In this study, we investigated the cell wall-anchored fibronectin-binding proteins SpsD and SpsL from the canine commensal and pathogen *Staphylococcus pseudintermedius* for their role in promoting bacterial invasion of canine progenitor epidermal keratinocytes (CPEK). Invasion was examined by the gentamicin protection assay and fluorescence microscopy. An Δ*spsD ΔspsL* mutant of strain ED99 had a dramatically reduced capacity to invade CPEK monolayers, while no difference in the invasion level was observed with single mutants. *Lactococcus lactis* transformed with plasmids expressing SpsD and SpsL promoted invasion, showing that both proteins are important. Soluble fibronectin was required for invasion, and an RGD-containing peptide or antibodies recognizing the integrin α5β1 markedly reduced invasion, suggesting an important role for the integrin in this process. Src kinase inhibitors effectively blocked internalization, suggesting a functional role for the kinase in invasion. In order to identify the minimal fibronectin-binding region of SpsD and SpsL involved in the internalization process, recombinant fragments of both proteins were produced. The SpsD<sub>526–846</sub> and SpsL<sub>538–823</sub> regions harboring the major fibronectin-binding sites inhibited S. *pseudintermedius* internalization. Finally, the effects of staphylococcal invasion on the integrity of different cell lines were examined. Because SpsD and SpsL are critical factors for adhesion and invasion, blocking these processes could provide a strategy for future approaches to treating infections.

The Gram-positive bacterium *Staphylococcus pseudintermedius* is a common commensal of dogs (1, 2). The bacterium is also the most common pathogen associated with canine otitis externa and pyoderma as well as surgical wound infections and urinary tract infections (3). Sporadic cases of human infection have also been reported, including some in individuals exposed to colonized household pets (4–7). Genome sequence analysis (8, 9) indicates that *S. pseudintermedius* could encode many potential virulence factors, including toxins, enzymes, and surface proteins, some of which can promote adhesion of the bacterium to the surface of epithelial cells (10–13) and to components of the extracellular matrix (14, 15).

Two cell wall-anchored surface proteins that are likely to be important in host tissue colonization and pathogenesis are SpsD and SpsL. (Fig. 1) (15). The primary translation product of SpsD from strain ED99 has an N-terminal secretory signal sequence and a C-terminal cell wall-anchoring domain (the sorting signal) comprising an LPXTG motif, a hydrophobic transmembrane domain, and a short sequence rich in positively charged residues. Residues at the N terminus of SpsD are 40% identical to the fibrinogen-binding A domain of FnBPA from *Staphylococcus aureus* and are predicted to fold into three subdomains: N1, N2, and N3. This domain is followed by a connecting region, region C, and a repeat region, region R. SpsL includes a signal sequence at the N terminus followed by an A domain with two IgG-like folds (N2 and N3), a domain containing seven tandem repeats with weak homology to the fibronectin binding repeats of FnBPA from *S. aureus*, and a C-terminal sorting signal.

SpsD and SpsL mediate bacterial adherence to fibrinogen (15) and fibrinogen (Fn) (15), while SpsD also binds to cytokertatin 10 and elastin (16). The binding site in fibrinogen for SpsD was mapped to residues 395 to 411 in the γ-chain, while a binding site for SpsD in Fn was localized to the N-terminal region. SpsD also binds to glycine- and serine-rich omega loops within the C-terminal tail region of cytokeratin 10 (16).

Another important Sps protein involved in host colonization is SpsO, which has been demonstrated to mediate adherence to *ex vivo* canine keratinocytes (12). However, the host ligand(s) interacting with SpsO remains to be determined (15).

The SpsO protein of *S. pseudintermedius* is also likely to be involved in colonization of the canine host. It promotes adhesion to *ex vivo* canine corneocytes, as does SpsD, although the ligand(s) recognized by SpsO remains to be identified. Invasive bacteria actively induce their own uptake by phagocytosis into normally nonphagocytic cells, where they establish a protected niche within which they can replicate (17). For example, *S. aureus*, usually considered an extracellular pathogen, can invade a variety of nonpro-

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fessional phagocytic cells, explaining its capacity to colonize mucosa and its persistence in tissue after bacteremia. The underlying major molecular mechanism of invasion involves the Fn-binding adhesins FnBPA and FnBPB (18, 19). Fn bridging between FnBPs and $\alpha_5\beta_1$ integrins on the host cell surface is sufficient to induce zipper-type uptake of staphylococci (18–20). The ternary complex promotes integrin clustering and a relay of signals that result in cytoskeletal rearrangements. The rearrangements are accompanied by endocytosis of S. aureus and internalization (17).

In this study, we wished to investigate whether S. pseudintermedius shares with S. aureus the ability to invade nonprofessional phagocytic cells and to determine the bacterial and host components that are involved. We reasoned that both SpS-D and SpS-L could be involved in the internalization of S. pseudintermedius by host cells. The objective of this study was to investigate internalization and its mechanistic basis. The analysis of this process will provide insights into the potential of a vaccine comprising components of SpS-D and SpS-L for the prevention of canine pyoderma.

MATERIALS AND METHODS
Bacterial strains and culture conditions. S. pseudintermedius strain ED99 (formerly M732/99) was isolated from a canine bacterial pyoderma case presented to the Dermatology Service of The Hospital for Small Animals, Division of Veterinary Clinical Sciences, The Royal (Dick) School of Veterinary Studies, The University of Edinburgh. S. pseudintermedius strains 264, 324, 326, 327, 328, and 329 were isolated from cases of canine pyoderma and were donated from Veterinary Studies, The University of Edinburgh, S. pseudintermedius strains 81852, 91180, 253834, 237425, and 235214/1 were isolated from cases of canine pyoderma and were donated from Instituto Zooprofilattico Sperimentale della Lombardia e della Emilia Romagna, Pavia, Italy. The strains were classified as S. pseudintermedius using standard phenotypic tests (21). S. pseudintermedius ED99 and its mutants were grown in brain heart infusion (BHI) broth (VWR International Srl, Milan, Italy) at 37°C with shaking. Transformants of Lactococcus lactis harboring plasmid pOrI23, pOrI23::spS-D, or pOrI23::spS-L (15) were grown in M17 medium (Difco, Detroit, MI, USA) supplemented with 10% lactose, 0.5% glucose, and 10 $\mu$g/ml $\times 100$ erthromycin at 30°C without shaking, Escherichia coli DC10B (22) and TOPP3 (Stratagene, La Jolla, CA) were grown in Luria agar (LA) and Luria broth (LB) (VWR International Srl).

Reagents, proteins, and antibodies. Human fibronectin was purified from plasma by a combination of gelatin- and arginine-Sepharose affinity chromatography. The purity of the protein was assessed by 7.5% SDS-PAGE and Coomasie brilliant blue staining. To exclude the possibility of trace amounts of contaminants, affinity-purified fibronectin was spotted onto nitrocellulose membranes at different concentrations and overlaid with antifibrinogen and antiplasminogen antibodies (23). The N-terminal fragment of Fn (N29), containing the five N-terminal type I modules, and the gelatin-binding domain (GBD), consisting of four type I modules and two type II modules, were isolated as previously reported (24). Unless stated otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-human Fn rabbit polyclonal IgG was purchased from Pierce (Rockford, IL, USA). The mouse monoclonal antibody JBS5 against the human integrin $\alpha_5\beta_1$ was purchased from Merck-Millipore (Darmstadt, Germany). The rabbit polyclonal antibody against the $\alpha_5$ chain of the $\alpha_5\beta_1$ integrin and the mouse monoclonal antibodies BV7 against the human $\beta_1$ chain and B212 against the human $\beta_3$ chain of the integrin $\alpha_5\beta_3$ were a generous gift from G. Tarone (University of Turin, Italy). Mouse polyclonal antibodies against region A of SpS-D and SpS-L were prepared as previously reported (16).

DNA manipulation. DNA encoding regions SpS-D164–523, SpS-D520–846, SpS-L444–960, SpS-L220–531, and SpS-L538–823 were amplified by PCR using S. pseudintermedius ED99 genomic DNA as the template. Oligonucleotides were purchased from Integrated DNA Technologies (Leuven, Belgium) (see Table S1 in the supplemental material). Restriction enzyme cleavage sites (see Table S1) were incorporated at the 5’ ends of the primers to facilitate cloning into plasmid pQE30 (Qiagen, Chatsworth, CA, USA). Restriction enzymes were purchased from New England BioLabs (Hertfordshire, United Kingdom). The integrity of cloned DNA was confirmed by sequencing (Primmibiotec, Milan, Italy).

Expression and purification of recombinant proteins. Recombinant proteins were expressed from pQE30 in E. coli TOPP3 (Stratagene). Overnight starter cultures were diluted 1:50 in LB containing ampicillin (100 $\mu$g ml$^{-1}$) and incubated with shaking until the culture reached an optical density at 600 nm (OD$_{600}$) of 0.4 to 0.6. Recombinant protein expression was induced by addition of isopropyl-1-thio-$\beta$-D-galactopyranoside (0.5 mM) and continued for 2 h. Bacterial cells were harvested by centrifuga-
tion and frozen at −80°C. Recombinant proteins were purified from cell lysates by Ni²⁺ affinity chromatography on a HiTrap chelating column (GE Healthcare, Buckinghamshire, United Kingdom). Protein purity was assessed to be 98% by SDS-PAGE, Coomassie brilliant blue staining, and densitometric analysis.

**ELISA-type solid-phase binding assays.** The ability of immobilized recombinant proteins to interact with soluble human Fn was determined using enzyme-linked immunosorbent assays (ELISAs). Microtiter wells were coated overnight at 4°C with 100 μl of 10 μg ml⁻¹ of bacterial protein in 50 mM sodium carbonate, pH 9.5. To block additional protein-binding sites, the wells were treated for 1 h at 22°C with 200 μl of 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The plates were then incubated for 1 h with increasing amounts of Fn. One microgram of the specific anti-Fn rabbit IgG (1:2,000) in PBS with 0.1% BSA was added to the wells, followed by incubation for 90 min. After washing, the plates were incubated for 1 h with peroxidase-conjugated secondary anti-rabbit IgG diluted 1:1,000. After washing, o-phenylenediamine dihydrochloride was added and the absorbance at 490 nm was determined. To calculate the relative affinity association constant (K_A) values of each bacterial protein for Fn the following equation was employed: $A = A_{max} [1 + K_A/1 + K_A/L]$, where [L] is the molar concentration of ligand. The dissociation constants (K_d) values were calculated as reciprocals of the K_A values. The assays were performed at least 3 times for each protein.

**Construction of spsD- and spsL-null mutants.** Allele replacement mutagenesis of spsD and spsL was performed using the thermosensitive plasmids pIMAY and pIMAY-Z (Table 1). For generation of the spsD-null mutation, approximately 500-bp fragments of DNA flanking the gene were PCR amplified using the AB and CD primers, spliced together, and inserted into the blunt-end ligation pSC-B vector (StrataClone, Agilent Technologies, Santa Clara, CA) before subcloning into pIMAY to produce the pIMAYΔspsD construct. The plasmid was transformed into E. coli DC10B before being electrotransformed into S. pseudintermedius ED99 at 28°C with selection on 10 μg ml⁻¹ m-chlorophenol, as previously described for S. aureus (25). For generation of the spsL-null mutation, a sequence ligation-independent cloning (SLIC) protocol was performed (26) using pIMAY-Z, a derivative of pIMAY with a constitutive lacZ marker, to construct pIMAY-ZΔspsL. Once the plasmids were transformed into ED99 at 28°C, growth at the restrictive temperature of 37°C selected for integrants. OUT primers, located outside the flanking regions and gene of interest, were used to determine if integration had occurred upstream or downstream of the chromosomal gene (22). A single colony from each site of integration was inoculated into broth and grown at 28°C and then diluted and grown at 37°C. The S. aureus antisense secY mechanism within pIMAY (27) was nonfunctional in S. pseudintermedius, and the lacZ marker was ineffective because plasmid-free cells expressed endogenous β-galactosidase activity. Allele exchange was confirmed by using OUT primer PCR and sequencing the resultant fragment (see Table S2 in the supplemental material).

**Release of surface proteins from S. pseudintermedius and L. lactis.** S. pseudintermedius and L. lactis cells were grown to an OD₆₀₀ of 0.4 to 0.6. Cells were harvested by centrifugation at 7,000 × g for 4°C for 15 min, washed 3 times with PBS, and resuspended at an OD₆₀₀ of 40 in lysis buffer (50 mM Tris-HCl, 20 mM MgCl₂, [pH 7.5]) supplemented with 30% raffinose. Cell wall proteins were solubilized from S. pseudintermedius by incubation with lysostaphin (200 μg ml⁻¹) and from L. lactis with mutanolysin (1,000 U/ml) and lysozyme (900 μg ml⁻¹) at 37°C for 20 min in the presence of protease inhibitors (Complete Mini; Roche Molecular Biochemicals, Indianapolis, IN, USA). Proteolysates were recovered by centrifugation at 6,000 × g for 20 min, and the supernatants were taken as the wall fractions. The material obtained from S. pseudintermedius ED99 and its mutants was adsorbed on IgG-Sepharose columns before Western immunoblotting analysis to remove IgG-binding proteins that would otherwise interfere with the specific antibody staining.

**SDS-PAGE and Western immunoblotting.** Samples for analysis by SDS-PAGE were boiled for 5 min in sample buffer (0.125 M Tris-HCl, 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 10% [vol/vol] β-mercaptoethanol, 0.002% [wt/vol] bromophenol blue) and separated on 10% (wt/vol) polyacrylamide gels. The gels were stained with Coomassie brilliant blue (Bio-Rad, Hercules, CA, USA). For Western immunoblotting, material was subjected to SDS-PAGE and then electroblotted onto a nitrocellulose membrane (GE Healthcare). The membrane was blocked overnight at 4°C with 5% (wt/vol) skim milk in PBS, washed, and incubated with mouse polyclonal antibody against region A of SpStD or SpS (1 μg ml⁻¹) for 1 h at 22°C. Following additional washings with 0.5% (vol/vol) Tween 20 in PBS (PBST), the membrane was incubated for 1 h with horseradish peroxidase-conjugated rabbit anti-mouse IgG. Finally, blots were developed using the ECL Advance Western blotting detection kit (GE Healthcare) and an ImageQuantTM LAS 4000 mini-biomolecular imager (GE Healthcare).

**Mammalian cell lines and culture conditions.** Canine progenitor epidermal keratinocytes (CPEK) were cultured in CnT-0.9 medium (CELLInTEC, Bern, Switzerland), without antibiotics at 37°C in 5% CO₂. The spontaneously immortalized keratinocytes (HaCaT) and the human epithelial cell line HEp-2 were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) ( Gibco BRL, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) ( EuroClone, Milan, Italy), 2% penicillin and streptomycin, 2% sodium pyruvate, and 2% l-glutamine at 37°C in 5% CO₂. Cells were cultured in T75 flasks to approximately 95% confluence, liberated with trypsin-EDTA (EuroClone), resuspended in invasion medium (growth medium without antibiotics), and plated as reported below for the cell invasion assay.

**Cell invasion assay.** Cell invasion assays were performed essentially as described previously (28). Briefly, cells were plated at 5 × 10⁴ in (0.4 ml invasion medium) into 24-well plates (Corning) and allowed to attach for 24 h at 37°C. Staphylococcal cultures were grown overnight in BHI at 37°C with shaking. L. lactis was grown overnight in M17 broth at 30°C without shaking. The following day, cultures were diluted 1:40 in fresh BHl or M17 medium, respectively, grown to an OD₆₀₀ of 0.4, washed 3 times in PBS, and diluted to obtain 10⁷ cells/ml in CnT-BM.2 medium supplemented with 10% FBS plus 2 mM L-glutamine. Bacterial suspensions (1 ml) were added to each well and the plates incubated for 2 h at 37°C. Monolayers were then washed 3 times in PBS to remove unattached bacteria. Medium containing antibiotics (200 μg ml⁻¹ gentamicin plus 2% penicillin and streptomycin) was added, and the plate was incubated for an additional 2 h to kill extracellular bacteria. The wells were washed again, and internalized bacteria were released by incubating with 200 μl of H₂O₂ containing 0.1% (vol/vol) Triton X-100. Serial dilutions of the cell lysates were plated on BHI agar, and CFU were counted after incubation. All assays were carried out in triplicate. Samples of monolayers were lysed prior to inoculation and plated on BHI agar, and the absence of staphylococcal colonies was noted.

**Inhibition of invasion.** The Src-family kinase inhibitors PP2, PP3, and CGP77675 (25 μM) were dissolved in dimethyl sulfoxide (DMSO), added to the cell medium at the appropriate concentrations, and preincubated with monolayers for 1 h at 37°C in 5% CO₂ before addition of bacteria. Likewise, wortmannin (20 nM), genistein (200 μM), and cy-

### Table 1: Plasmids used for the construction of spsD- and spsL-null mutants

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>pIMAY</td>
<td>Thermosensitive plasmid for allelic exchange</td>
<td>21</td>
</tr>
<tr>
<td>pIMAYΔspsD</td>
<td>pIMAY with fragments flanking spsD</td>
<td>This paper</td>
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<tr>
<td>pIMAY-Z</td>
<td>pIMAY derivative with a constitutive lacZ marker</td>
<td>25</td>
</tr>
<tr>
<td>pIMAY-ZΔspsL</td>
<td>pIMAY-Z with fragments flanking spsL</td>
<td>This paper</td>
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tchalasin D (50 μM) were dissolved in PBS and incubated with cells for 60 min prior to the addition of bacteria. Gentamicin protection assays were then performed as described above, except that no intermediate washing was carried out. To test cell viability during exposure to the Src inhibitors, the compounds were added to cell monolayers for 3 h at 37°C. Then the cells were gently washed with DMEM, trypsinized, and mixed with an equal volume of trypan blue (0.5% [vol/vol] in PBS) for 5 min. Ten microliters of the mixture was placed on a Neubauer chamber, and stained cells were counted by light microscopy. The percentage of dead cells was calculated by dividing the mean number of dead (stained) cells by the total number of cells in 50 microscopic fields and multiplying the result by 100.

Fluorescence microscopy. Bacteria were grown to an OD_{600} of 0.3 (S. pseudintermedius) or 0.4 (L. lactis), centrifuged, and resuspended in 100 μl PBS. Then, 0.5 μl 10 mM calcein-AM (Molecular Probes, Eugene, OR, USA) was added and incubated for 1 h at 37°C (S. pseudintermedius) or 2 h at 30°C (L. lactis). Stained bacteria were washed 3 times with PBS and resuspended in 1 ml PBS. Suspensions (100 μl) were added to CPEK monolayers and incubated for 2 h at 37°C to allow internalization. Cells were washed with PBS, counterstained for 1 to 3 min with ethidium bromide (10 μg ml^{-1}), and washed again. Fluorescence microscopy (Olympus BX51; Olympus, Segrate, Italy) was performed using a green filter, a red filter, and white light. Images were captured with a charge-coupled device (CCD) camera and assembled using Adobe Photoshop Creative Suite 2.

Staining of monolayers. Mammalian cells were stained with Giemsa stain modified solution (Sigma) according to the manufacturer’s instructions and observed under a light microscope at a magnification of ×20.

Invasion assays with formaldehyde-fixed staphylococci. To perform invasion assays with killed bacteria, staphylococci were fixed in 0.5% formaldehyde in PBS for 1 h, stained with calcein-AM, and subjected to fluorescence microscopy. Alternatively, to analyze the effect of formaldehyde-fixed staphylococci on cell survival, monolayers were stained with Giemsa and observed as reported above.

MTT assay. The MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] tetrazolium reduction assay was performed according to the manufacturer’s instructions (Sigma).

Statistical methods. Continuous data were expressed as means and standard deviations. Two-group comparisons were performed by Student’s t test. One-way analysis of variance, followed by Bonferroni’s post hoc tests, was exploited for comparison of three or more groups. Analyses were performed using Prism 4.0 (GraphPad). Two-tailed P values of 0.001 were considered statistically significant.

RESULTS

SpSD and SpSL binding to fibronectin. To localize the Fn-binding sites in SpSD and SpSL, recombinant domains were obtained following PCR amplification of genomic DNA from strain ED99. The cloned SpSD domains included the minimum fibrinogen-binding region (residues 164 to 523) (SpSD164–523), a connecting region (region C, residues 520 to 846) (SpSD520–846), and a repeat region (region R, residues 844 to 960) (SpSD844–960). Two recombinant SpSD domains were expressed: the N-terminal region (region R, residues 844 to 960) (SpSD844–960). SpSD520–846 and SpSL538–823 bound Fn in the low nanomolar range (SpSD520–846, K_D = 1.7 ± 0.38, SpSL538–823, K_D = 0.81 ± 0.02 nM), while SpSD164–523 gave a half-maximal binding of 2.19 ± 0.47 μM (data not shown).

Invasion of mammalian cells by S. pseudintermedius. Several S. pseudintermedius isolates were found to invade canine keratino-

FIG 2 Dose-dependent binding of fibronectin to SpSD and SpSL fragments in an ELISA-type assay. Microtiter wells were coated with SpSD520–846, SpSL844–960, SpSL220–531, and SpSL538–823. The wells were probed with increasing amounts of Fn, followed by incubation with rabbit anti-Fn IgG and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG. The graph is representative of three experiments, with each point representing the average for triplicate wells.

When bacteria were preincubated with increasing amounts of the N29 fragment of Fn and then tested for adherence to or invasion of CPEK, we observed an almost complete inhibition of bacterial internalization. Conversely, no effect was observed when the invasion assay was performed with a high concentration of the GBD of Fn (Fig. 4B). Together, these findings indicate that the N29 domain is specifically involved in adhesion to and invasion of CPEK.
Invasion of CPEK by ED99 mutants and \textit{L. lactis} expressing SpsD or SpsL. Mutants of \textit{S. pseudintermedius} ED99 deficient in SpsD and SpsL were tested for their ability to attach to surface-coating Fn. Mutants defective in either SpsD or SpsL alone adhered as well as the parental strain, while the double mutant defective in both proteins did not bind at all (data not shown). The absence of SpsD or SpsL proteins was confirmed by testing material solubilized from the cell wall with lysostaphin by Western blotting and probing with antibodies against region A of SpsD or SpsL. Both the proteins were absent from the double mutant (Fig. 5A). Conversely, SpsL was expressed normally by the SpsD mutant and vice versa.

The SpsD- and SpsL-defective mutants were also tested for invasiveness. Single mutants retained the ability to invade CPEK at the same level as the wild type, while the double mutant lacking both SpsD and SpsL invaded at a much lower level (Fig. 5B).

In order to test whether coreceptors are required for SpsD- or SpsL-mediated invasion, we expressed both \textit{S. pseudintermedius} proteins individually in \textit{Lactococcus lactis} (Fig. 6A). Transformants of \textit{L. lactis} carrying plasmid pOri23::spsD and pOri23::spsL (Fig. 6A) showed invasiveness similar to that of the wild-type strain ED99 (Fig. 5B), while very low internalization by CPEK was observed with \textit{L. lactis} harboring the empty plasmid (Fig. 6B).

Reduced invasion by the double mutant of ED99 was also assessed by visualizing uptake into CPEK by fluorescent imaging. Bacteria were stained with calcein-AM (green) prior to CPEK invasion, and at the assay endpoint, the fluorescence of external bacteria was visualized with ethidium bromide (red). As shown in Fig. 5C, the wild type and the single mutant strains were observed inside CPEK, while no green fluorescence, indicative of the internalized bacteria, was detected when the double mutant was tested. \textit{L. lactis} expressing SpsL or SpsD behaved similarly (Fig. 6C). Together these results demonstrate that expression of a single adhesin (SpsD or SpsL) is sufficient to confer efficient uptake of bacteria into CPEK.

Localization of Sps domains promoting invasion of CPEK. To identify the domains of SpsD and SpsL that are involved in invasion, recombinant fragments were assessed for inhibition of \textit{S. pseudintermedius} ED99 uptake into CPEK. We found that SpsD520 – 846 (Fig. 7A) and SpsL538 – 823 (Fig. 7B) strongly inhibited internalization, whereas SpsD164 – 523 showed a weak inhibitory effect. The inhibitory effects exhibited by these proteins correlate with their affinities for fibronectin. SpsL220 – 631 and SpsD523 – 846 used at the same concentrations did not interfere with staphylococcal invasion.

Dependence of invasion on integrin $\alpha_5\beta_1$. Immunofluores-
cent antibodies that specifically bind to the $\alpha_5$ subunit of the human Fn-binding $\alpha_5\beta_1$ integrin stained CPEK, suggesting that the canine cells express an $\alpha_5\beta_1$ integrin that is closely related to the human integrin (Fig. 8A, inset). To test the role of the $\alpha_5\beta_1$ integrin in invasion, CPEK were preincubated with function-blocking anti-$\alpha_5\beta_1$ IgG prior to the addition of *S. pseudintermedius*. Antibodies recognizing the $\alpha_5$ and the $\beta_1$ chains both reduced internalization of *S. pseudintermedius* by more than 80%, whereas antibodies against the $\beta_3$ chain of the human $\alpha_5\beta_3$ integrin did not alter invasion (Fig. 8A). This indicates that the $\alpha_5\beta_1$ integrin on canine CPEK is responsible for Fn-mediated bacterial invasion.

**Inhibition of invasion by an RGD-containing peptide.** The $\alpha_5\beta_1$ integrin recognizes the tripeptide sequence RGD within the cell-binding domain of Fn (29, 30). To test the role of this interaction in invasion of *S. pseudintermedius*, the effect of the RGDS peptide was analyzed. Incubation of CPEK with the RGDS peptide reduced the level of invasion by strain ED99 in a dose-dependent manner, while a control peptide RGES had no inhibitory effect (Fig. 8B). This suggests that the interaction of $\alpha_5\beta_1$ with Fn is necessary for efficient invasion of CPEK.

**Protein phosphorylation during *S. pseudintermedius* invasion.** To identify changes in host cell signaling associated with staphylococcal invasion, the assay was performed in the presence of protein tyrosine phosphorylation inhibitors. Genistein, a tyrosine kinase inhibitor, strongly inhibited internalization, whereas wortmannin, an inhibitor of the phosphatidylinositol-3-phosphate kinase, did not (Fig. 8C). We also tested Src kinase inhibitors and found that both CGP77675 and PP-2 inhibited *S. pseudintermedius* internalization into CPEK. While both inhibitors effectively blocked internalization, CGP77675 appeared to be a more potent inhibitor than PP-2. PP-3, a compound similar to PP-2 but with no significant Src inhibitory activity, had no effect on internalization (Fig. 8C). At the concentrations used, the inhibitors did not affect bacterial adhesion to the CPEK or cause loss of viability, as shown by trypan blue staining (data not shown). To investigate a possible role for actin cytoskeletal rearrangements in *S. pseudintermedius* invasion, we tested cytochalasin D, which interferes with F-actin polymerization. Pretreatment of CPEK with 1 $\mu$g/ml cytochalasin D almost completely abolished invasion, demonstrating the involvement of actin cytoskeletal rearrangements (Fig. 8C).

**Invasion of Hep-2 and HaCaT cell lines by *S. pseudintermedius*.** Human-derived Hep-2 and HaCaT cells were efficiently invaded by *S. pseudintermedius*, and invasion was dependent on Sps proteins. In addition, as reported for CPEK, internalization required the presence of Fn and involved the $\alpha_5\beta_1$ integrin (see Fig. S1 and S2 in the supplemental material).

**Alterations to cell monolayers following internalization by *S. pseudintermedius*.** To investigate alterations to CPEK, Hep-2, and HaCaT cells following *S. pseudintermedius* invasion, cell monolayers were infected with *S. pseudintermedius* strain ED99 for 2 h prior to gentamicin treatment. Then the cells were incubated for 4 h and 36 h, fixed, and analyzed for morphological changes by light microscopy. A remarkable difference in mor-
Phylogeny was observed between infected and uninfected cell monolayers. Internalization of bacteria by CPEK caused cell detachment and a reduction of the cell density (Fig. 9A). Incubation of Hep-2 cells with strain ED99 for 36 h resulted in rounding and detachment of the cells (see Fig. S3A in the supplemental material). In contrast, HaCaT cells showed a pattern similar to that exhibited by uninfected cells (see Fig. S3C).

The assessment of the cell growth and survival of infected cells by the MTT assay showed that staphylococcal invasion substantially reduced the viability of CPEK (Fig. 9B) and Hep-2 (see Fig. S3B in the supplemental material), whereas HaCaT cells survived to a level comparable to that of the uninfected cells (see Fig. S3D). To further investigate the contribution of bacterial invasion to cell damage, all the cell lines were incubated with the ΔspsDΔspsL double mutant. Both morphological observations and MTT assays showed that the double mutant did not affect the viability of cells.

**DISCUSSION**

In this study, we analyzed the molecular mechanism by which *S. pseudintermedius* adheres to and invades canine keratinocytes (CPEK) and the effects of internalization on the viability of the mammalian cells. We found that all strains of *S. pseudintermedius* tested invaded CPEK efficiently. Importantly, we found that both the cell wall-anchored surface proteins SpsD and SpsL efficiently promoted invasion of strain ED99. Single mutants defective in either SpsD or SpsL alone showed no reduction in invasion. Only the double mutant lacking both proteins was defective. Conversely, both SpsD and SpsL promoted efficient uptake of the non-invasive surrogate host *L. lactis* when expressed ectopically from recombinant plasmids. Subdomains within SpsD and SpsL were expressed as recombinant proteins, which allowed identification of regions with a high affinity for Fn and which also strongly inhibited bacterial invasion. Invasion of CPEK was dependent on the presence of Fn, as demonstrated by the markedly reduced uptake upon removal of Fn from the cell culture medium (fetal bovine serum) and the restoration of invasion by supplementation with purified human Fn. We recognize the limitation of using human and bovine Fn to assess the role of this protein in bacterial invasion of a canine cell line. However, it should be noted that there is a high level of similarity between human, bovine, and canine Fn (93 to 94% identity, 98% similarity), so that the use of human or bovine Fn is valid.

In *S. aureus* Fn-binding proteins FnBPA and FnBPB both promote invasion of mammalian cells, where Fn acts as a bridge between the bacterial surface protein which binds to the N-terminal N29 domain by the tandem β zipper mechanism and the αβ1 integrin, which recognizes an RGD motif within C-terminal repeat 10 of Fn (18, 31). The finding that the N-terminal region of Fn and an RGD-containing peptide inhibited *S. pseudintermedius* invasion of CPEK strongly suggests that the same mechanism is employed and involves the Fn binding domains of SpsD and SpsL. Inhibition of invasion of CPEK by monoclonal antibodies recognizing epitopes in the human αβ1 integrin strongly suggests that the canine CPEK express an immuno-cross-reactive integrin that is responsible for bacterial adhesion and invasion.
The Fn bridging mechanism for attachment to and invasion of mammalian cells results from integrin-initiated actin polymerization stimulated by receptor clustering and cell signaling events involving Src (32, 33). Here, invasion of *S. pseudintermedius* was strongly reduced by the Src-specific inhibitors CGP77675 and PP-2, implying that similar mechanisms are responsible. Similar results were obtained when human Hep-2 epithelial cells and HaCaT keratinocytes were tested for invasion by *S. pseudintermedius* ED99. However, certain differences were observed compared to invasion of CPEK. A 10-fold-smaller inoculum was needed for efficient invasion of Hep-2 cells than for HaCaT and CPEK. We speculate that the differences in invasion efficiencies might be due to variations in the density of the /H92515/H92521 integrins, although other factors could be involved. Together, these data demonstrate that *S. pseudintermedius* employs a mechanism of host cell invasion similar to that used by *S. aureus* that involves bacterial surface proteins binding to Fn and uptake mediated by integrin /H92515/H92521.

Invasion of CPEK and Hep-2 cells resulted in cells detaching and losing viability, whereas HaCaT cells remain unchanged. Thus, in the first two cell lines, invasion by *S. pseudintermedius* triggers a reduction in cell viability. Formaldehyde-killed bacterial cells were actively internalized by the mammalian cells, suggesting that no active expression of invasion-promoting factors was necessary to achieve invasion. Conversely, the lack of effects on host cell survival by killed bacterial cells indicates that additional factors such as secreted toxins are required to induce cell death.

Membrane-damaging toxins that are expressed by intracellular *S. aureus* are major factors in promoting apoptosis (34). *S. pseud-

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**FIG 7** Effect of SpsD and SpsL fragments on invasion of CPEK by *S. pseudintermedius*. CPEK monolayers were incubated with increasing concentrations of SpsD164-523, SpsD520-846 or SpsD844-960 (A) or SpsL220-531 or SpsL538-823 (B) prior to addition of bacteria. Invasion is expressed as a percentage of that observed in the absence of potential inhibitors (control; 2.2 × 10^5 CFU). Error bars show SD of the means from three independent determinations performed in triplicate. Statistically significant differences are indicated (Student’s two-tailed t test; *, *P < 0.05; **, *P < 0.001).

**FIG 8** (A) Effect of antiintegrin antibodies on invasion of CPEK by *S. pseudintermedius*. CPEK monolayers were incubated with antibodies against /H92515/H92521 and /H9251v/H92523 integrins prior to the addition of bacteria. After incubation, internalized bacteria were quantified as described above. Invasion is expressed as a percentage of that observed in the absence of antibodies (control; 2.8 × 10^5 CFU). Bars and error bars represent the means and SD from three independent determinations performed in triplicate. Statistically significant differences are indicated (Student’s two-tailed t test; *, *P < 0.05). The inset shows expression of /H92515/H92521 integrin by CPEK by staining with immunofluorescent antibodies that specifically bind to the /H92515 subunit of the /H92515/H92521 integrin. Bar, 40 μm. (B) Effect of an RGD-containing peptide on invasion of CPEK by *S. pseudintermedius*. CPEK were incubated with increasing concentrations of the RGDS or RGES peptide prior to addition of bacteria. After incubation, internalized bacteria were quantified as described above. Invasion is expressed as a percentage of that observed in the absence of peptides (control; 2.3 × 10^5 CFU). Values are means from three independent determinations performed in triplicate. (C) Effect of kinase inhibitors on invasion of CPEK by *S. pseudintermedius*. CPEK were exposed to genistein, CGP77675, PP2, PP3, wortmannin, and cytochalasin D for 1 h before addition of bacteria. Invasion assays were performed on inhibitor-treated cells three times with similar results. Invasion is expressed as a percentage of that observed in the absence of inhibitors (control; 2.1 × 10^5 CFU). Bars and error bars represent the means and SD from three independent determinations performed in triplicate. Statistically significant differences are indicated (Student’s two-tailed t test; *, *P < 0.05; **, *P < 0.001).
intermedius has the potential to express a bicomponent leukotoxin, Luk-I, which is similar to the Panton-Valentine leukocidin (PVL) of S. aureus (35), as well as a homologue of β-toxin and a putative hemolysin (hemolysin III) (8). It can be hypothesized that at least one of these factors is responsible for inducing cell death in CPEK. Indeed, PVL facilitates escape of S. aureus from human keratinocyte endosomes and induces apoptosis (34), which might indicate a role here for Luk-I. Studies with mutants lacking one or more of the toxins will help clarify this point.

The initiation of the skin infection canine pyoderma is probably related to the ability of S. pseudintermedius to adhere to corneocytes on the surface of the stratum corneum as well as to invade the underlying keratinocytes. S. pseudintermedius adheres more strongly to corneocytes from regions of inflamed skin of dogs with atopic dermatitis than to noninflamed areas, suggesting that ligands for bacterial surface protein adhesins are present at higher levels (36). Both SpsD and SpsO mediate bacterial adherence to canine corneocytes, but the host ligands involved are not known (12). In addition, fibronectin is present in the stratum corneum of atopic human skin, where it could provide an abundant ligand, whereas it was not detected in healthy skin (37). Thus, Fn could promote colonization of the stratum corneum as well as invasion of keratinocytes.

In conclusion, we have identified and characterized two fibronectin-binding proteins of S. pseudintermedius which are required for adhesion to and invasion of keratinocytes. An appropriate animal model will be required to assess the significance of SpsD and SpsL in the pathogenesis of canine pyoderma and to establish whether these antigens are suitable candidates for a multicomponent vaccine to combat the disease.

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