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CELLULAR MICROBIOLOGY:
PATHOGEN-HOST CELL MOLECULAR INTERACTIONS

Identification and Characterization of *Staphylococcus delphini* Internalization Pathway in Nonprofessional Phagocytic Cells

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ABSTRACT The intracellular lifestyle of bacteria is widely acknowledged to be an important mechanism in chronic and recurring infection. Among the *Staphylococcus* genus, only *Staphylococcus aureus* and *Staphylococcus pseudintermedius* have been clearly identified as intracellular in nonprofessional phagocytic cells (NPPCs), for which the mechanism is mainly fibronectin-binding dependent. Here, we used bioinformatics tools to search for possible new fibronectin-binding proteins (FnBP-like) in other *Staphylococcus* species. We found a protein in *Staphylococcus delphini* called *Staphylococcus delphini* surface protein Y (SdsY). This protein shares 68% identity with the *Staphylococcus pseudintermedius* surface protein D (SpsD), 36% identity with *S. aureus* FnBPA, and 39% identity with *S. aureus* FnBPB. The SdsY protein possesses the typical structure of FnBP-like proteins, including an N-terminal signal sequence, an A domain, a characteristic repeated pattern, and an LPXTG cell wall anchor motif. The level of adhesion to immobilized fibronectin was significantly higher in all *S. delphini* strains tested than in the fibronectin-binding-deficient *S. aureus* DU5883 strain. By using a model of human osteoblast infection, the level of internalization of all strains tested was significantly higher than with the invasive-incompetent *S. aureus* DU5883. These findings were confirmed by phenotype restoration after transformation of DU5883 by a plasmid expression vector encoding the SdsY repeats. Additionally, using fibronectin-depleted serum and murine osteoblast cell lines deficient for the β_1 integrin, the involvement of fibronectin and β_1 integrin was demonstrated in *S. delphini* internalization. The present study demonstrates that additional staphylococcal species are able to invade NPPCs and proposes a method to identify FnBP-like proteins.

KEYWORDS fibronectin, fibronectin-binding proteins (FnBPs), integrin $\alpha5\beta1$, nonprofessional phagocytic cells, *Staphylococcus*, host cell invasion

Bacteria belonging to the genus *Staphylococcus*, which comprises more than 50 species, are responsible for a variety of diseases from benign to serious infections originating from both community-acquired and nosocomial sources (1, 2). Staphylococcal infections are associated with high rates of morbidity and mortality, and the latter reaches 30% in cases of bacteremia and up to 66% in cases of endocarditis (3, 4). Within the *Staphylococcus* genus, although *Staphylococcus aureus* is the most prevalent

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and virulent pathogen, *Staphylococcus* non-*aureus* (SNA) species, classically considered less virulent, opportunistic pathogens that are primarily responsible for chronic infections, have become major nosocomial pathogens (1, 5, 6). This could be explained by the increased numbers of immunocompromised patients, as well as by the use of inserted foreign bodies (7–9). However, the physiopathology of SNA-related infections remains far from being understood.

Conversely, various pathophysiological mechanisms have been identified to explain the development of chronic *S. aureus* infections, including the formation of biofilm, the secretion of specific virulence factors, and internalization into nonprofessional phagocytic cells (NPPCs) (7–13). The latter mechanism allows the bacteria to evade the host innate immune system and antibiotics and thus survive inside a wide variety of mammalian cells, which leads to chronic and recurring infection (14, 15). A plethora of studies have described this mechanism for *S. aureus*, showing that this bacterium can invade and persist within various NPPCs, such as epithelial cells (16), osteoblasts (17), and fibroblasts (18). The internalization of *S. aureus* into host cells is an active phenomenon from the cell side, controlled by the actin cytoskeleton, but is a passive phenomenon from the bacterial side. The integrin $\alpha_5\beta_1$ binds to fibronectin of the extracellular matrix via the Arg-Gly-Asp (RGD) motif (19). Bacterial surface fibronectin-binding proteins (FnBPs), belonging to microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), bind the fibronectin to trigger bacterial internalization (20–23). Although alternative invasion mechanisms involving other MSCRAMMs have been proposed in the literature, the FnBP-fibronectin-$\alpha_5\beta_1$ integrin pathway is predominant (24). FnBPs are characterized by a Sec-dependent secretory signal sequence at the N terminus, an N-terminal A domain comprising three separately folded subdomains (N1, N2, and N3), a variable fibronectin-binding repeat region, and a C-terminal sorting signal allowing covalent anchorage to the cell wall peptidoglycan by sortase. Two homologous FnBPs have been described for *S. aureus*, FnBPA and FnBPB, encoded, respectively, by the *fnbA* and *fnbB* loci, with very similar domain organizations and sequences (25). Although there is no doubt as to the capacity of *S. aureus* to be internalized in NPPCs and the pathophysiological consequences of this mechanism for infections, the question of whether SNA can also act as facultative intracellular pathogens in NPPCs is still debated. For instance, only a few MSCRAMMs involved in cellular internalization have been identified for the SNA species. We and others have described SpsD and SpsL, two FnBP-like proteins, as being sufficient for internalization of *S. pseudintermedius* in bone and epithelial cells via an interaction with fibronectin (22, 26). In addition to having strong homology with *S. aureus* FnBPs, SpsD and SpsL share the same characteristic motifs. For the other SNA species, the little data available are contradictory; for instance, while Hussain et al. suggested the involvement of the autolysin AtlL in the internalization process of *Staphylococcus lugdunensis* (27), other authors highlighted the incapacity of *S. lugdunensis* to be internalized in NPPCs using a panel of clinical and reference strains (26, 28).

To identify other species that can be internalized into NPPCs, we sought to identify FnBP-like proteins involved in the invasion process of NPPCs. Using *in silico* analysis and *in vitro* cellular models, we identified a new protein homologous to *S. aureus* and *S. pseudintermedius* fibronectin-binding proteins, named *Staphylococcus delphini* surface protein Y (SdsY), that is expressed by strains belonging to the species *S. delphini* that are responsible for human and animal infections (29–31).

RESULTS

***In silico* analysis and identification of new *fnb* gene homologs among clinical isolates.** *In silico* searches allowed us to identify the hypothetical protein WP_019165356.1, which is encoded by the *S. delphini* strain 8086. This hypothetical protein, called *Staphylococcus delphini* surface protein Y (SdsY), presented 68% identity with the SpsD protein from the *S. pseudintermedius* strain ED99. Furthermore, this protein also had 36% identity with FnBPA and 39% identity with FnBPB from *S. aureus* Mu50. The SdsY protein encoded by *S. delphini* DSM 20771^T has 77% identity with the

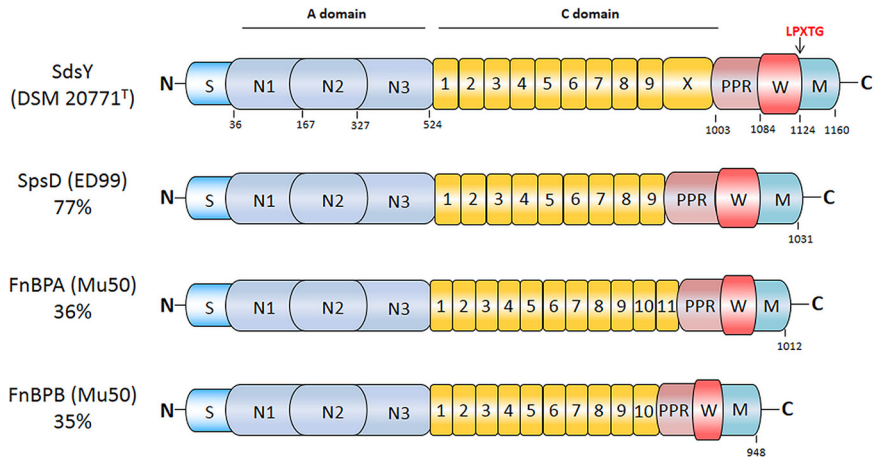

FIG 1 Schematic representation of SdsY from *S. delphini* DSM 20771^T. The SdsY protein includes a signal sequence (dark blue; S) at the N terminus, followed by an A domain spanning residues 37 to 524 (light blue; N1, N2, N3), a connecting domain in yellow containing nine tandem repeats with an immunoglobulin-like domain X (C; residues 525 to 1003), a proline-rich repeat spanning residues 1004 to 1084 (light red; PPR), and the wall (red; W) and membrane (cyan; M) spanning domains at the extreme C terminus residues 1085 to 1160 (22). The A domain of SdsY has 70% identity with SpsD, 38% identity with FnBPA, and 43% identity with FnBPB. The putative fibronectin binding region of SdsY has 73% identity with SpsD, 43% identity with FnBPA, and 41% identity with FnBPB.

SpsD protein of the *S. pseudintermedius* strain ED99 and possesses the typical motifs of MSCRAMM proteins, including an N-terminal signal sequence, an A domain, a characteristic repeat region, and an LPXTG cell wall anchor motif (Fig. 1). PCR amplification targeting *sdsY* revealed the presence of the gene in all *S. delphini* isolates ($n = 7$).

Capacity to bind human fibronectin and to invade osteoblasts. *In vitro* fibronectin binding assays found that all *S. delphini* strains tested adhered significantly more (mean $\pm$ standard deviation [SD], 50.0% $\pm$ 12.4% to 110.5% $\pm$ 15.3% relative to *S. aureus* 8325-4) than the negative-control *S. aureus* DU5883 lacking the *fnbA* and *fnbB* genes (29.4% $\pm$ 6.0%; all $P < 0.001$; Fig. 2A). In addition, the internalization levels in MG63 osteoblasts for all *S. delphini* strains were similar to or higher than that for *S. aureus* 8325-4 (Fig. 2B). In order to confirm the key role of the SdsY repeats for fibronectin binding and host cell invasion, we expressed in the invasive-incompetent *S. aureus* DU5883 strain a chimeric protein composed of the backbone (N-terminal A domain and C-terminal domain) of FnBPA of *S. aureus* with the predicted repeats of SdsY. The *S. aureus* DU5883 strain transformed with the pFnBPR0fnbA::*sdsY* (Hyb) showed significantly higher fibronectin adhesion and MG63 internalization than the *S. aureus* DU5883 strain carrying the empty plasmid pFnBPR0 (Fig. 3A and B). The expression of the SdsY repeats in the *S. aureus* DU5883 strain enabled recovery of adhesiveness and invasiveness similar to that of *S. delphini* DSM 20771.

Characterization of the cellular pathways involved in the internalization process of *S. delphini*. To determine whether internalization of *S. delphini* in osteoblasts involves the soluble plasma-derived fibronectin, fetal bovine serum (FBS) was fibronectin-depleted using gelatin-Sepharose affinity chromatography. The depletion of fibronectin (Fn-depleted FBS) reduced the invasion level by 45% ($P < 0.0001$) compared with control conditions (FBS; Fig. 4A). These observations suggest that *S. delphini* DSM 20771 uses soluble plasma-derived Fn in the internalization process. Of note, we observed that the capacity of *S. delphini* to invade osteoblasts was not totally abolished without plasma-derived Fn. We suggest that it could be explained by the presence of fibronectin produced by osteoblasts. To investigate the role of cellular integrin $\alpha_5\beta_1$ in the internalization mechanism of *S. delphini* in osteoblasts, an *in vitro* model comparing two murine osteoblast cell lines, floxed OB-β_1 (OB-$\beta_1^{fl/fl}$) and OB-$\beta_1^{-/-}$, was used. We investigated the internalization capacity of *S. aureus* 8325-4 (positive control), *S. aureus* DU5883 (negative control), and *S. delphini* DSM 20771^T (reference strain). As expected,

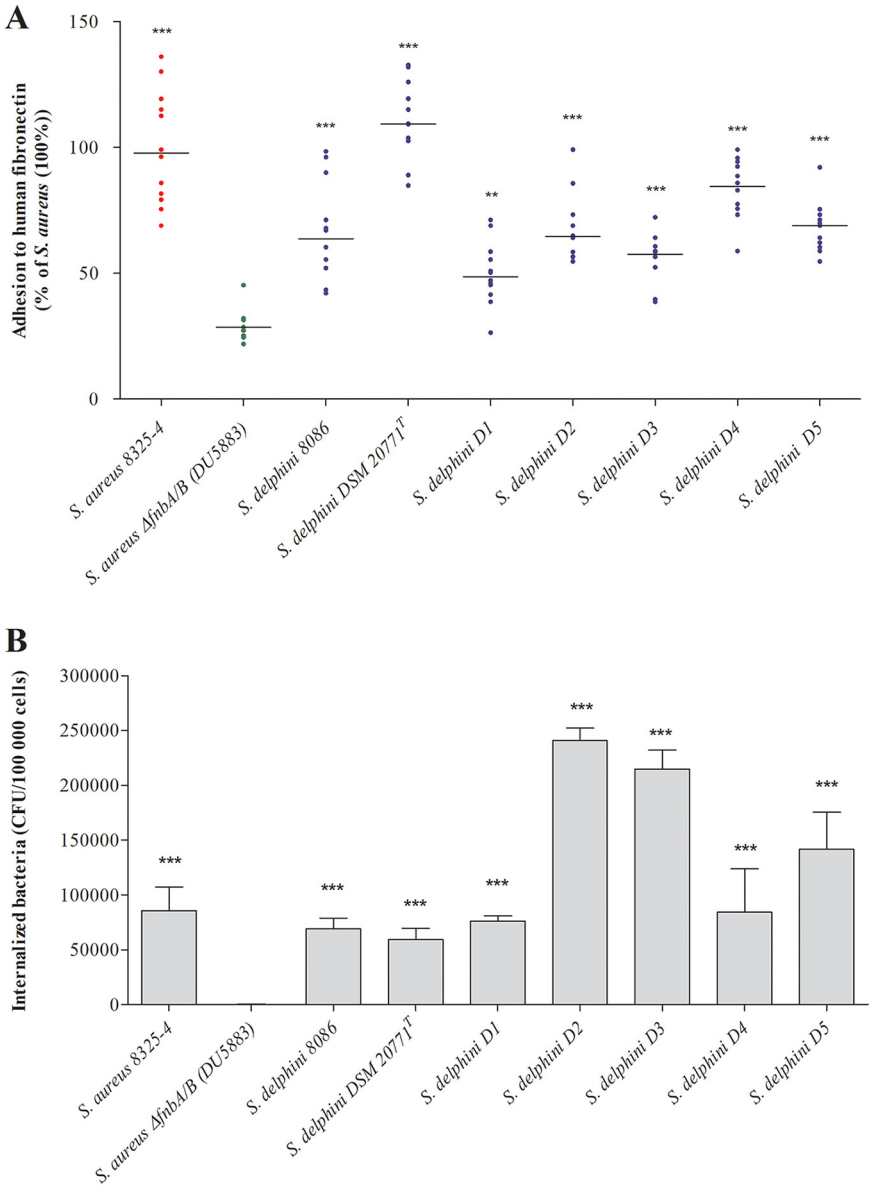

FIG 2 Determination of the capacity of *S. delphini* to adhere to human fibronectin *in vitro* and to be internalized in MG63 cells. (A) Quantification of the fibronectin adhesion capacity of seven *S. delphini* strains. All results are expressed as the proportion of the values obtained for the *S. aureus* 8325-4 strain. The horizontal bars indicate the mean derived from three independent experiments performed in quadruplicate, and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test using *S. aureus* Δ*fnb*A/B as a control with an α risk of 0.05 (**, $P \leq 0.001$; ***, $P \leq 0.0001$). (B) Quantification of *S. delphini* internalization in osteoblast MG63 cells. Bars represent the mean ± standard deviation derived from three experiments performed in triplicate, and the results are expressed as internalized bacterial CFU/100,000 cells. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple-comparison test using *S. aureus* Δ*fnb*A/B as a control with an α risk of 0.05 (***, $P \leq 0.0001$).

a complete loss of internalization of *S. aureus* 8325-4 was observed for OB-$\beta_1^{-/-}$ cells compared to OB-$\beta_1^{fl/fl}$ cells ($P < 0.0001$). A similar abolition was obtained for the *S. delphini* DSM 20771^T strain comparing the two cell lines ($P < 0.0001$; Fig. 4B), which suggests that the *S. delphini* internalization process was widely mediated by the β_1 integrin.

DISCUSSION

The intracellular lifestyle provides various advantages for bacterial pathogens. After internalization in NPPCs, bacteria become inaccessible to humoral and complement-

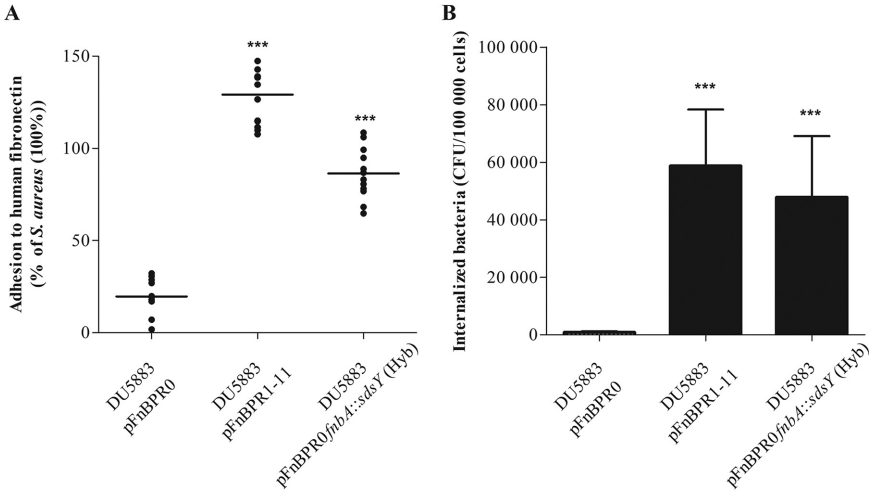

FIG 3 Determination of the capacity of the DU5883 strain complemented with pFnBR0, pFnBR1-11, or pFnBR0*fnbA*::*sdsY* (Hyb) to adhere to human fibronectin *in vitro* and to be internalized in MG63 cells. (A) Quantification of the fibronectin adhesion capacity of *S. aureus* DU5883 expressing pFnBR1-11 or pFnBR0*fnbA*::*sdsY* (Hyb). All results are expressed as the proportion of the values obtained for the *S. aureus* 8325-4 strain. The horizontal bars indicate the mean derived from three independent experiments performed in quadruplicate. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple-comparison test using DU5883 pFnBPR0 as a control with an α risk of 0.05 (***, $P \leq 0.0001$). (B) MG63 invasion by *S. aureus* DU5883 expressing pFnBR1-11 or pFnBR0*fnbA*::*sdsY* (Hyb). Internalized bacteria were measured as indicated above. Bars represent the mean $\pm$ standard deviation from three independent experiments performed in triplicate. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple-comparison test using DU5883 pFnBPR0 as a control with an α risk of 0.05 (***, $P \leq 0.0001$).

mediated attack; they avoid shear stress-induced clearance, and they evade antibiotic activities. Thus, bacterial intracellular location allows persistence of bacteria leading to chronic and recurrent infection (32). Host cell entry is the key step of the invasion process. Identifying the *Staphylococcus* species able to be internalized in host cells is crucial to better characterize a contributing pathophysiological mechanism related to SNA infections but also to provide new approaches for avoiding or preventing cellular invasion.

The *in silico* screening approach allowed us to identify a hypothetical protein, called SdsY, within the *S. delphini* genomes, which has identity with staphylococcal FnBPs/FnBP-like proteins. This protein has a high homology with the SpsD protein of *S. pseudintermedius* and shares all the characteristic domains of the FnBP type, including a secretory signal sequence at the N terminus, an N-terminal A domain, a C-terminal repeat region, and a C-terminal peptidoglycan-binding motif (LPXTG) (33). The analysis also found the presence of similar proteins already defined as FnBPs in the *Staphylococcus argenteus* and *Staphylococcus schweitzeri* genomes; both species have been recently described and belong to a complex closely related to *S. aureus* (34).

The screening approach used to detect FnBP-like protein may present some limitations. The results of this *in silico* screening method depend entirely on the sequences available in public databases, which are constantly evolving. For several staphylococcal species, only unassembled genomes are available, and as a consequence, the genes of interest can be truncated if they are located at the beginning or the end of a contig. Moreover, the identity and coverage thresholds, from which a protein may be considered FnBP-like, were set relatively high, and therefore it cannot be excluded that other proteins of interest have been missed using this approach. This is illustrated by the study reported by Ben Zakour et al., who used a different approach (BlastP with a minimum identity of 40% and minimum sequence coverage of 80%) to screen the distribution of virulence factors, including FnBP-like protein in eight staphylococcal species, including *S. delphini*, *S. intermedius*, *S. epidermidis*, and *S. haemolyticus*; they found no orthologous protein-coding sequences for FnBPA/B or for SpsD/L in the

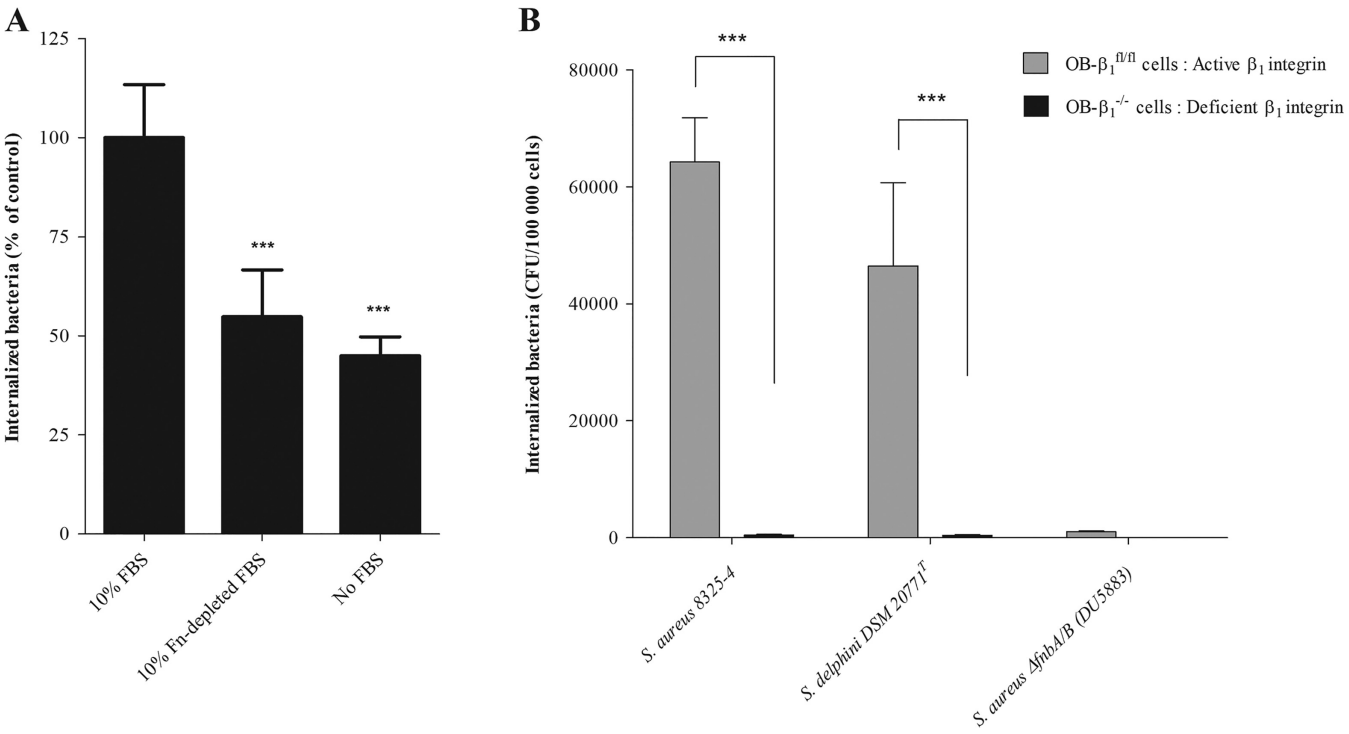

FIG 4 Characterization of the cellular pathways involved in the internalization process of *S. delphini*. (A) Role of soluble fibronectin in invasion of *S. delphini*. The invasion assay was performed in the presence of 10% FBS (control condition), in the presence of 10% fibronectin-depleted FBS, and in the absence of FBS. Invasion is expressed as a percentage of that observed in the presence of 10% FBS. The horizontal bars indicate the mean derived from three independent experiments performed in quadruplicate. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple-comparison test using 10% FBS as a control with an α risk of 0.05 (***, $P \leq 0.0001$). (B) Evaluation of the involvement of the β_1 integrin in the *S. delphini* internalization process using murine osteoblast cell lines (OB-$\beta_1^{fl/fl}$ and OB-$\beta_1^{-/-}$) with functional and nonfunctional β_1 subunits, respectively. The internalization capacity of *S. aureus* 8325-4 (positive control), DU5883 *S. aureus* Δ*fnb*A/B (negative control), and the reference strain *S. delphini* DSM 20771^T in OB-$\beta_1^{fl/fl}$ and OB-$\beta_1^{-/-}$ osteoblasts was measured 3 hours postinfection from three experiments performed in triplicate. The two-tailed Student's *t* test was used to compare the data obtained for the OB-$\beta_1^{fl/fl}$ and OB-$\beta_1^{-/-}$ osteoblasts with an α risk of 0.05 (***, $P < 0.0001$).

various genomes analyzed (35). However, high thresholds were chosen owing to the high functional redundancy of MSCRAMMs that have evolved to play multiple roles and so contain similar motifs that are, in fact, able to recognize variable and different proteins of the extracellular matrix (33, 36).

Using a panel of seven *S. delphini* isolates, we confirmed by PCR that all of the tested isolates were positive for the *sdsY* gene. Investigation of the capacity of *S. delphini* to be internalized into NPPCs found homogeneous behavior among the seven tested strains. In line with *in silico* findings, the *in vitro* results revealed that all strains were able to be internalized in the intracellular compartment of human osteoblasts. Currently, among staphylococci, only *S. aureus* and *S. pseudintermedius* are reported as intracellular pathogens; in both, fibronectin is involved in the internalization process by forming a bridge between the $\alpha_5\beta_1$ integrin on the cellular side and FnBP-like protein (FnBPA/B and SpsD/L) on the bacteria (22, 26). Additionally, using fibronectin-depleted serum and murine osteoblast cell lines deficient for β_1 integrin, the involvement of fibronectin and β_1 integrin was demonstrated in *S. delphini* internalization. Especially described in animal infections such as *Equidae* and *Mustilidae*, a recent study demonstrates the involvement of *S. delphini* in human infections (29–31). Considering the complexity of identifying *Staphylococcus intermedius* group (SIG) species in a routine diagnostic laboratory, the prevalence of *S. delphini*-related infections is likely to be underestimated (37). Like *S. aureus* and *S. pseudintermedius*, *S. delphini* has the ability to invade NPPCs using an FnBP-fibronectin-$\alpha5\beta1$ integrin pathway. The results of the present study suggest that these 3 species that belong to the coagulase-positive staphylococcus (CoPS) group share a pathophysiological mechanism that can explain the genesis of infections. The results also prompt further studies of the various species

TABLE 1 Strains used in the present study

Species	Strain code	Origin	Reference or source
S. aureus	8325-4	Human	54
S. aureus Δ*fnb*A/B	DU 5883	Human	55
S. aureus Δ*fnb*A/B	DU 5883 (pFnBPR1-11)	Human	51
S. aureus Δ*fnb*A/B	DU 5883 (pFnBPR0)	Human	51
S. aureus Δ*fnb*A/B	DU 5883 (pFnBPR0*fnbA*::*sdsY* [Hyb])	Human	This study
S. delphini	8086	Horse	35
S. delphini	DSM 20771^T	Dolphin	43
S. delphini	HT 20030674 (D1)	Camel	42
S. delphini	HT 20030676 (D2)	Camel	42
S. delphini	HT 20030677 (D3)	Camel	42
S. delphini	HT 20030679 (D4)	Camel	42
S. delphini	HT 20030680 (D5)	Camel	42

belonging to the *Staphylococcus* genus to accurately investigate the ability of these species to be internalized.

MATERIALS AND METHODS

***In silico* analysis of fibronectin-binding protein homologs.** The search for FnBP homologs was performed using the BlastX search algorithm (translated nucleotide protein query against protein database; https://blast.ncbi.nlm.nih.gov/Blast.cgi) and NCBI's nonredundant (nr) protein database (38). We used as query sequences the *fnb* genes currently characterized in the literature, *fnbA* and *fnbB* of *S. aureus* Mu50 and *spsD* and *spsL* of *S. pseudintermedius* ED99 (39, 40). The search was restricted to the *Staphylococcus* group (taxid: 90964); the *S. aureus* (taxid: 1280) and *S. pseudintermedius* (taxid: 283734) species were excluded from the search. All results with a minimum identity of ≥ 50% and a minimum sequence coverage of ≥ 80% were considered positive. The identification of a hypothetical SdsY in the genome of *S. delphini* strain 8086 (GenBank accession number NZ_CAIA01000117.1) led us to sequence the genome of the reference strain, *S. delphini* DSM20771^T (ATCC 49171), and deposit it in the GenBank database under the BioProject number PRJNA389509. The predicted FnBP-like proteins were further characterized by searching for functional domains using EMBL-EBI InterPro Scan (http://www.ebi.ac.uk/interpro) (41).

Bacterial strains and culture media. Seven *S. delphini* isolates were included in the study: five clinical strains isolated from camelids (42), the strain *S. delphini* 8086 isolated from *Equidae* (35), and the reference strain *S. delphini* DSM 20771^T, isolated from a dolphin (43) (Table 1). As described by Gharsa et al., the identification was carried out using PCR-restriction fragment length polymorphism of the *pta* gene with the MboI enzyme (supplemental material S1) (31, 44). The strain *S. aureus* 8325-4, a strain well characterized for its ability to bind fibronectin and to invade osteoblasts, was used as a positive control in each experiment. Its isogenic mutant, DU5883 Δ*fnbA/B* (inactivated for the *fnbAB* genes and therefore unable to adhere to or invade osteoblasts), was used as a negative control (Table 1). Prior to the assays, strains were incubated overnight in brain heart infusion medium (BHI; bioMérieux, Marcyl'Etoile, France) aerobically at 36°C for 18 hours.

Illumina sequencing and *de novo* assembly. *S. delphini* DSM 20771^T genomic DNA was extracted using a QIAcube extraction kit (Qiagen, Hilden, Germany). The Nextera XT DNA preparation kit (Illumina, San Diego, CA, USA) was used to generate sequencing libraries from 1 ng of DNA. Whole-genome sequencing was done using an Illumina NextSeq 500 sequencer to generate 150-bp paired-end reads. The raw paired-end reads were cleaned of potential contamination by the adapters during the Nextera XT protocol using Trimmomatic v0.36 (45). After being quality filtered, the reads were assembled with SPAdes v3.9.0 using the "careful" option and with the SPAdes error correction (BayesHammer module) turned on (46).

Detection of target genes in *S. delphini* isolates. The consensus sequence for the *sdsY* gene was obtained using Seaview software v4.5.4 after aligning the sequences obtained from the two public whole-genome sequences (WGS) of *S. delphini* strains (strains 8086 [GenBank accession number NZ_CAIA01000117.1] and DSM 20771^T [BioProject number PRJNA389509]) (47). Specific primers selected from the consensus sequence of the gene of interest (*sdsY*) were designed using Primer 3 software and are listed in Table S1 (48). The reaction mixture and the PCR program used for amplification are available in Tables S2 and S3.

Expression of the SdsY repeats into *S. aureus* lacking *fnbAB* genes. The plasmids pFnBR0 (which expresses an FnBPA variant containing no fibronectin-binding repeats) and pFnBR1-11 (which expresses the full length of FnBPA) were kindly provided by Ruth Massey (49). We inserted the putative binding domain of SdsY (residues 525 to 1003) into the plasmid pFnBPR0 using a prolonged overlap extension-PCR (POE-PCR) strategy (50, 51). Briefly, the insert corresponding to the repeats of SdsY was amplified from the genomic DNA of the *S. delphini* strain DSM20771, using Q5 HotSart polymerase (NEB, Ipswich, MA, USA) with the primers Rfor (5′-GCGAACGGAAATGAGAAAAATACTTATTCTCCTGTAATGACTTTCCA-3′) and Rrev (5′-GGCGTTGGTGGCACGATTGGTTCG/ATTTTCTTTAGGATCTTTTGGCT-3′). The pFnBR1-11 plasmid was opened with inverse PCR using primers that were designed to delete the repeats of FnBPA (5′-TGGAAAGTCATTACAGGAGAATAAGTATTTTTCTCATTTCCGTTCGC-3′) (Nrev) and (5′-AGCCAAAAGATCC

TAAAGAAAATGAACCAATCGTGCCACCAACGCC-3′) (Cfor) to obtain the open plasmid pFnBPR0. After being cleaned up using the MinElute PCR purification kit (Qiagen), the products of each PCR were mixed in a 1:1 molar ratio at a concentration of 2 ng/μl for the insert (repeats of SdsY), and the POE-PCR was run as previously described (50). Then, *Escherichia coli* DH5α was transformed with 5 μl of the POE-PCR product, and positive clones containing pFnBR0*fnbA*::*sdsY* (Hyb) were selected on LB plus ampicillin (100 μg/ml). After plasmid purification from *E. coli* using a miniprep kit (Qiagen), the *S. aureus* RN4220 strain was transformed by electroporation, and positive clones were selected on Trypticase soy agar (TSA; bioMérieux) plus chloramphenicol (10 μg/ml) as previously described (51). Plasmids were recovered using a combination of lysostaphin and the Qiagen miniprep kit and retransformed into the invasive-incompetent *S. aureus* DU5883 strain.

Microplate adhesion to fibronectin assay. The fibronectin adhesion assay was performed *in vitro* in 96-well flat-bottom plates as previously described (52). Briefly, the wells were coated with 200 μl of human fibronectin (Dutscher SAS, Brumath, France) at 50 μg/ml (18 h, 4°C). They were then washed three times (20 min, 37°C) with PBS supplemented with 1% fetal bovine serum (FBS). Meanwhile, after an overnight broth culture in BHI at 36°C, bacterial suspensions were adjusted to an optical density at 620 nm (OD_{620}) of 1 ± 0.05, corresponding to approximately 1×10^9 cells/ml. Then, 1 ml of each suspension was centrifuged (12,000 $\times$ *g*, 2 min), and pellets were washed and suspended in PBS. Next, 100 μl of each bacterial suspension was incubated in the fibronectin-coated plate for 30 min at 37°C with mild shaking. Wells were then washed 3 times with PBS to remove nonadherent bacteria. Adherent bacteria were fixed with glutaraldehyde (2.5% vol/vol in 0.1 M PBS for 2 h at 4°C) and stained with crystal violet (0.1% mass/vol) for 30 min at room temperature. After 3 PBS washes, the total remaining stain impregnating the adherent bacteria was solubilized using 100 μl Triton X-100 solution (0.2% vol/vol H_2O; Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 30 min. Quantification of adherent bacteria to fibronectin was assessed by measuring the OD_{620} for each well using a spectrophotometer (Auto Reader model 680; Bio-Rad, Hercules, CA, USA). The results were expressed as the mean of OD_{620} in 3 experiments performed in quadruplicate. The values were normalized to the reference strain, *S. aureus* 8325-4.

Cell culture. All cell culture reagents were obtained from Gibco (Paisley, UK). Human MG63 osteoblastic cells (LGC Standards, Teddington, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine and 25 mM HEPES, supplemented with 10% FBS and 100 U/ml penicillin and streptomycin ("growth medium with antibiotics"). Two murine osteoblastic cell lines were used for the quantification of the internalization process: the OB-$\beta1^{fl/fl}$ cell line, expressing the functional integrin β_1 subunit, and the OB-$\beta1^{-/-}$ cell line, deficient in the expression of the β_1 integrin subunit after the conditional deletion of the *itgb1* gene. As previously described, both cell lines were derived from calvaria of transgenic mice bearing itgb1-floxed alleles. OB-$\beta1^{fl/fl}$ cells were then immortalized via retroviral infection with the SV40 large T-antigen; OB-$\beta1^{-/-}$ cells were obtained from the parental OB-$\beta1^{fl/fl}$ cell line after expressing Cre recombinase (53). All cells were maintained at 1 passage per week.

Determination of the invasion capacity of *S. delphini* strains. Osteoblasts were seeded at 80,000 cells per well into 24-well tissue culture plates (Falcon, Le Pont-de-Claix, France) in 1 ml of growth medium with antibiotics. One day later, cells were washed twice with 1 ml of DMEM before the addition of bacteria. Bacterial suspensions in growth medium without antibiotics or in growth medium fibronectin-depleted (Fn-depleted FBS) were added to the cell culture wells at a multiplicity of infection (MOI) of 100 as previously described (17) (see supplemental material S1). After 2 h of coculture of bacteria and osteoblasts in a 37°C/5% CO_2 incubator, the wells were washed twice with 1 ml of DMEM. They were then incubated for 1 h in medium containing 200 μg/ml gentamicin to specifically kill the extracellular bacteria. To evaluate the internalization rate, osteoblasts were then lysed by osmotic shock using water, and dilutions of cell lysates were plated using the easySpiral instrument (Interscience, Saint Nom, France) in duplicate on Trypticase soy agar (TSA; bioMérieux) plates before being incubated at 36°C to quantify the number of colonies corresponding to intracellular bacteria.

Statistical analysis. Statistical comparisons were made using one-way ANOVA, followed by Dunnett's multiple-comparison test for multiple comparison and Student's *t* test for two-group comparison; *P* values below 5% were considered statistically significant. All analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.01 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.

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