FEMS Microbiology Letters, 363, 2016, fnw164

doi: 10.1093/femsle/fnw164
Advance Access Publication Date: 7 July 2016
Research Letter

RESEARCH LETTER – Pathogens & Pathogenicity

Capsule of *Streptococcus equi* subsp. *zooepidemicus* hampers the adherence and invasion of epithelial and endothelial cells and is attenuated during internalization

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One sentence summary: Capsule breakdown in cell invasion by *Streptococcus equi* subsp. *zooepidemicus*.
Editor: Akio Nakane

ABSTRACT

Direct interaction between pathogens and host cells often is a prerequisite for colonization, infection and dissemination. Regulated production of capsular polysaccharide (CPS), which is made of hyaluronic acid, is essential for the pathogenicity of *Streptococcus equi* subsp. *Zooepidemicus* (SEZ). Here, we constructed a CPS-deleted mutant and analyzed it along with the parental wild-type strain in attachment and invasion of mammalian epithelial and endothelial cell lines. The CPS-deleted mutant exhibited significant increase in adherence and invasion by several orders of magnitude compared with the wild-type strain through quantitative analysis and electron microscopy observation. After the wild-type strain was recovered from invaded cells, its morphology was analyzed by visual methods and scanning electron microscopy, which revealed that its capsule was almost completely absent. Capsule measurements showed a similar result in which CPS production was nearly attenuated to the same extent as in the CPS-deleted mutant. qPCR assays revealed a marked reduction in the transcriptional levels of the CPS biosynthesis genes, *has* operon. Moreover, the repression in capsular production was stable inheritance. Our findings indicate that SEZ is a facultative intracellular bacterium, capsule attenuation in SEZ contributes to attachment and invasion in interactions with host cells, and the active regulation of capsule breakdown is controlled by SEZ during internalization.

Keywords: *Streptococcus equi* subsp. *zooepidemicus*; capsule; invasion; adherence; attenuation; morphology

INTRODUCTION

Streptococcus equi subsp. *zooepidemicus* (SEZ), a group C *streptococcus*, is an important conditioned pathogen that infects various animals, especially horses, pigs, dogs and sometimes humans (Pesavento *et al.* 2008; Abbott *et al.* 2009; Eyre *et al.* 2010). SEZ generally inhabits the mucous membranes and skin of animals, and causes septicemia, meningitis, endocarditis and arthritis. In the equine and swine industries, these infections lead to serious welfare problems and economic losses (Feng and Hu 1977; Waller 2010). Occasionally, humans are infected with SEZ via

Received: 19 March 2016; **Accepted:** 23 June 2016

zoonotic transmission from contact with infected domestic animals or by ingesting unpasteurized animal products, such as milk and cheese; this may lead to septicemia and meningitis (Minces, Brown and Veldkamp 2010).

Despite the clinical relevance of SEZ, the factors that contribute to its virulence are not well known. Some of them have been identified in preliminarily reports. SzP, SzM and capsular polysaccharide (CPS) all contribute to defense against opsonophagocytosis and complement deposition. Additionally, SzP and SzM exhibit fibrinogen-binding activity (Ma *et al.* 2012; Wei *et al.* 2012; Velineni and Timoney 2013). The fibronectin-binding protein has fibronectin- and collagen-binding activities (Hong 2005). C5a peptidase acts as an invasin for host epithelial cells (Wei *et al.* 2013). Streptolysin S exhibits hemolytic effects (Ma *et al.* 2015). The main component of CPS is hyaluronic acid (HA), for which the production is regulated by the hyaluronic acid synthesis (*has*) operon that contains five genes—*hasA*, *hasB*, *hasC*, *glmU* and *pgi* (Blank, Hugenholtz, and Nielsen 2008).

Bacterial survival resulting from resistance to phagocytosis and complement-mediated killing in deep tissues and blood circulation is essential for SEZ infection; these properties are conferred by maximal expression of CPS (Wei *et al.* 2012). However, invasion of the mucous membranes, migration into different tissues and penetration of the blood–brain barrier are indispensable for the transition from infection to invasive disease. In these pathogenic processes, bacteria generally attach to and invade different epithelial and endothelial cells. The roles of the CPS of SEZ in these processes have not yet been well defined. In studies of some bacteria, such as *Streptococcus pneumoniae*, *S. suis*, *Neisseria meningitides* and *Klebsiella pneumoniae*, they demonstrated that the capsule impeded adhesion to and invasion of epithelial cells (Sahly *et al.* 2000; Benga *et al.* 2004; Hammerschmidt *et al.* 2005; Bartley *et al.* 2013). Moreover, studies of *S. pneumoniae* determined that CPS production is decreased in the interaction between the bacteria and epithelial cells (Hammerschmidt *et al.* 2005). As the capsule components of SEZ are different from those of the bacteria mentioned above, whether the CPS plays similar roles when SEZ attach to and internalize in epithelial or endothelial cells remains to be determined and will be the subject of this present study.

MATERIALS AND METHODS

Bacterial strains and cell lines

SEZ ATCC35246 was purchased from the American Type Culture Center (ATCC, Manassas, Virginia, US) and was cultured in Todd–Hewitt (BD, Franklin Lakes, New Jersey, US) medium at 37°C (Fan *et al.* 2009). *Escherichia coli* DH5α was used as host for the temperature-sensitive shuttle vector pSET4s (Takamatsu, Osaki and Sekizaki 2001) and was cultured at 37°C in Luria–Bertani medium (Sigma-Aldrich, St. Louis, Missouri, US). For construction of the recombinant plasmid and the selection of mutants, spectinomycin was added to the medium at 100 μg mL^{-1} for SEZ and 50 μg mL^{-1} for *E. coli*.

The human colon epithelial cell line CACO-2 (ATCC, Manassas, Virginia, US; HTB-37), human epithelial cell line HEp-2 (ATCC, Manassas, Virginia, US; CCL-23) and mouse brain endothelial cell line bEnd.3 (ATCC, Manassas, Virginia, US; CRL-2299) were cultured in Dulbecco's modified Eagle medium (DMEM) high glucose (GIBCO, Waltham, Massachusetts, US) supplemented with 10% fetal bovine serum (GIBCO, Waltham, Massachusetts, US) at 37°C under 5% CO_2.

Construction of a SEZ capsule mutant

The primers used in this experiment are listed in Table 1. For construction of a *hasB* deletion mutant, the upstream and downstream flanking sequences of *hasB*, including the sequence of overlapping part between *hasB* and *hasC*, were amplified and integrated together by PCR. Then, the product was digested with restriction endonucleases (SalI and BamHI, TaKaRa, Kusatsu, Shiga, Japan) and ligated into the pSET4s with T4 DNA Ligase (TaKaRa, Kusatsu, Shiga, Japan). The recombinant plasmid

Table 1. Primers used in this study[a].

Name	Oligonucleotide sequence (5′ - 3′)	Product
Construction of mutant		
HASB-UPSTREAM-F	CGCGTCGACCTTAACACGCTTGACAGA	Upstream of *hasB*
HASB-UPSTREAM-R	TTCATTTTTTCCTTTGATACCTTAG	Upstream of *hasB*
HASB-DOWNSTREAM-F	AGGTATCAAAGGAAAAAATGAAATGTCTTTGGAAGAGACTAGTCAGA	Downstream of *hasB*
HASB-DOWNSTREAM-R	GCCGAATTCCCTCTAGTATCCCACCTTA	Downstream of *hasB*
HASB-TEST-F	TCCTAGACCTAAATGCGTCAG	Part of *hasB*
HASB-TEST-R	CCAATATCCTCGCCAAGC	Part of *hasB*
qRT-PCR		
HASA-F	ATAGATACATCAACCAGACCTTCCT	Part of *hasA*
HASA-R	TGCTGCTTCAAGTAAGTAGACATC	Part of *hasA*
HASB-F	TGTAGCAGGCTCAGGATATGTC	Part of *hasB*
HASB-R	TGTCGGAGTAGCAATGATAATAAGG	Part of *hasB*
HASC-F	GAGAAGCCAAGTCCAGATGAG	Part of *hasC*
HASC-R	GAGCATAGTCAAGTGAGGTCTT	Part of *hasC*
GLUM-F	TTCTCACAGCCAATGCCAAG	Part of *glum*
GLUM-R	TCTGCCTCATTAGCGTCCTT	Part of *glum*
PGI-F	GCTCGTAAGGACCTGTCATCA	Part of *pgi*
PGI-R	GCCAGTTGCTTCCACCATTC	Part of *pgi*
C5A-F	TCGGATTCCTTATCAGCGG	Part of C5a peptidase gene
C5A-R	CTCCAGCAACCAGCAAAACA	Part of C5a peptidase gene
GAPDH-F	CGGTCGTATCGGTCGTCTT	Part of *gapdh*
GAPDH-R	AAGCGTCCTTGAGTTGAATCAT	Part of *gapdh*

[a]Underlined portions of the primers correspond to restriction enzyme recognition sites. GTCGAC, SalI; GGATCC, BamHI.

pSET4s-*hasBUD* was introduced into strain SEZ ATCC35246 by electroporation. Transformants were cultured at 37°C on TH agar plates in the presence of spectinomycin. A single colony was continuously cultured by serial passage at 28°C in TH broth without spectinomycin. Then, the bacterial solution was diluted and plated on TH agar. Colonies were picked and plated on agar in the presence or absence spectinomycin. Spectinomycin-sensitive colonies were characterized for deletion of the *hasB* gene by PCR using two pairs of primers, HASB-TEST-F/HASB-TEST-R, which were utilized to amplify part of the *hasB* gene, and HASB-UPSTREAM-F/ HASB-DOWNSTREAM-R, which were utilized to amplify the locus of the *hasB* gene including its upstream and downstream flanking sequences. Then the PCR product was sequenced.

Adherence and invasion assays

The epithelial/endothelial cells mentioned above were grown to confluence (Caco-2 and HEp-2 ~5.0×10^5 cells/well, bEnd.3 ~ 2.0×10^5 cells/well) in 24-well plates and used for assays. Bacteria in logarithmic growth phase were harvested by centrifugation (5000 g, 5 min), washed with DMEM and added to confluent cells at a multiplicity of infection (MOI) of ~100, 10 or 1. After 2 h of incubation at 37°C under 5% CO_2, cells were washed with phosphate buffered saline (PBS). In parallel, cells were added to double-distilled water to get cells lysed and recover the total number of cell-associated bacteria (intracellular and extracellular), or were added to DMEM containing 5 μg mL^{-1} penicillin and 200 μg mL^{-1} gentamicin for another 1 h of incubation to kill extracellular bacteria before recovery of the intracellular bacteria according to the same cell lysis method. Recovered bacteria were diluted and plated on TH agar. All adherence or invasion assays were repeated in three independent experiments.

Capsule measurements

Capsule production by SEZ was analyzed using a quantitative assay for uronic acids, as described previously (Blumenkrantz and Asboe-Hansen 1973). Briefly, bacteria grown to an OD_{600} of 0.5 in TH broth were harvested by centrifugation. Each bacterial pellet from 4 mL of broth was washed once with 150 mM Tris-HCl (pH 7.0) and resuspended in 200 μL of the same buffer. After the addition of 1.2 mL 12.5 mM tetraborate in 96% sulfuric acid, the suspension was incubated for 5 min at 99°C. Then, the sample was mixed with 20 μL 0.5% NaOH with 0.15% m-hydroxydiphenyl, or without m-hydroxydiphenyl as a blank control for subtraction. Changes in color were determined spectrophotometrically at 520 nm. Experiments were repeated at least three times.

qPCR assays

Total RNA of SEZ was extracted using the RNAiso Plus procedure (TaKaRa, Kusatsu, Shiga, Japan). Any remaining genomic DNA was digested using Recombinant DNase I (TaKaRa, Kusatsu, Shiga, Japan). cDNA synthesis was performed using the PrimeScript RT reagent kit (TaKaRa, Kusatsu, Shiga, Japan), and mRNA levels were measured with the SYBR® *Premix Ex Taq* kit (TaKaRa, Kusatsu, Shiga, Japan) according to the manufacturer's instructions. Primers used for the various qPCR assays are listed in Table 1. The GAPDH gene was amplified as a reference gene (Ma *et al*. 2015). Relative changes in gene transcription were calculated using the comparative CT method (Livak and Schmittgen 2001). Each set of qPCR was repeated at least three times with independent RNA preparations.

Electron microscopy

Cells and bacteria samples were fixed using a lysine-acetate based formaldehyde/glutaraldehyde ruthenium red-osmium fixation (LRR fixation) procedure, as described previously (Hammerschmidt *et al*. 2005). Briefly, samples were fixed successively with 2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, 0.09 M sucrose; pH 6.9) containing 0.075% ruthenium red and 0.075 M lysine-acetate for 20 min on ice, 2% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer with 0.075% ruthenium red for 3 h, and 1% osmium tetroxide in cacodylate buffer with 0.075% ruthenium red for 1 h at room temperature. Between each fixation procedure, samples were washed sufficiently with cacodylate buffer containing 0.075% ruthenium red. After final washing, samples were dehydrated with a graded series of ethanol on ice for 30 min for each step. Subsequently, the samples were viewed using a scanning electron microscope (ZEISS, Oberkochen, Germany) at a magnification of 4000–15000× and an acceleration voltage of 20 kV.

Statistical analysis

Data were analyzed and plotted using GraphPad Prism (version 5.0; GraphPad Software, USA [http://www.graphpad.com]). Data are expressed as means with standard errors (SEM). Statistical analyses of data were performed using Student's *t*-test. A confidence interval with a value of $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Construction and identification of non-capsulated mutant of SEZ

No amplicon was observed from a *hasB*-knockout mutant of SEZ by PCR using primer pairs HASB-TEST-F/HASB-TEST-R. The sequenced PCR product from the *hasB*-knockout mutant, which was amplified by PCR using primer pairs HASB-UPSTREAM-F/HASB-DOWNSTREAM-R, lacked complete nucleotide sequence of *hasB* (except the sequence of overlapping part between *hasB* and *hasC*) compared with wild-type SEZ. To investigate whether or not expression of downstream genes of the *has* operon is affected by the deletion of *hasB*, qPCR assays were performed. As a result, the transcriptional levels of the rest *has* operon genes were not significantly decreased when the *hasB*-knockout mutant was compared with the wild-type SEZ (Fig. 1A). A research has shown that invasion of SEZ into Hep-2 cells is mediated by the invasin C5a peptidase (Wei *et al*. 2013). The transcriptional level of the C5a peptidase gene was analyzed. There was no significant difference in the mRNA level of this gene when the mutant was compared with the wild-type SEZ (Fig. 1A).

The wild-type SEZ produced large, translucent, moist colonies, reminiscent of sticky droplets. After extended growth for more than 16 h, closely spaced colonies of wild-type SEZ would blend together (Fig. 2A). In contrast, colonies of the *hasB-knockout* mutant strain were small, milky, opaque and desiccated. The mutant colonies did not readily integrate, even if they were adjacent to each other (Fig. 2B and H). Furthermore, when the wild-type SEZ was grown in TH broth, the culture was characterized by uniform turbidity (Fig. 2K). In contrast, when the mutant was grown in TH broth, it always sank to the bottom of the culture, and the culture liquid remained clear (Fig. 2L).

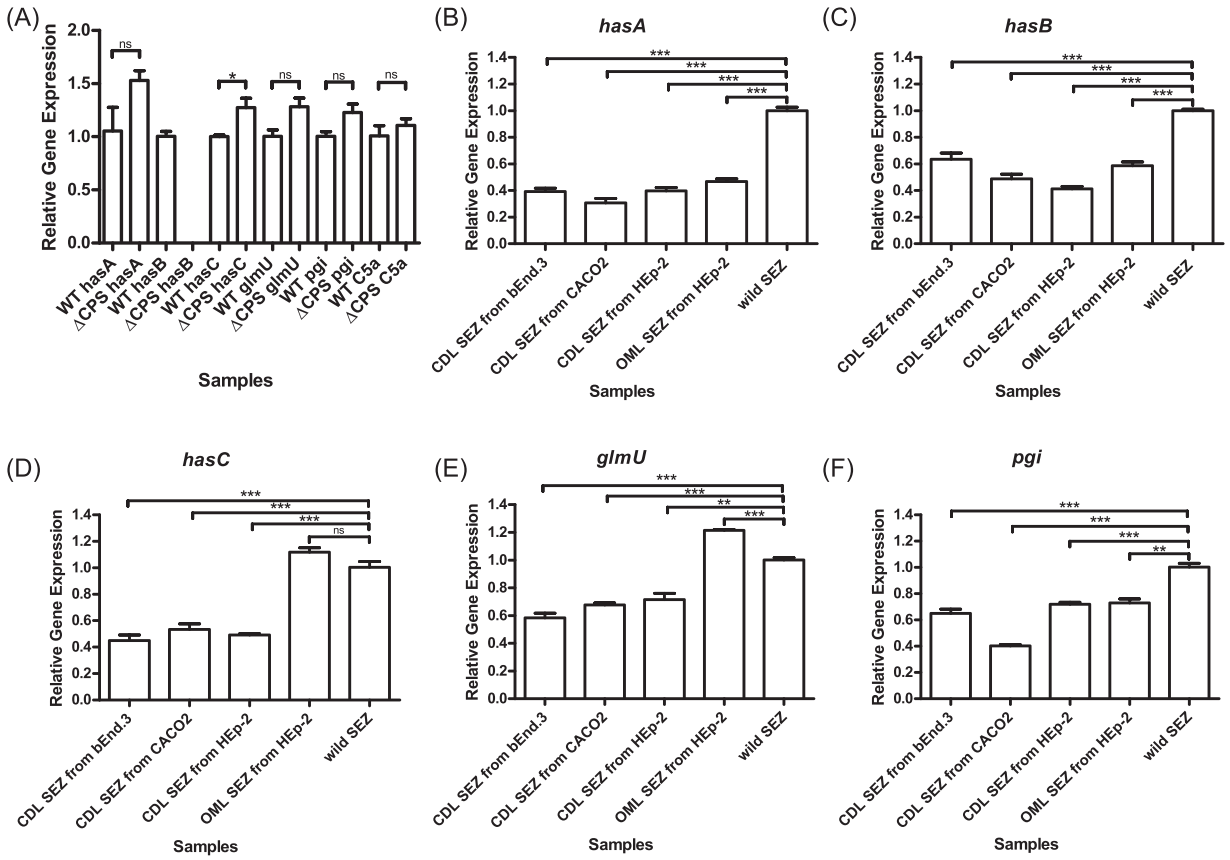

Figure 1. qPCR assays. (**A**) Transcriptional levels of all *has* operon genes and C5a peptidase gene in wild-type SEZ and capsule-deleted mutant grown to logarithmic phase in TH broth. (**B–F**) Transcriptional levels of all *has* operon genes in capsule-deleted-like (CDL) wild-type SEZ recovered from invasion, original-morphology-like (OML) wild-type SEZ recovered from invaded HEp-2 cells and the untreated wild-type SEZ grown 24 h on TH agar. Data are presented as mean values and standard errors from three independent experiments. Statistical significance is assessed using Student's *t*-test (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Adherence and invasion by the wild-type SEZ and its capsule-deleted mutant

In adherence assays, at each MOI, the capsule-deleted mutant displayed a significantly increased adherence (by at least two orders of magnitude, $P < 0.001$) to bEnd.3 and CACO-2 cells relative to wild-type SEZ (Fig. 3A and C). However, this significant increase was only a ~10-fold ($P < 0.001$) in HEp-2 cells when the mutant was compared with wild-type SEZ (Fig. 3E). The number of bacteria that adhered to bEnd.3 or CACO-2 per 10^5 cells was almost identical at each MOI for the same type of strain (i.e. differences were within 2-fold, Fig. 3A and C). However, wild-type SEZ adhered to HEp-2 at a level more than 30-fold higher compared with its adherence to bEnd.3 or CACO-2 at each MOI. For both wild-type SEZ and its capsule-deleted mutant, a 10-fold higher MOI resulted in an increase in bacterial adherence to bEnd.3, CACO-2 and HEp-2 of ~2-5-fold (Fig 3A, C and E).

In invasion assays, almost no wild-type SEZ invaded bEnd.3 at a MOI of 1 or CACO-2 at a MOI of 10 or 1 (Fig. 3B and D). Even at a MOI of 100, fewer than 100 wild-type bacteria invaded bEnd.3 (per 10^5 cells) (Fig. 3B). In stark contrast, the numbers of capsule-deleted mutant bacteria that invaded bEnd.3 or CACO-2 were significantly higher than for the wild-type bacteria at each MOI ($P < 0.001$) (Fig. 3B and D). In assays using HEp-2 cells, hundreds or thousands of wild-type SEZ invaded per 10^5 cells (Fig. 3F). When the capsule-deleted mutant interacted with HEp-2 cells, the number of internalized bacteria was reduced ~2- to 7-fold compared with the number of adherent bacteria (Fig. 3E and F). Ratios of the number of adherent bacteria to the number of invaded bacteria were ~4–21 folds and ~33–216 folds when the capsule-deleted mutant interacted with bEnd.3 (Fig. 3A and B) and CACO-2 cells (Fig. 3C and D), respectively.

HEp-2 monolayers infected with wild-type SEZ or capsule-deleted mutant were fixed with an LLR-fixation procedure, and viewed using scanning electron microscopy. The images revealed that the wild-type SEZ had made contact with microvilli of cells still present the capsule (Fig. 4A). SEZ adherence stimulated elongation and massive recruitment of neighboring microvilli (Fig. 4A). An individual wild-type SEZ was much bigger than capsule-deleted mutant (Fig. 4A and B). Next, we emphatically observed interaction between HEp-2 monolayers and capsule-deleted mutant; the mutant had higher adherence and invasion rates. The recruited microvilli surrounded bacteria, generated large pseudopod-like structures, fused to lamellipodia and then engulfed bacteria into the cells (Fig. 4C–F).

Morphological analyses and capsule content assays of wild-type SEZ recovered from invaded cells

In invasion assays of wild-type SEZ, we found that almost all the bacteria recovered from invaded bEnd.3 or CACO-2 cells had a similar colony morphology to the capsule-deleted mutant (Fig. 2C and D). The colonies with an appearance similar to the

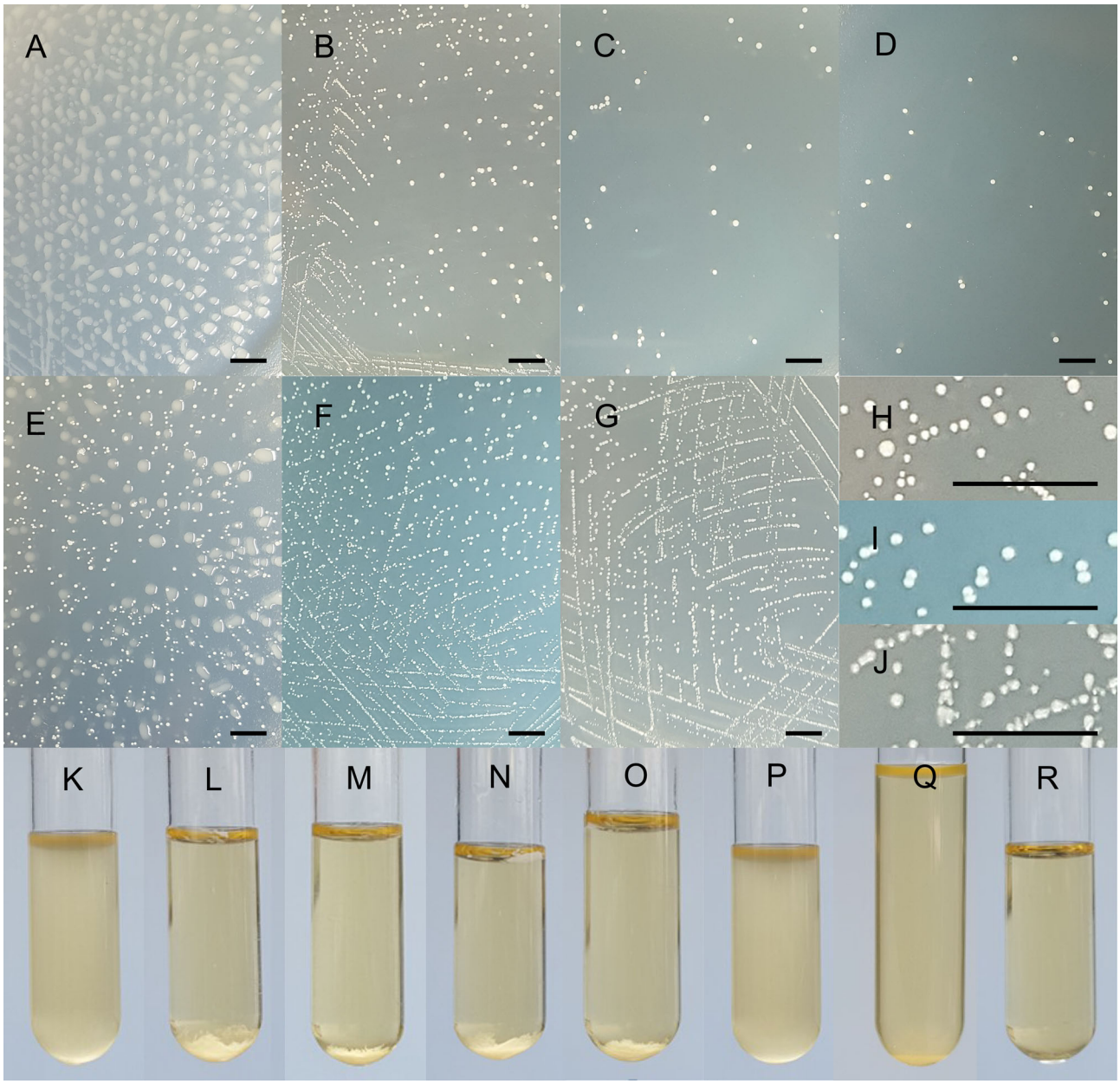

Figure 2. Observation of bacterial colony morphology and culture solutions. Untreated wild-type SEZ (**A**, **K**); capsule-deleted mutant (**B**, **H**, **L**); capsule-deleted-like wild-type SEZ recovered from invaded bEnd.3 (**C**, **M**), CACO-2 (**D**, **N**, and HEp-2 (**E**, **O**); original-morphology-like wild-type SEZ recovered from invaded HEp-2 (**E**, **P**; and the capsule-deleted-like wild-type SEZ continuously cultured by serial passage on TH agar for 15 times (**F**, **I**) or in TH broth for 30 times (**G**, **J**, **Q**), were grown on TH agar or cultivated in TH broth. Sterile TH broth was used as a control (R). Bars = 1 cm.

input grown on TH agar accounted for only 10%–20% of the total colonies after wild-type SEZ was recovered from invaded HEp-2 cells and plated (Figs 2E and 3G). To establish whether these bacteria were capsule deficient and to determine the relationship between the capsule and internalization in barrier cells by wild-type SEZ, we designed a series of assays. From here on, our analysis refers to four types of SEZ: (i) wild-type SEZ not used in invasion or adherence assays, and also used as untreated wild-type SEZ; (ii) the capsule-deleted mutant; (iii) 'capsule-deleted-like' wild-type SEZ recovered from invaded cells; and (iv) 'original-morphology-like' wild-type SEZ recovered from invaded cells.

First, we picked colonies of untreated wild-type SEZ, its capsule-deleted mutant and wild-type SEZ recovered from invaded cells and cultivated them in TH broth. As a result, the bacterial solution containing capsule-deleted-like wild-type SEZ recovered from cells was clear, and the bacteria sank to the bottom, just like in cultures of the capsule-deleted mutant (Fig. 2M, N and O). However, the turbidity of the solution containing original-morphology-like wild-type SEZ recovered from invaded cells reached the level observed in cultures of untreated wild-type SEZ (Fig. 2P). We continuously cultured the capsule-deleted-like wild-type SEZ by serial passage on TH agar 24 h at 37°C for 15 times or in TH broth for 12 h at 37°C for 30 times. After serial passage, the capsule-deleted-like wild-type SEZ grown on TH agar remained the same morphology as before (Fig. 2F and I). However, the culture medium containing capsule-deleted-like wild-type SEZ was turbid, while part of bacteria still sank to the bottom (Fig. 2Q). We subcultured this SEZ on TH agar for 24 h

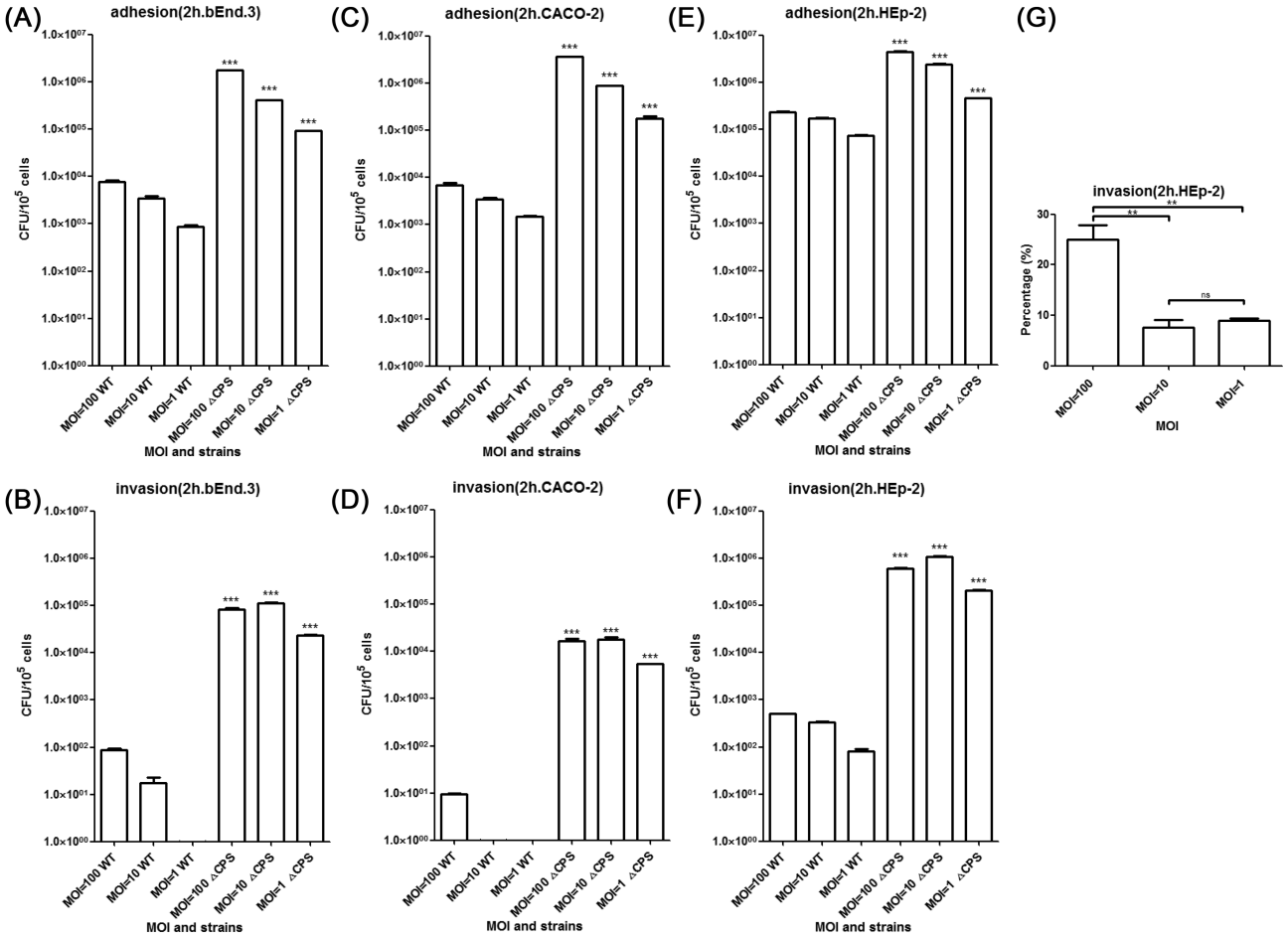

Figure 3. Adhesion and invasion assays. The graphs show number of CFU of wild-type SEZ or capsule-deleted mutant per 1×10^5 mammalian cells recovered from adherence or invasion of bEnd.3 (**A**, **B**), CACO-2 (**C**, **D**) and HEp-2 (**E**, **F**), respectively. The final graph shows the percentage of encapsulated cells among wild-type SEZ recovered from invaded HEp-2 (**G**). In each group of experiments, three different MOIs were used, ~100, 10 and 1. All values for the non-capsulated mutant are significantly higher ($P < 0.001$, labeled with ***) than those for the corresponding encapsulated parent strain. Data shown represent mean values and standard errors of at least three independent experiments for each type of assay. Statistical significance is assessed using Student's *t*-test (ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

at 37°C, and found colonies were still small, milky, opaque, but moist (Fig. 2G and J).

Next, the amount of CPS in the various strains cultured above was quantified. The capsule production in capsule-deleted-like wild-type SEZ recovered from all three types of mammalian cells, including after serial passage of them on TH agar was an average of 95.33% lower than that in the parental strain ($P < 0.001$), and at almost the same level as the capsule-deleted mutant. However, original-morphology-like wild-type SEZ recovered from invaded HEp-2 cells contained only an average of 17.04% less polysaccharides than the untreated wild-type strain ($P < 0.001$). The capsule production of the capsule-deleted-like wild-type SEZ after serial passage in TH broth was significantly higher than that before serial passage ($P < 0.001$), but still less than a quarter compared with the untreated wild-type SEZ ($P < 0.001$) (Fig. 5).

Meanwhile, the transcriptional levels of capsule synthesis-related genes in the various strains were estimated by qPCR. The mRNA levels of all of the *has* operon genes, including *hasA*, *hasB*, *hasC*, *glmU* and *pgi*, were significantly decreased when the capsule-deleted-like wild-type strain recovered from invaded cells was compared with untreated wild-type SEZ. In original-morphology-like wild-type SEZ recovered from invaded HEp-2 cells, the transcriptional levels of *hasA*, *hasB* and *pgi* were significantly lower than in untreated wild-type SEZ, although the *hasC* level not significant changed, and *glmU* level was significantly higher (Fig. 1B–F).

Finally, untreated wild-type SEZ, capsule-deleted mutant and capsule-deleted-like wild-type SEZ recovered from invaded HEp-2 cells were fixed and viewed using scanning electron microscopy. Even though the capsular structure was partially lost after washed with PBS by centrifugation and resuspension, and collapsed during fixation, untreated wild-type SEZ was covered with a layer of capsular structure (Fig. 4G). However, the wild-type SEZ recovered from invaded cells was completely capsule deficient (Fig. 4I), just like cells of the capsule-deleted mutant (Fig. 4H).

DISCUSSION

It is necessary for bacterial pathogens to penetrate the host mucous membranes or other cell barriers to cause infections by using a transcellular and/or paracellular route (Pezzicoli *et al*. 2008; Backert *et al*. 2013; Kim and Bhunia 2013). To identify whether a particular bacterium follows a transcellular infection route

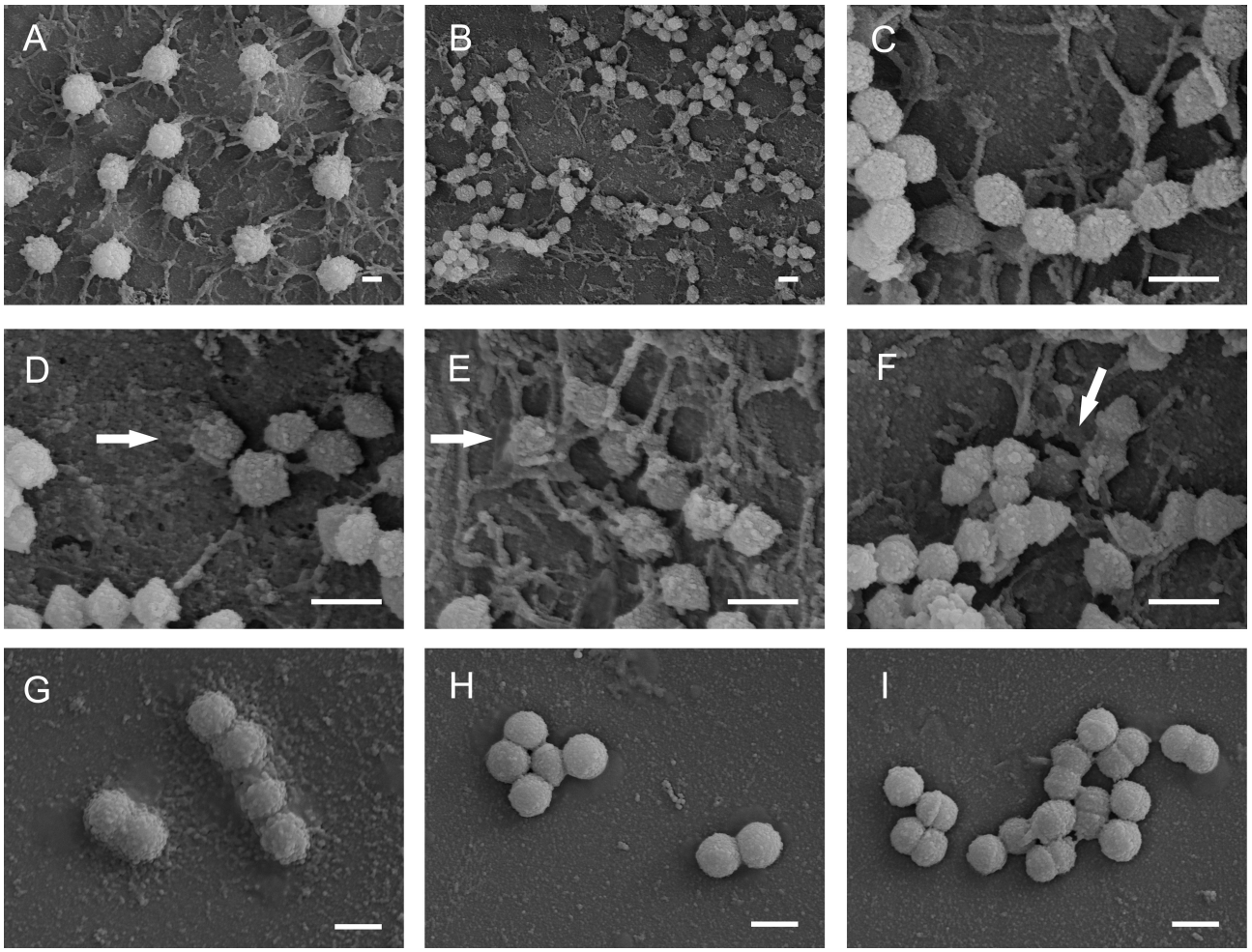

Figure 4. Scanning electron microscopic analysis. HEp-2 monolayers infected with wild-type SEZ (**A**) or capsule-deleted mutant (**B–F**) for 2 h, untreated wild-type SEZ (**G**), capsule-deleted mutant (**H**) and capsule-deleted-like wild-type SEZ recovered from invaded HEp-2 cells (**I**) were fixed with an LLR-fixation procedure and viewed using a scanning electron microscope. Arrows in panels **D** and **E** show that the bacteria were surrounded by lamellipodia. Arrow in panel **F** shows that the membrane of HEp-2 cell under the lamellipodia-adhered bacteria was sunken, which indicates the bacterium being engulfed into cell. Bars = 1 μm.

or whether it is a facultative intracellular bacterium, research about adherence to, and especially invasion of, non-phagocytic type barrier cells is indispensable (Ferrando *et al*. 2015). SEZ, as a classic extracellular pathogen, was shown here to be internalized by non-phagocytic cells. Meanwhile, we also found that the attachment and internalization abilities of wild-type SEZ were notably impeded by CPS on the bacterial surface. This idea was supported by the following findings. First, the encapsulated wild-type SEZ barely invaded bEnd.3 and CACO-2 cells. Second, the non-capsulated mutant of SEZ exhibited significantly higher (~1000-fold) invasion ability with these two cells types. Third, wild-type SEZ recovered from invaded cells was shown to generally lack the capsule. Moreover, even after wild-type SEZ recovered from invaded cells had been continuously cultured by serial passage on TH agar or broth, the morphology and capsule measurements of bacteria still indicated capsule deficiency. Besides, our other research during the same period has shown that the SEZ is capable of survival in HEp-2 cells (Xu *et al*. 2016).

Notably, the transcriptional levels of all *has* operon genes were reduced when the capsule-deleted-like wild-type strain recovered from invaded cells was compared with untreated wild-type SEZ. These data indicate that there is an active regulatory mechanism whereby wild-type SEZ reduces the transcript levels of *has* operon genes to decrease the amount of CPS generated during the process of internalization, irrespective of whether a capsule digestion pathway exists that is regulated by cells or/and bacteria. The mRNA levels of *hasA*, *hasB* and *pgi* of the original-morphology-like wild-type SEZ were low as with those of the capsule-deleted-like wild-type SEZ (Fig. 1B, C and F); nevertheless, original-morphology-like wild-type SEZ showed only 17.04% less polysaccharide compared with the untreated wild-type strain (Fig. 5). This indicates a complex regulatory mechanism, which is related to the content of CPS on the surface of bacteria, must exist.

The encapsulated wild-type SEZ could relatively easily invade HEp-2 cells. Additionally, 10%–20% of the wild-type SEZ recovered from invaded HEp-2 were encapsulated, compared with almost none of the wild-type SEZ recovered from invaded bEnd.3 and CACO-2 were encapsulated, suggesting that the hampering influence of the capsule in intimate contact between SEZ and HEp-2 is less than with the rest two mammalian cell lines. Our data imply that, despite the influence of the capsule, cells derived from different tissues, which express various types or amounts of cell surface receptors and signaling molecules, also influence adherence of and invasion by bacteria. However, the non-capsulated mutant of SEZ still showed significantly higher

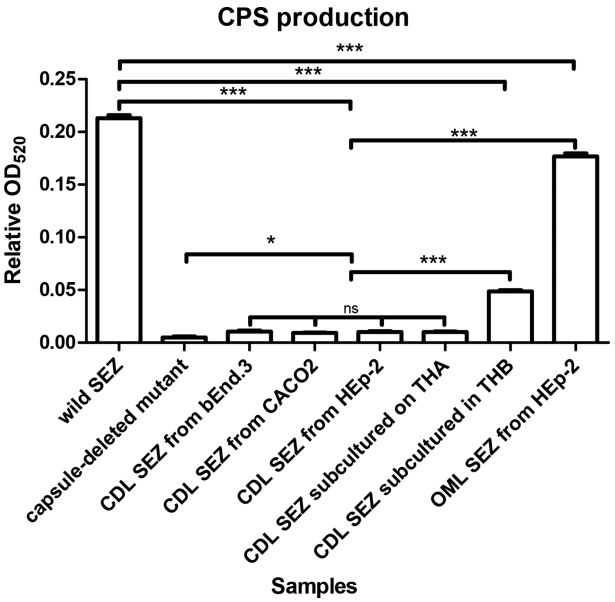

Figure 5. Quantification of capsule production by wild-type SEZ, its capsule-deleted mutant, capsule-deleted-like (CDL) and original-morphology-like (OML) wild-type SEZ recovered from invasion assays, and the CDL wild-type SEZ after continuously cultured by serial passage on TH agar for 15 times or in TH broth for 30 times. Statistical significance was determined using Student's *t*-test (ns, not significant; *, $P < 0.05$; ***, $P < 0.001$).

invasion of HEp-2 than wild-type SEZ (increase of at least three orders of magnitude) and reached the same degree of internalization as in bEnd.3 and CACO-2 cells.

The reduction in capsule production contributes to adherence and invasion, but without the protection of capsule, SEZ is vulnerable to phagocytosis and complement-mediated killing, which impacts its survival ability in host. As a result, the non-capsulated mutant is highly attenuated in virulence and can easily be eliminated in serum (Wei *et al*. 2012). Hence, the conversion between encapsulated and non-capsulated SEZ must be carefully regulated to enable colonization, survival and dissemination of the pathogen within the host. It is also possible that non-capsulated SEZ can easily invade host cells, and may it be beneficial to them to evade the immune response.

Furthermore, the wild-type SEZ ATCC35246 used in this study is originally an isolate from infected pig in Sichuan, China. This and other swine isolates preserved in our laboratory remain the same morphology in variety of environmental conditions, such as pH (6.0–8.0), CO_2 level (atmospheric to 5%), temperature (20°C–37°C) and even recovered from challenged mouse. This is entirely different from equine isolates of SEZ in different temperature conditions. Most clinical equine isolates produce small, dry colonies at 37°C, and produce big, mucoid colonies at temperatures below 35°C (Velineni and Timoney 2015). After continuously cultured by serial passage on TH agar or in TH broth, the capsule-deleted-like wild-type SEZ recovered from invaded cells still could not restore the morphology of untreated wild-type SEZ, which indicates the changes in morphology, especially the repression in capsular production are stable inheritance. In *Streptococcus pneumoniae* type 3, spontaneous generated random tandem duplications of 11–239 bp segments within the *cap3A* gene of the type 3 capsule locus are responsible for capsule phase variation (Waite, Struthers and Dowson 2001). So we sequenced the whole *has* operon including promoter and terminator regions of the capsule-deleted-like wild-type SEZ. However, any type of genetic change could not be found. The factor, which is responsible for hereditary stability in severe capsular insufficiency, is still unknown.

In conclusion, our research systematically provided evidence about SEZ invasion of non-phagocytic cells, the effect of the capsule in impeding this process and active regulation of capsule attenuation mediated by SEZ during invasion; this provides the basis for studying the mechanism of transcellular and/or paracellular routes of infection and the influence of the capsule on these processes. As infection is quite a complex process, *in vivo* and *in vitro* experiments and studies of other bacterial pathogens show a wide variety of penetration mechanisms, the influence of the internalization of SEZ into non-phagocytic cells on infection and the mechanism that induces capsule breakdown during cell invasion by SEZ will be the subjects of further research.

FUNDING

This study was supported by the National Natural Science Foundation of China [31272581, 31172319]; Program from the National Basic Research Program (973) of China [2012CB518804]; the National Transgenic Major Program [2014ZX0800946B]; the Jiangsu Agriculture Science and Technology Innovation Fund [CX (15)1056]; and the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Conflict of interest. None declared.

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