Characterization and Functional Analysis of \textit{atl}, a Novel Gene Encoding Autolysin in \textit{Streptococcus suis}

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\textit{Streptococcus suis} serotype 2 (\textit{S. suis} 2) is an important swine and human pathogen responsible for septicemia and meningitis. A novel gene, designated \textit{atl} and encoding a major autolysin of \textit{S. suis} 2 virulent strain HA9801, was identified and characterized in this study. The \textit{Atl} protein contains 1,025 amino acids with a predicted molecular mass of 113 kDa and has a conserved \textit{N}-acetylmuramoyl-L-t-alanine amidase domain. Recombinant \textit{Atl} was expressed in \textit{Escherichia coli}, and its bacteriolytic and fibronectin-binding activities were confirmed by zymography and Western affinity blotting. Two bacteriolytic bands were shown in the sodium dodecyl sulfate extracts of HA9801, while both were absent from the \textit{atl} inactivated mutant. Cell chains of the mutant strain became longer than that of the parental strain. In the autolysis assay, HA9801 decreased to 20% of the initial optical density (OD) value, while the mutant strain had almost no autolytic activity. The biofilm capacity of the \textit{atl} mutant was reduced ~30% compared to the parental strain. In the zebrafish infection model, the 50% lethal dose of the mutant strain was increased up to 5-fold. Furthermore, the adherence to HEp-2 cells of the \textit{atl} mutant was 50% less than that of the parental strain. Based on the functional analysis of the recombinant \textit{Atl} and observed effects of \textit{atl} inactivation on HA9801, we conclude that \textit{Atl} is a major autolysin of HA9801. It takes part in cell autolysis, separation of daughter cells, biofilm formation, fibronectin-binding activity, cell adhesion, and pathogenesis of HA9801.

Bacteria produce several peptidoglycan hydrolases, some of which are autolysins able to disintegrate their own peptidoglycan sacculs and lead to bacterial cell lysis in unfavorable conditions (44). Autolysins have been implicated in various biological functions, such as cell wall turnover, cell separation, cell division and antibiotic-induced autolysis (48, 55). In addition to their biological functions, bacterial autolysins are also involved or implicated in the pathogenicity of Gram-positive bacteria. Intact autolytic function is required for full virulence in \textit{Streptococcus pneumoniae} (7). Autolysin-deficient mutants, including a LytA mutant of \textit{S. pneumoniae} (8), an AtlE mutant of \textit{Staphylococcus epidermidis} (46), and Ami (36), Auto (11), p60 (41), and MurA (28) mutants of \textit{Listeria monocytogenes} are less virulent in animal models than their parental strains.

\textit{Streptococcus suis} is an important zoonotic pathogen that causes a variety of serious diseases, including meningitis, arthritis, and septicemia and even sudden death in pigs and humans (35, 49). Among the 35 serotypes of \textit{S. suis} that have been described, \textit{S. suis} serotype 2 (\textit{S. suis} 2) is the most virulent and most frequently isolated serotype. Although a set of virulence factors have been identified, the pathogenic mechanisms of \textit{S. suis} 2 are still unclear. Autolysins are believed to play an important role in cell wall metabolism and in the pathogenicity of bacteria (7). However, the concentration of autolysins in \textit{S. suis} 2 is rather low. Recently, a novel \textit{S. suis} 2 gene (GenBank accession no. EF563971) was identified using in vivo-induced antigen technology (IVIAT) (17). When analyzed by software, the protein designated \textit{Atl} was found to contain six repeated GBS_Bsp-like domains and one \textit{N}-acetylmuramoyl-L-alanine amidase domain.

In this study, we cloned the autolysin gene, \textit{atl}, from the \textit{S. suis} 2 virulent strain HA9801 and characterized its activity and function using an \textit{atl} deletion mutant and complementation strain compared with the wild type. The bacteriolytic and fibronectin-binding activity as well as the biofilm capacity of the three strains were evaluated. In addition, the in vitro adherence to eukaryotic HEp-2 cells and in vivo virulence in zebrafish were determined.

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial strains and cell culture.} Bacterial strains and plasmids used in this study are listed in Table 1. \textit{S. suis} 2 strains were grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, MI) medium or plated on THB agar containing 5% (vol/vol) sheep blood. \textit{Escherichia coli} strains were cultured in Luria broth (LB) liquid medium or plated on LB agar. \textit{S. suis} 2 strains were grown in THB supplemented with 2% yeast extract (THY) for preparation of competent cells. When necessary, antibiotics were added to the plate or broth at the following concentrations: chloramphenicol (Sigma, St. Louis, MO), \(5 \mu\text{g/ml for } S. suis, 10 \mu\text{g/ml for } E. coli\); spectinomycin (Sigma), 100 mg/ml for \textit{S. suis}, 50 mg/ml for \textit{E. coli}; ampicillin (Sigma), 100 \mu\text{g/ml for } E. coli.

\textbf{Cloning, expression, and purification of recombinant protein.} The \textit{atl} sequence (residues 673 to 3081), including the six repeated GBS_Bsp-like domains and the \textit{N}-acetylmuramoyl-L-alanine amidase domain, was amplified by PCR using HA9801 genomic DNA as the template and \textit{Atl}\textsuperscript{\textminus}F and \textit{Atl}\textsuperscript{\textplus}R as the primers (Table 2). The PCR product was cloned in-frame into the pET32a vector to generate the expression plasmid pET32a::\textit{atl}. \textit{E. coli} BL21 competent cells were transformed with the resulting plasmid and cultured in 100 ml of LB broth containing ampicillin until the optical density at 600 nm (OD\textsubscript{600}) reached 0.6. At that time, the recombinant \textit{E. coli} was induced by adding 1 mM isopropyl-\textbeta-\textgamma-thio-galactoside (IPTG) and was cultured for another 5 h. The cells were then harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended in 8 ml of 50 mM sodium phosphate (pH 7.5) containing 500 mM NaCl and 20 mM imidazole. The bacterial cells were

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Table 1} & \textbf{Bacterial Strains} & \textbf{Plasmids} \\
\hline
HA9801 & \textit{S. suis} 2 & pET32a::\textit{atl} \\
\hline
HA9801 \textit{atl} \textsuperscript{\textminus} & \textit{S. suis} 2 & pET32a::\textit{atl} \\
\hline
HA9801 \textit{atl} \textsuperscript{\textplus} & \textit{S. suis} 2 & pET32a::\textit{atl} \\
\hline
\end{tabular}
\caption{Bacterial strains and plasmids used in this study.}
\end{table}
disrupted by sonication on ice and the lysate centrifuged. The supernatant was saved for subsequent purification of the 6×His-tagged fusion proteins using a HisTrap FF column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer’s instructions.

**Preparation of purified cell wall and bacteriolytic enzyme.** Purified cell walls were prepared as described previously (6), with some modifications. Briefly, bacteria were grown in 500 ml of THB and harvested at an OD_{500} of 0.8. The cells were sedimented and washed twice with PBS. The pellets were suspended in a 4% (wt/vol) sodium dodecyl sulfate (SDS) solution in distilled water, boiled for 30 min, and the SDS heat-treated cell walls were washed six times with distilled water and lyophilized. The SDS-stable bacteriolytic enzyme of *S. suis* 2 was prepared as follows: SDS cell extracts were obtained from harvested cells by centrifugation and suspending them in 4% (wt/vol) SDS (0.6 ml/g [wet weight] of cells) for 2 h at room temperature.

**Zymography analysis.** Recombinant Atl and SDS-stable bacteriolytic enzymes were analyzed by SDS-polyacrylamide renaturing gel electrophoresis, as previously described (6, 15). Briefly, an SDS-polyacrylamide gel containing 0.1% (wt/vol) purified HA9801 cell walls was used for detection of lytic activity. Following electrophoresis, the gel was soaked for 30 min in 200 ml of distilled water at room temperature with gentle shaking. After incubation, the gel was washed in distilled water carefully, stained with 0.1% methylene blue in 0.01% KOH for 4 h, and destained in distilled water. Lytic activity appeared as a transparent band on the blue background.

**Western affinity blotting.** A Western affinity blotting assay was carried out as described previously (4). Proteins (*S. suis* SDS extracts and *E. coli* lysates) were transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Amersham, Bucks, England) using a semi-drying blotting apparatus (Amersham Pharmacia Biotech). The blotting buffer contained 2.5 mM Tris-HCl, 192 mM glycine, and 15% methanol. Membranes were incubated in Tris-buffersaline (TBS) blocking agent (20 mM Tris-HCl, 500 mM NaCl [pH 7.5 to 8.0]) containing 2.5% bovine serum albumin (BSA). Binding proteins were detected by incubating the membranes with 30 μg fibronectin ml⁻¹ for 1 h at room temperature and then with rabbit anti-human fibronectin polyclonal antibody. Antibodies were detected with the DAB peroxidase substrate kit (Tiangen, Beijing, China) and SPA-horseradish peroxidase conjugate.

**MS analysis.** Proteins corresponding to the lytic bands in the zymograms were excised from the SDS-polyacrylamide gels and sent to Nanjing Medical University for tryptic in-gel digestion and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis. Data from MALDI-TOF MS acquisitions were used in a combined search against the NCBI nr protein database using Mascot (M-

### TABLE 1 Summary of bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristic or function</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA9801</td>
<td>Virulent strain of S22 isolated from a pig that died from acute septicaemia</td>
<td>Collected in our laboratory</td>
</tr>
<tr>
<td>HA9801 Δatl mutant</td>
<td>Deletion mutant of atl with HA9801 background, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>Δatl complement strain</td>
<td>HA9801 Δatl complemented with the vector pSET2::atl, Cm&lt;sup&gt;+&lt;/sup&gt; Spc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>Cloning host for maintaining the recombinant plasmids</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>E. coli</em> BL21</td>
<td>Host for expressing the recombinant protein Atl</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSET2::Atl</td>
<td>Recombinant vector with the pSET2 background, containing the promoter followed by the full-length atl ORF</td>
<td>This study</td>
</tr>
<tr>
<td>pET32a::Atl</td>
<td>pET32a inserted in-frame with the atl gene for expressing autolysin Atl</td>
<td>This study</td>
</tr>
<tr>
<td>pSET4S::Atl</td>
<td>Recombinant vector with the pSET4S background, designed for knockout of Atl, Cm&lt;sup&gt;+&lt;/sup&gt; Spc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

### TABLE 2 Primers used for PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’–3’)</th>
<th>Restriction site</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA-F</td>
<td>CAGAAGCTTGAATAAGTCTACAGGAGATG</td>
<td>HindIII</td>
<td>Upstream border of atl</td>
</tr>
<tr>
<td>LA-R</td>
<td>CCGCCTGAGCTCTCTTTGTATGATTTAACATG</td>
<td>XhoI</td>
<td>Downstream border of atl</td>
</tr>
<tr>
<td>RA-F</td>
<td>ATCCGTCGAGAATTGCGAGTACAGGTTTATG</td>
<td>PstI</td>
<td>The fragment within atl near the start codon</td>
</tr>
<tr>
<td>RA-R</td>
<td>CCGGAATTGTACGAGCTTAAATGTTGTCACAC</td>
<td>EcoRI</td>
<td>The fragment within atl near the stop codon</td>
</tr>
<tr>
<td>Cm-F</td>
<td>GGCCTGAGCTGTAGAAAGTTGTTTGTA</td>
<td>XhoI</td>
<td>Cm&lt;sup&gt;+&lt;/sup&gt; gene cassette</td>
</tr>
<tr>
<td>Cm-R</td>
<td>GGCCTGAGTTAAGATGACAGTCGACAT</td>
<td>PstI</td>
<td></td>
</tr>
<tr>
<td>Atl5’-F</td>
<td>AGGTATATTCTTTTCTTCTTCTTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atl5’-R</td>
<td>GGTGAGCTCTTTTCTTCTTCTTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atl3’-F</td>
<td>GGGCGAGACTCGGCTGTCTTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atl3’-R</td>
<td>AACTCGAGGATTATTTTCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atl&lt;sup&gt;+&lt;/sup&gt;-F</td>
<td>ACCGAATTTGCTCTTTTCTTCTTCTTCTT</td>
<td>EcoRI</td>
<td>A fragment for expressing recombinant Atl</td>
</tr>
<tr>
<td>Atl&lt;sup&gt;+&lt;/sup&gt;-R</td>
<td>AGAAGGCTTCAATGTTTACACCTTGTTGCGCA</td>
<td>HindIII</td>
<td></td>
</tr>
<tr>
<td>Atl-F</td>
<td>ATATCGAGCGACAGATGTGATGACATACACTT</td>
<td>PstI</td>
<td></td>
</tr>
<tr>
<td>Atl-R</td>
<td>AGTGGATCCCGCTCGAAGGATAGAATTACG</td>
<td>BamHI</td>
<td></td>
</tr>
</tbody>
</table>

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TABLE 3 Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide sequence (5′–3′)</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atl-up-F</td>
<td>ATGGTTTGTGAAAAAAAAGAAGCG</td>
<td>Fragment starts from start codon of atl</td>
</tr>
<tr>
<td>Atl-up-R</td>
<td>AACTGAGATGAGAAACTTG</td>
<td>Fragment ends with terminator of atl</td>
</tr>
<tr>
<td>Atl-down-F</td>
<td>ATATCCCTTGCGAGGTGCAAAGG</td>
<td></td>
</tr>
<tr>
<td>Atl-down-R</td>
<td>CTACATTTTCCTCAAGCAGCACC</td>
<td></td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>CTTGTGATCTTCCGAAGATGAAGG</td>
<td></td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>TCATAGCAGCGTTTACTCTTACG</td>
<td></td>
</tr>
</tbody>
</table>

Microscopy. Microscopy was performed as described previously (42). Cells were grown to an OD<sub>600</sub> of 0.8 in THB at 37°C, and Gram stained cells were examined with a light microscope equipped with a 100× oil immersion objective. Furthermore, wild, mutant, and complemented strains were cultured in THB at 37°C for 16 h before the bottoms of the tubes were photographed.

Autolysis assay. The autolysis assay was carried out as previously described (19, 62). Cells were grown to an OD<sub>600</sub> of 0.8 in THB at 37°C and pelleted by centrifugation. The cells were washed once and resuspended in 50 mM Tris-HCl (pH 7.0) containing 0.05% Triton X-100 to an OD<sub>600</sub> of 0.6. The cell suspensions were incubated at 37°C with gentle shaking. The decrease in optical density was monitored every hour.

Biofilm assays. The biofilm assay was performed as previously described (9, 16). An overnight culture of S. suis 2 was diluted in fresh broth to an OD<sub>600</sub> of 0.2. Samples (100 μl) were added to the wells of a 96-well polystyrene tissue culture plate containing 100 μl of culture medium supplemented with 4 mg/ml porcine fibrinogen. After incubation for 24 h at 37°C, medium and free-floating bacteria were removed by aspiration, and the wells were washed three times with 50 mM PBS (pH 7.4). The biofilms were stained with 0.04% crystal violet (100 μl) for 10 min. The wells were washed three times with PBS to remove unbound crystal violet dye and dried for 2 h at 37°C. After adding 100 μl of 95% (vol/vol) ethanol to each well, the plate was shaken for 10 min to release the stain from the biofilms, and the absorbance at 575 nm (A<sub>575</sub>) was recorded. Wells with sterile broth medium served as controls. All biofilm assays were run in triplicate, and the mean values of three independent experiments were calculated.

LD<sub>50</sub> determinations. Care and feeding of zebrafish (Pearl River Fishery Research Institute, Chinese Academy of Fishery Science) were performed with some modifications, as described previously (38). Challenge with S. suis 2 strains in zebrafish was followed as described previously (60, 61). Briefly, log phase cultures of S. suis 2 strains were centrifuged at 10,000 × g for 10 min at 4°C. The pellets were washed twice in PBS (pH 7.4). Zebrafish were anesthetized with tricaine methanesulfonate (MS-222) (Hangzhou Animal Medicine Factory) at a concentration of 90 mg/liter. Several groups of 10 zebrafish were intraperitoneally injected with 10-fold serially diluted suspensions containing 10<sup>-1</sup> to 10<sup>-6</sup> CFU of bacteria in sterile PBS. Control fish were injected with sterile PBS. The fish were observed for 7 days until mortality was reached. The 50% lethal dose (LD<sub>50</sub>) values were calculated by the Reed and Muench method (43).

Adherence assay. The adherence assay was performed as previously described (5, 12), with some modifications. Log phase bacteria were pelleted, washed twice with PBS (pH 7.4), and resuspended in fresh cell culture medium without antibiotics. Bacteria (10<sup>5</sup> CFU) were added to wells of a 24-well tissue culture plate containing a monolayer (10<sup>5</sup> cells) of HEP-2 cells in 0.2 ml of medium (multiplicity of infection [MOI] of 100 bacteria/cell). The plates were incubated for 3 h at 37°C in 5% CO<sub>2</sub> to allow cell adherence by the bacteria. The monolayers were then washed five times with PBS, digested with 100 μl of trypsin for 15 min at 37°C, and then disrupted by the addition of 900 μl of sterile deionized water following repeated pipetting to release all bacteria. Aliquots (100 μl) diluted 1:100 and 1:1,000 in PBS were used for quantitative plating. All assays were performed in duplicate and repeated three times.

Statistical analysis. The data were analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL). The difference between mean values among groups was first evaluated by one-way analysis of variance (ANOVA) and then by pairwise comparison of the mean values between the two groups, followed by Tukey’s student rank test. Differences with a P value of <0.05 were considered significant, and a P value of <0.001 was considered greatly significant.

RESULTS

Cloning, expression, and purification of recombinant protein. The atl open reading frame (ORF) was amplified by PCR from HA9801 genomic DNA. The identity of the insert and the fidelity of the amplification were confirmed by DNA sequencing. To fa-
cililitate the high-level inducible expression of the cloned fusion
gene, plasmid pET32a::atl was transformed into E. coli BL21. The
Atl fusion protein was extracted from a mid-log culture, which
had been induced with 1 mM IPTG for 5 h. The IPTG-inducible
expression plasmid pET32a contained a start codon followed by a
6×His tag sequence, allowing purification of the recombinant au-
tolysin by using a HisTrap FF column. As shown in Fig. 1, an
apparent protein band of approximately 96 kDa appeared in the
tolysin by using a HisTrap FF column. The arrows indicate the recombinant Atl.

**FIG 1** Analysis of recombinant Atl expressed from E. coli in an SDS polyacryl-

demic gel stained with Coomassie brilliant blue R-250. Lane M, protein mo-


taneous mass marker; lane 1, crude extract from uninduced cells carrying ex-


pression vector; lanes 2, 3, 4, and 5, crude extracts from cells induced for 2, 3,


4, and 5 h, respectively; lane 6, crude extract from cells transformed with an


ew empty expression vector; lane 7, purified 6×His-tagged fusion proteins using


a HisTrap FF column. The arrows indicate the recombinant Atl.

Zymography and Western affinity blotting. The purified pro-

ctein was assayed using zymography to confirm its lytic activity. On


the zymogram of purified HA9801 cell walls, a transparent band


was detected near the 96 kDa protein standard, which was consis-


tent with the hypothesis that Atl has typical autolysin activity (Fig.


2). There were two lytic bands with the wild-type strain, which


were absent with the atl mutant strain. The complementation


strain could restore both lytic bands, regardless of the antibiotic


pressure; however, the larger band appeared very weak compared


with the smaller one (Fig. 2). The SDS extracts of S. suis 2 (including


wild-type, mutant, and complemented strains) and crude cell


lysates of IPTG-induced E. coli were tested to determine whether


Atl could adhere to fibronectin. After the chromogenic reaction,


one protein band corresponding to Atl in the recombinant E. coli


lysates gave a strong fibronectin-binding signal, while there was


no such signal in the negative control E. coli (Fig. 3). Two protein


bands gave a strong fibronectin-binding signal in the wild-type


strain, while they were absent in the atl mutant strain. The com-


plemented strain also had the two positive bands, although the


signal of the larger one was very weak (Fig. 3).

**MS analysis.** Two lytic protein bands appeared in the wild-type

strain, which were not present in the atl mutant strain. The two

protein bands were excised from the SDS-PAGE gel and charac-


terized by MALDI-TOF MS. The data were compared to those in


the NCBI sequence database. The probability score for the match,


molecular weight (MW), number of peptide matches, and the


percentage of the total translated ORF sequence covered by the


peptides were used for protein band identification. The Mascot


search results are summarized in Table 4. The two proteins corre-


sponding to the lytic bands were both identified as N-acetyl-


muramoyl-1-l-alanine amidase in S. suis 05ZYH33. Furthermore,


the matched peptides of the larger lytic protein started from


the 7th amino acid, while the smaller one started from the 111th


amino acid of the N-acetylmuramoyl-1-l-alanine amidase protein.

Construction of S. suis atl mutant. To determine the roles of


atl in S. suis 2, a homologous suicide plasmid, pSET::atl, was con-


structed with a Cm’ cassette (Fig. 4A) and electrotransformed into


HA9801 competent cells. Transformants were screened on THB


agar plates under chloramphenicol pressure. Colony PCR was


performed to detect whether atl was still present in the genome as


previously described (29). One candidate mutant in which the atl


gene failed to be amplified was further verified. The allelic replace-


ment of atl genes by the Cm’ cassette in the candidate mutant was


confirmed by multiple PCR analysis with specific primers (Fig.


4B). The results indicated that an isogenic knockout mutant of atl


(the HA9801 Δatl mutant) was successfully constructed.

Expression profiling of the atl gene. When we first carried out the

zymography analysis, the complementation strain could only
restore the smaller autolysin, although the entire atl gene was complemented (data not shown). Therefore, we examined the transcriptional profile of the full-length atl by qRT-PCR. The expression levels of the Atl-up (containing start codon of atl) and Atl-down (containing terminator of atl) were quantified by qRT-PCR. Total RNA was extracted at the exponential phase with an OD600 value of 0.8. The results indicated that the mRNA levels of Atl-up and Atl-down were the same. The mRNA levels of Atl-up and Atl-down in the atl mutant, the /H9004atl complement strain supplemented with antibiotic, the /H9004atl complement strain in the absence of antibiotic pressure were decreased by 1.0, 0.26, and 0.40, respectively, compared with the parental strain. The results confirmed the entire atl gene was transcribed after complementation regardless of the antibiotic pressure.

Microscopy. The growth curve of S. suis 2 was not affected by the atl mutation (data not shown). However, the mutant cells formed longer cell chains, and the cell chain lengths were nearly restored to that of the wild-type strain by atl complementation (Fig. 5A). This result suggests that the longer chains formed in the mutant arose from inactivation of the atl gene encoding autolysin of HA9801.

Autolysis assay. The autolytic activities of HA9801, the Δatl mutant, and the Δatl complement strain were measured. The autolysis assay results showed a decrease in optical density over time for whole-cell suspensions in buffer. There was an obvious difference among the three autolysis curves. HA9801 decreased to 20% of the initial OD value, the /H9004atl complement strain came to 58% of the initial OD value, while the mutant strain had almost no autolysis ability after 48 h of incubation (Fig. 6).

Biofilm assays. The capacities of HA9801, the Δatl mutant, and the Δatl complement strain to form biofilms in 96-well microtiter plates were evaluated. Biofilm formation by the atl mutant in THB supplemented with fibrinogen was significantly decreased compared to that of the parental strain HA9801, which was 0.73-fold of HA9801 (P < 0.05). Furthermore, the biofilm formation

### TABLE 4 Proteins corresponding to the lytic band identified by MALDI-TOF MS

<table>
<thead>
<tr>
<th>Protein no.</th>
<th>Identified protein</th>
<th>Theoretical mol mass (Da)</th>
<th>Mascot score</th>
<th>No. of peptides matched</th>
<th>Coverage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gi</td>
<td>146318948</td>
<td>N-Acetylmuramoyl-t-alanine amidase/Streptococcus suis 05ZYH33</td>
<td>113,193</td>
<td>78</td>
</tr>
<tr>
<td>2</td>
<td>gi</td>
<td>146318948</td>
<td>N-Acetylmuramoyl-t-alanine amidase/Streptococcus suis 05ZYH33</td>
<td>113,193</td>
<td>118</td>
</tr>
</tbody>
</table>

*1, protein corresponding to the lytic band with the larger molecular mass; 2, protein corresponding to the lytic band with the smaller molecular mass.*

*a* gi number in NCBI.

*c* Protein score greater than 76 was considered significant in this study (P < 0.05).

*d* Number of peptides that matched the predicted protein sequence.

*e* Percentage of predicted protein sequence covered by matched peptides.

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**FIG 4** Construction and confirmation of the HA9801 Δatl knockout mutant strain. (**A**) Strategy for deletion mutagenesis of the Δatl mutant in S. suis HA9801 by allelic exchange with a chloramphenicol resistance cassette and schematic representation of the chromosomal structures before (left) and after (right). The dotted lines represent the chromosomal sequences flanking the left and right arms of atl. (**B**) Multiple PCR analysis of the Δatl mutant. The primer combinations used in the PCR were as follows: lanes 1 and 2, LA-F/Atl5'-R; lanes 3 and 4, Atl3'-F/RA-R; lanes 5 and 6, Atl3'-F/Atl3'-R; lanes 7 and 8, Cm-F/Cm-R; lanes 9 and 10, LA-F/Cm-R; lanes 11 and 12, Cm-F/RA-R; lanes 13 and 14, LA-F/RA-R. Genomic DNA from the following strains was used as the template: wild-type strain HA9801 (lanes 1, 3, 5, 7, 9, 11, and 13) and the Δatl mutant (lanes 2, 4, 6, 8, 10, 12, and 14). The 5 kb DNA ladder marker is shown on the left (M).
by the \( \Delta \text{atl} \) complement strain was restored, but it did not reach the level of the parental strain (Fig. 7). The results revealed that the inactivation of \( \text{atl} \) reduced the biofilm formation of \( \text{S. suis} \) 2 HA9801.


TABLE 5 LD₅₀ of wild-type, complementation, and atl mutant *S. suis* 2 strains

<table>
<thead>
<tr>
<th>Dose</th>
<th>HA9801</th>
<th>HA9801 Δatl complement strain</th>
<th>HA9801 Δatl mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 × 10⁷ CFU/fish</td>
<td>15/15</td>
<td>15/15</td>
<td>15/15</td>
</tr>
<tr>
<td>2.0 × 10⁸ CFU/fish</td>
<td>13/15</td>
<td>12/15</td>
<td>10/15</td>
</tr>
<tr>
<td>2.0 × 10⁹ CFU/fish</td>
<td>9/15</td>
<td>8/15</td>
<td>6/15</td>
</tr>
<tr>
<td>2.0 × 10¹⁰ CFU/fish</td>
<td>7/15</td>
<td>6/15</td>
<td>4/15</td>
</tr>
<tr>
<td>2.0 × 10¹¹ CFU/fish</td>
<td>2/15</td>
<td>0/15</td>
<td>0/15</td>
</tr>
<tr>
<td>LD₅₀ CFU/fish</td>
<td>5.28 × 10⁴</td>
<td>1.15 × 10⁵</td>
<td>3.37 × 10⁵</td>
</tr>
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</table>

**LD₅₀ determinations.** To study the effect of the atl mutation on *S. suis* virulence, we injected zebrafish via the intraperitoneal route with either HA9801, the Δatl mutant, or the Δatl complementation strain. The mortality of zebrafish was observed within 7 days after the challenge. As shown in Table 5, the LD₅₀ was 3.37 × 10⁵ CFU/fish for the Δatl mutant, 5.28 × 10⁴ CFU/fish for HA9801, and 1.15 × 10⁵ CFU/fish for the Δatl complementation strain. These results indicated a reduction in the virulence of *S. suis* 2 in the mutant strain (*P < 0.001*), while that of the complementation strain was restored to 2-fold higher than the mutant strain. The results suggested that the inactivation of *atl* could attenuate the virulence of *S. suis* 2 in zebrafish to some extent.

**Adherence assay.** The capacity of adhesion to HEp-2 cells was compared between the parental, mutant, and complementation strains under the same conditions. Consistent with the hypothesis that autolysin of *S. suis* 2 may be involved in cell adherence, mutation of *atl* was shown to affect adherence to HEp-2 cells. As shown in Fig. 8, there was a reduction of 50% in the adherence of the Δatl mutant strain compared with the parental strain HA9801 (*P < 0.001*). The adherence of the Δatl complementation strain was approximately 1.5 times that of the Δatl mutant, which was about 75% of the parental strain.

**DISCUSSION**

Autolysins have been reported to play important roles in various biological functions, such as cell wall turnover, cell separation, cell division, and antibiotic-induced autolysis in a large range of bacterial species (48, 55). In addition to their biological functions, such as cell wall turnover, cell separation, cell division, and antibiotic-induced autolysis in a large range of bacterial species (48, 55), autolysins are also involved or implicated in the pathogenicity of Gram-positive bacteria. However, the function of the *atl* gene in *S. suis* 2 is still unclear. Thus, this study was conducted to explore the multiple roles of Atl in *S. suis* 2.

![FIG 8 Effects of *atl* on *S. suis* 2 adherence to HEp-2 cells (MOI, 100). Results are presented as percent adherence recovered from the inoculums. The data are representative of three independent experiments carried out in triplicate. The error bars indicate standard deviations. The adherence of the parental strain HA9801 and the Δatl complementation strain was significantly different compared with that of the Δatl mutant strain (*P < 0.001*).](http://jb.asm.org)
teolytic enzyme HtrA in *E. coli*. Generally, chaperones and proteases are thought to bind to exposed hydrophobic sequences, present in unfolded or misfolded proteins in *E. coli*, and protein quality control is carried out by a protein network, comprised of chaperones and proteases (14). A critical question that immediately arises is how the unfolded larger autolysin can survive in the host cell. The most likely explanation is that the activity of the proteolytic enzyme is tightly regulated in the host cell, being carried out by the proteasomes in response to some specific signal (59). Therefore, the levels of the larger autolysin and the smaller autolysin can be rigidly controlled in the wild-type strain. After functional complementation with the recombinant plasmid pSET2:atl, it is interesting to note that the ratio of the larger autolysin to the smaller autolysin changed greatly compared with the bands in the SDS extracts, we can conclude that the Atl plays a major role in *HA9801* autolysis when suspended in lysis buffer, the OD$_{600}$ of HA9801 was reduced to 20% of the initial value, and the complementation strain had partially restored autolysis capacity (Fig. 2). The results revealed that Atl plays a major role in *HA9801* autolysis when bacteria are in adverse conditions. Having observed that recombinant Atl demonstrated peptidoglycan hydrolysis activity and that the inactivation of *atl* causes the disappearance of two lytic bands in the SDS extracts, we can conclude that the *atl* gene cloned in this study encodes the major autolysin of *S. suis* 2 and plays a major role in HA9801 autolysis.

Light microscopic observations revealed that the *atl* mutant cells connected to each other to form very long cell chains compared with those formed by the parental strain (Fig. 5A). The inactivation of the *lytB* gene also leads to the formation of long chains of cells in *S. pneumoniae* (13), and the inactivation of *atlA* causes the formation of long chains in *S. mutans* and *Staphylococcus aureus* (47). In this study, most *atl* mutant cells sedimented when grown overnight, while the broth culture of wild-type cells was turbid, with fewer sedimented cells (Fig. 5B). As the growth rate of the cells was not affected in the *atl* mutant (data not shown), the difference in sedimented cells could be attributed to its longer cell chain length.

In addition to the bacteriolytic activity, some staphylococcal autolysins have adhesive functions. The *S. epidermidis* autolysin AtlE mediates the primary attachment to polystyrene and binds vitronectin, whereas its binding to fibronectin is very weak and not reproducible (19). The *Staphylococcus saprophyticus* autolysin Aas binds fibronectin and sheep erythrocytes, and the binding domain has been mapped to the central region containing the repeats (20). Whether *S. suis* 2 autolysin Atl also has such an adhesive function needs to be explored. Here, we used the SDS extracts of *S. suis* (wild-type, mutant, and complemented strains) and crude cell lysates of IPTG-induced *E. coli* to test whether Atl could adhere to fibronectin. Both the natural and recombinant autolysin gave strong positive fibronectin-binding signals (Fig. 3), revealing the fibronectin-binding activity of Atl. The *atl* mutant strain showed greatly reduced adherence to the HEp-2 eukaryotic cells in vitro. The primary attachment of the *atl* mutant to HEp-2 was reduced by approximately 2-fold (*P < 0.05*) in comparison to that of the wild-type strain, indicating that Atl can facilitate bacterial adherence to the host cell. The other autolysins, including Aas of *S. saprophyticus* (22), AtIC of *S. caprae* (3), AtlE of *S. epidermidis* (21), and Ami of *L. monocytogenes* (36), have also been shown to be involved in bacterial adhesion. Binding to fibronectin has been suggested to play an important role in adherence of the group A *Streptococcus pyogenes* to host epithelial cells (20). The finding that Atl could bind to fibronectin may explain how autolysin Atl affects the *S. suis* 2 adherence to HEp-2 cells.

Compared with the parental strain, biofilm formation of the *atl* mutant was significantly decreased in a microtiter plate assay. The complementation strain restored biofilm formation at a specific concentration. In *Enterococcus faecalis*, the autolysin (*atl*) mutant also causes reduction in biofilm formation compared with the parental strain (37). Previous studies have indicated that cells in mutant biofilms (e.g., the *comC* mutant, the *brpA* mutant, and the *hr11* and *rr11* mutants) with abnormal structures tend to form long chains, suggesting that the variation in biofilm structure may result from the formation of extremely long chains of cells (30, 31, 56). Biofilm formation is thought to be a two-step process that requires the adhesion of bacteria to a substrate surface followed by cell-cell adhesion, forming the multiple layers of the biofilm. *S. suis* biofilm formation on a polystyrene surface is stimulated in a dose-dependent manner when fibrinogen is added to the culture medium (9). Perhaps fibrinogen promotes the biofilm formation by strengthening cell-cell adhesion. In this study, we found that autolysin Atl could bind to fibrinogen and contribute to the fibrinogen-mediated cell-cell adhesion, which is the main step of biofilm formation. The hypothesis was supported by the fact that the *atl* isogenic mutant strain had greatly reduced biofilm formation compared with the parent strain.

In *vivo* studies with animal models (mice, rats, and guinea pigs) of infection have shown that mutant strains defective in autolysin synthesis, including AtlE of *S. epidermidis* (45), p60, Ami and AutoL of *L. monocytogenes* (11, 36, 41), and LytA of *S. pneumoniae* (8), are less virulent than the wild-type strains. Autolysin also plays crucial roles in the pathogenesis of pneumococcal meningitis (24). In the last few years, zebrafish has been used as a model for human disease, cancer and immunological studies, and some experimental infections have been conducted with several streptococcal species such as *Streptococcus iniae*, *S. pyogenes* (34, 38), and *Streptococcus agalactiae* (40). The fact that zebrafish have adaptive and innate cellular immune defenses that are not unlike those of the mammalian species most often used to model infectious diseases of humans (52) makes them highly useful as a model host organism. Fish have immunoglobulins, antigen-processing cells, T cells, and B cells (18, 57, 58) as well as complement and phagocytic cells and leukocytes capable of producing reactive species of oxygen and nitrogen (23). Thus, it is likely that the underlying principles of host-pathogen relationships in fish will be very similar to those in mammalian species. Upon infection with *S. suis*, the transcriptional responses in zebrafish have shown clear conservation with host responses detected in porcine cells, human cells, or
mammalian models, including induction of genes involved in the immune response, inflammatory response, complement activation, and defense response, and provided further validation of this model for *S. suis* infection (61). That Atl may affect the virulence of *S. suis* was supported by observations of the LD₅₀ in zebrafish. A 5-fold reduction of virulence was observed *in vivo* for the *atl* mutant compared with the wild type. Given that the *atl* mutant strain had attenuated adherence to HEP-2 cells and lower virulence in zebrafish and that the *atl* complementation strain could restore these phenotypes to some extent, we concluded that autolysin Atl contributes to the virulence of *S. suis* 2.

The mechanism by which autolysin Atl affects the virulence of *S. suis* 2 may be explained by the following observations of our work and others. LytA of *S. pneumoniae* and MurA and p60 of *L. monocytogenes* are thought to mediate the release of cytoplasmic toxins or proinflammatory degraded cell wall components resulting in subsequent tissue injuries (25, 28, 33, 53). When it comes to the *atl* mutant strain, the release process may be hindered for the loss of autolysis capacity.

In Gram-positive bacteria, the presentation or release of virulent factors is crucially dependent on the continuous remodeling of the cell wall (10), and a defect in this process in the *atl* mutant could have contributed somewhat to the attenuated virulence. Adherence of pathogenic microorganisms to the cell surface is also a key event during infection (36). Previous findings have suggested that certain autolysins may contribute directly to the pathogenicity of Gram-positive bacteria by mediating bacterial adherence (21). It stands to reason that the *atl* mutation reduced the bacterial adherence and therefore weakened the virulence of *S. suis* 2. AtlA, an autolysin of *Streptococcus mutans*, is a fibronectin-binding protein that contributes to bacterial survival in the bloodstream and virulence, causing infective endocarditis (26). The *suis* 2 autolysin Atl could also bind fibronectin and thereby escape the host cell defense.

The results presented herein provide insights into novel roles of Atl, a suspected autolysin of *S. suis* that takes part in the cell autolysis, separation of daughter cells, biofilm formation, fibronectin-binding activity, cell adhesion, and pathogenesis. The expression, distribution, and activation of the autolysin are likely to be tightly controlled in *S. suis*. Understanding this mechanism could provide unique insights into the pathogen and facilitate the development of new strategies for the treatment of *S. suis* infection.

**ACKNOWLEDGMENTS**

This work was financially supported by the Fund of the National Natural Science Foundation of China (U0931002) and the Special Fund for Public Welfare Industry of Chinese Ministry of Agriculture (20083016).

We thank Tsutomu Sekizaki and Daisuke Takamatsu for kindly providing plasmid pSET4s for this study.

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38. Reference deleted.

March 2012 Volume 194 Number 6 jb.asm.org 1473

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