was prepared. The truncated *pflB* (Ca. 900 bp) was constructed by EcoRV. The truncated construct and pIMAY were cut with the same restriction enzymes, ligated, and transformed into *E. coli* DC10B. The pIMAY-$pflB_{trunc}$ was then transformed into *S. aureus* through electroporation, integrated into the chromosome, and the *S. aureus pflB* mutation was confirmed through PCR. Compared to the wild-type, the invasion efficiency of the mutant strain into the Caco-2 cells decreased by 60% ($p < 0.05$) (Fig. 2). This result indicates that, although Pfl, known as an oxygen sensitive enzyme, was involved in the conversion of pyruvate to formate, it is crucial for *S. aureus* invasion into Caco-2 cells (Fig. 2). In a previous study, it was proved that *pflB* had important role in deeper layer of biofilm formation as Pfl supplies formate on such anaerobic and nutrient-deficient conditions[16]. Biofilm formation and adhesion are usually first step of *S. aureus* infection for persistence of bacteria in host, and the colonization and persistence on around of the invaded cells is probably important in the invasion procedure of bacterial cells. Taken together, it can be suggested that *pflB* may have a role in the intestinal cell invasion of *S. aureus* in the process of biofilm formation.

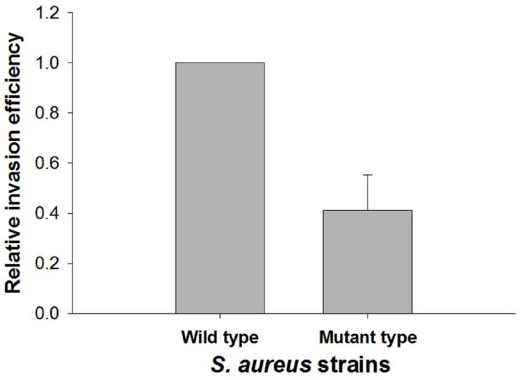

Fig. 2. Relative invasion efficiencies of the *pflB* mutant to wild type *Staphylococcus aureus* KACC10768.

CONCLUSION

In conclusion, although *S. aureus* is known to cause foodborne illness by release of toxins, and the mechanism of intestinal cell invasion remains unclear, certain *S. aureus* strains can invade the intestinal cells due to the expression of the *pfl* gene. Thus, the chronic effect of invasive *S. aureus* in the intestine should be further researched.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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