Role of *Staphylococcus agnetis* and *Staphylococcus hyicus* in the Pathogenesis of Buffalo Fly Skin Lesions in Cattle

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**ABSTRACT** Buffalo flies (*Haematobia irritans exigua*) are hematophagous ectoparasites of cattle causing production and welfare impacts in northern Australian herds. Skin lesions associated with buffalo fly infestation and *Stephanofilaria* nematode infection are manifested as focal dermatitis or ulcerated areas, most commonly on the medial canthus of the eye, along the lateral and ventral neck, and on the abdomen of cattle. For closely related horn flies (*Haematobia irritans irritans*), *Staphylococcus aureus* has been suggested as a contributing factor in the development of lesions. To investigate the potential role of bacterial infection in the pathogenesis of buffalo fly lesions, swabs were taken from lesions and normal skin, and bacteria were also isolated from surface washings of buffalo flies and surface-sterilized homogenized flies. Bacterial identification was conducted by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and strain typing by repetitive sequence-based PCR (rep-PCR) and DNA sequencing to determine species similarity and virulence factors. Of 50 bacterial isolates collected from lesions, 38 were identified as *Staphylococcus agnetis* and 12 as *Staphylococcus hyicus*, whereas four isolates from normal skin were *S. hyicus* and one was *Mammallicoccus sciuri*. Of the *Staphylococcus* isolates isolated from buffalo flies, five were identified as *S. agnetis* and three as *S. hyicus*. Fifty percent of the buffalo fly isolates had rep-PCR genotypic patterns identical to those of the lesion isolates. Genome sequencing of 16 *S. agnetis* and four *S. hyicus* isolates revealed closely similar virulence factor profiles, with all isolates possessing exfoliative toxin A and C genes. The findings from this study suggest the involvement of *S. agnetis* and *S. hyicus* in buffalo fly lesion pathogenesis. This should be taken into account in the development of effective treatment and control strategies for lesions.

**IMPORTANCE** Skin lesions in cattle associated with feeding by *Haematobia* fly species are a significant welfare issue in Australia, North and South America, and Europe. The development of these lesions has been attributed to a number of causal factors, but the exact etiology and pathogenesis were unclear. This study characterized *Staphylococcus agnetis* and *Staphylococcus hyicus* strains from cattle skin lesions and in vector flies and demonstrated their role in the pathogenesis of these lesions. These findings will aid the development of targeted and more effective treatment and control strategies for lesions associated with fly infestation in cattle.

**KEYWORDS** *Staphylococcus agnetis*, *Staphylococcus hyicus*, *Haematobia*, buffalo fly lesions, cattle, exfoliative toxin
Australia and Asia and other parts of Oceania, while HFs are prevalent in South and North America and Europe (4). In Australia, cattle skin lesions associated with BF feeding are termed BF lesions. These lesions can range from raised, dry, alopecic, hyperkeratotic, or scab-encrusted areas to severe open suppuring wounds occurring mainly near the medial canthus of the eye, neck, and ventral midline (5). Although these lesions are associated with BF feeding, Sutherst et al. reported a low correlation between BF counts and lesion development (6).

An unnamed species of Stephanoﬁlaria nematode has been implicated in the development of these lesions (5), but nematodes were detected in only 40% of skin lesions (7). Naseem et al. suggested that Stephanoﬁlaria sp. infection might not be essential for BF lesion development as their study found only 10.83% of lesions infected with Stephanoﬁlaria sp., with no nematodes found in either lesions or BFs in some regions of Australia despite the frequent occurrence of lesions (8). Horn ﬂies are reported as vectors for Stephanoﬁlaria stilesi nematodes, which have also been implicated in the development of skin lesions in cattle in North and South America (1). However, hypersensitivity to HF feeding and the involvement of Staphylococcus aureus have also been suggested as contributing causes in the development of these lesions (9, 10). These ﬁndings suggest that other factors might be involved in the development of BF lesions.

In the United States, HFs have been identiﬁed as vectors of Staphylococcus aureus bacteria, which have been isolated from lesions on the teats and udders of dairy cattle (9). Nickerson et al. showed that dairy farms using HF control presented lower rates of S. aureus intramammary infection than herds without control (11), and later, Gillespie et al. conﬁrmed that S. aureus isolates from HF had DNA ﬁngerprints identical to 95% of S. aureus isolates from mammary secretions and streak canal swabs (12). In addition, S. aureus, Staphylococcus saprophyticus, Staphylococcus hyicus, and Mammaliicoccus sciuri have been identiﬁed in the microbiome of HF (13).

Staphylococcus hyicus has also been isolated from fresh, encrusted, dry, and old healing skin lesions on the back, shoulder, and root of the tail of cattle, and experimental inoculation with S. hyicus produced lesions with a similar clinical appearance (14). Hazarika et al. also isolated S. hyicus from skin lesions around the eye, forehead, neck, shoulder, hump, and trunk of cattle and reproduced skin lesions in rabbit skin by experimental inoculation with isolated S. hyicus (15). Staphylococcus hyicus has also been identiﬁed as the causative agent of skin lesions in horses and goats and exudative epidermitis (greasy pig disease) in swine (16–19). In addition, S. hyicus isolates from exudative epidermitis of pigs were found to produce epidermolytic exfoliative toxins, which damaged the superficial layer of the skin (20, 21). In all of these studies, phenotypic methods were used for the identiﬁcation and differentiation of staphylococcal species. Adkins et al. (22) developed the ﬁrst PCR assay to differentiate S. hyicus from S. agnetis, revealing that the majority of their previously identiﬁed S. hyicus isolates from cattle were S. agnetis. It is likely that previously identiﬁed S. hyicus from skin lesions of cattle may have also been misidentiﬁed.

The foregoing observations led to the hypothesis that bacterial infections could also have a role in the pathogenesis of BF lesions. In this study, we isolated and identiﬁed Staphylococcus spp. from BFs and BF lesions from different north Australian beef herds, sequenced the genomes of selected Staphylococcus isolates, and investigated the presence of virulence factors in various isolates to assess the potential role of bacteria in the development of BF lesions.

RESULTS

Bacterial isolation. Forty-two lesion swabs were collected from 34 cattle, with two swabs from two separate lesions from eight animals. All lesion swabs produced small, round, white, nonhemolytic, Gram-positive staphylococcus-like colonies on blood agar. Swabs from active lesions yielded pure cultures, while swabs from partially active lesions produced mixed cultures with dominant growth of staphylococcus-like colonies.

All six lesion swabs from herd 1 (H1) produced growth of Staphylococcus spp., with four swabs yielding a pure culture. Swabs from normal skin of the H1 heifers did not produce...
any bacterial growth resembling that seen with the lesion swabs. Buffalo fly surface rinses from one animal yielded three colonies of *Staphylococcus* spp., while pure cultures of *Staphylococcus* spp. were isolated from homogenized BFs plated from two animals with lesions. All eight lesion swabs from H2 also yielded *Staphylococcus* growth, with pure cultures obtained from six lesions. Three swabs from normal skin of H2 steers (including one from a steer without lesions) produced one to two colonies of *Staphylococcus* spp. with abundant environmental contaminants, whereas one swab from the normal skin of an animal with lesions yielded two colonies of *M. sciuri*. A pure culture of *Staphylococcus* spp. was isolated from homogenized BFs collected from two steers (one with lesions and one without lesions). Twelve swabs from H3 produced *Staphylococcus* colonies, with very heavy growth from four lesion swabs. *Staphylococcus*-like colonies of various sizes were present on seven swabs from H3. Multiple colonies (one representative from each size variant) were purified by subculture. Eight lesion swabs collected from H4 also yielded staphylococcal growth in blood agar, and the bacteria were isolated as pure cultures from three swabs. A normal skin swab from one H4 animal produced only one colony of *Staphylococcus* sp., while BF washings from one animal and homogenized BFs from two animals yielded two *staphylococcus*-like colonies and heavy *staphylococcus*-like growth, respectively. All swabs collected from H1 and H2 in 2021 had *staphylococcus*-like growth, with pure cultures grown from two swabs.

In initial identification by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF), 43 of 44 isolates from 2020 were identified as *S. hyicus*, and one normal skin isolate was identified as *M. sciuri*. Since MALDI-TOF was unable to differentiate *S. hyicus* and *S. agnetis*, identification of these staphylococcal isolates to the species level was inconclusive at this point. The MALDI-TOF technique was not used to identify any isolates from the 2021 sampling from H1, H2, and H4. All the 2020 and 2021 isolates were reidentified by PCR, and no bacterial growth was observed following plating on MacConkey agar.

**Strain typing by rep-PCR.** Strain typing by repetitive sequence-based PCR (rep-PCR) was completed on all 44 isolates collected in 2020 from lesions, BFs, and unaffected skin which had been identified by MALDI-TOF as described above. From these 44 isolates, 21 different banding patterns (cluster/pattern types 1 to 21) were identified at a 90% similarity cutoff value. Overall, 10 clusters contained a single isolate, five clusters contained two isolates, and the remaining six clusters contained more than two isolates. Thirty-five isolates from the lesions belonged to 17 different clusters, of which six clusters consisted of a single isolate. Five isolates from the BFs were placed in three clusters, with three isolates being in a cluster with a lesion isolate, while two isolates belonged to single isolate clusters. Four isolates from normal skin showed four different strain types, one of which was *M. sciuri*, as distinguished based on a banding pattern different from those of most of the other isolates. Only one isolate from the normal skin had a similar strain pattern, with one lesion isolate from the same animal.

Among the 19 isolates collected in 2021, 15 different patterns were observed (cluster/pattern types 22 to 36), of which 13 were identified only once. Upon comparison, none of the 2021 isolates showed strain similarity with any of the 21 strain types isolated in 2020. The same pattern was observed from normal skin on one animal, one BF, and two lesion isolates from 2021 collections. There were no differences between the strain types of isolates from different herds. The details for the cluster/pattern type of each isolate are provided in Table 1.

**Genome assemblies and annotation.** To genetically characterize and determine the role of the bacterial isolates associated with lesion development, 21 isolates (one representative from each cluster/pattern type) from the 2020 sampling were selected for whole-genome sequencing and virulence factor analysis (Table 1). In this study, we generated draft de novo genome assemblies for 12 representative isolates (8 from lesions, 2 from BFs, and 2 from normal skin) from 2 × 150-bp paired Illumina reads. For the rest of the 9 representative isolates (8 from lesions and 1 from BFs), we generated complete, finished de novo genome assemblies by hybrid assembly approaches using
| Herd | Animal ID | Yr | Isolate ID | Sample type | Cluster/pattern (rep-PCR) | PCR/sequencing identification |
|------|-----------|----|------------|-------------|--------------------------|-----------------------------|
| H1   | C1        | 2020 | BR2785     | Eye lesion, R | 3 | S. agnetis |
| H1   | C2        | 2020 | BR2788     | Eye lesion, R | 5 | S. agnetis |
| H1   | C3        | 2020 | BR2789     | Eye lesion, L | 5 | S. agnetis |
| H1   | C4        | 2020 | BR2786\(^a\) | Eye lesion, L | 8 | S. agnetis |
| H1   | C5        | 2020 | BR2787\(^a\) | Belly lesion | 5 | S. agnetis |
| H1   | C6        | 2020 | BR2795\(^a\) | Belly lesion | 6 | S. agnetis |
| H1   | C7        | 2021 | BR2910     | Eye lesion, R | 31 | S. agnetis |
| H1   | C8        | 2021 | BR2906     | Eye lesion, R | 32 | S. agnetis |
| H1   | C9        | 2021 | BR2911     | Eye lesion, R | 33 | S. agnetis |
| H1   | C10       | 2021 | BR2908     | Eye lesion, L | 34 | S. agnetis |
| H1   | C8        | 2021 | BR2909     | Shoulder lesion | 32 | S. agnetis |
| H1   | C4        | 2020 | BR2804\(^a\) | Homogenized BF | 3 | S. agnetis |
| H1   | C3        | 2020 | BR2806     | Homogenized BF | 3 | S. agnetis |
| H1   | C3        | 2020 | BR2807     | BF washings   | 3 | S. agnetis |
| H2   | C11       | 2020 | BR2824     | Eye lesion, L | 7 | S. agnetis |
| H2   | C12       | 2020 | BR2816\(^a\) | Eye lesion, L | 13 | S. agnetis |
| H2   | C13       | 2020 | BR2820\(^a\) | Eye lesion, R | 17 | S. hyicus |
| H2   | C14       | 2020 | BR2823\(^a\) | Eye lesion, R | 19 | S. hyicus |
| H2   | C15       | 2020 | BR2824     | Eye lesion, L | 19 | S. hyicus |
| H2   | C16       | 2020 | BR2825     | Eye lesion, R | 19 | S. hyicus |
| H2   | C17       | 2021 | BR2917     | Eye lesion, L | 35 | S. hyicus |
| H2   | C18       | 2021 | BR2918     | Eye lesion, R | 36 | S. hyicus |
| H2   | C12       | 2020 | BR2815     | Dewlap lesion | 13 | S. agnetis |
| H2   | C18       | 2020 | BR2821     | Normal skin   | 17 | S. hyicus |
| H2   | C19       | 2020 | BR2827     | Normal skin   | 19 | S. hyicus |
| H2   | C16       | 2020 | BR2831\(^a\) | Normal skin   | 21 | S. hyicus |
| H2   | C14       | 2020 | BR2822\(^a\) | Normal skin   | 18 | M. scuri |
| H2   | C16       | 2020 | BR2828\(^a\) | Homogenized BF | 4 | S. agnetis |
| H2   | C20       | 2020 | BR2814\(^a\) | Homogenized BF | 14 | S. agnetis |
| H3   | C21       | 2020 | BR2841     | Eye lesion, R | 1 | S. agnetis |
| H3   | C21       | 2020 | BR2842     | Eye lesion, R | 1 | S. agnetis |
| H3   | C22       | 2020 | BR2846     | Eye lesion, R | 3 | S. agnetis |
| H3   | C23       | 2020 | BR2847\(^a\) | Eye lesion, R | 1 | S. agnetis |
| H3   | C23       | 2020 | BR2848     | Eye lesion, R | 1 | S. agnetis |
| H3   | C23       | 2020 | BR2849     | Eye lesion, L | 1 | S. agnetis |
| H3   | C24       | 2020 | BR2851     | Eye lesion, L | 1 | S. agnetis |
| H3   | C24       | 2020 | BR2852     | Eye lesion, R | 1 | S. agnetis |
| H3   | C25       | 2020 | BR2845\(^a\) | Eye lesion, L | 2 | S. agnetis |
| H3   | C25       | 2020 | BR2862\(^a\) | Eye lesion, R | 9 | S. agnetis |
| H3   | C26       | 2020 | BR2863     | Eye lesion, R | 9 | S. agnetis |
| H3   | C27       | 2020 | BR2858\(^a\) | Eye lesion, L | 10 | S. agnetis |
| H3   | C25       | 2020 | BR2844\(^a\) | Eye lesion, L | 11 | S. agnetis |
| H3   | C26       | 2020 | BR2864\(^a\) | Eye lesion, L | 12 | S. agnetis |
| H3   | C26       | 2020 | BR2865     | Eye lesion, L | 12 | S. agnetis |
| H3   | C28       | 2020 | BR2859\(^a\) | Eye lesion, L | 15 | S. agnetis |
| H3   | C28       | 2020 | BR2860     | Eye lesion, L | 15 | S. agnetis |
| H3   | C28       | 2020 | BR2861     | Eye lesion, L | 15 | S. agnetis |
| H3   | C29       | 2020 | BR2855\(^a\) | Eye lesion, R | 16 | S. agnetis |
| H3   | C27       | 2020 | BR2857     | Eye lesion, R | 16 | S. agnetis |
| H4   | C30       | 2021 | BR2885     | Eye lesion, L | 22 | S. agnetis |
| H4   | C31       | 2021 | BR2894     | Eye lesion, L | 28 | S. agnetis |
| H4   | C32       | 2021 | BR2886     | Eye lesion, R | 23 | S. hyicus |
| H4   | C33       | 2021 | BR2890     | Eye lesion, L | 25 | S. hyicus |
| H4   | C35       | 2021 | BR2892     | Eye lesion, R | 26 | S. hyicus |
| H4   | C36       | 2021 | BR2893     | Eye lesion, R | 27 | S. hyicus |
| H4   | C37       | 2021 | BR2895     | Neck lesion   | 26 | S. hyicus |
| H4   | C34       | 2021 | BR2888     | Belly lesion  | 24 | S. agnetis |
| H4   | C34       | 2021 | BR2889     | Normal skin   | 26 | S. hyicus |
| H4   | C32       | 2021 | BR2899     | Homogenized BF | 29 | S. hyicus |
| H4   | C33       | 2021 | BR2897     | Homogenized BF | 30 | S. hyicus |
| H4   | C33       | 2021 | BR2896     | BF washings   | 26 | S. hyicus |

\(^a\)R, right eye; L, left eye.
\(^b\)Sequenced with Illumina NovaSeq 6000.
\(^c\)Sequenced with both Illumina NovaSeq 6000 and MinION (ONT).
Illumina and Oxford Nanopore Technologies (ONT) reads. Assembly details (assembly status, number of contigs, genome size, number of CDS, rRNA, and tRNA) for each isolate sequenced are provided in Table 2. The draft genome assemblies ranged from 38 to 115 contigs, comprising 2.40 to 2.78 Mbp. The hybrid assemblies comprised a single circular contig and range from 2.41 to 2.50 Mbp. The numbers of coding sequences (CDS), rRNAs, and tRNAs varied between isolates and ranged from 2,334 to 2,769, 3 to 19, and 42 to 75, respectively (Table 2).

### Pangenome analysis, read mapping, and multilocus sequence analysis

The initial pangenome analysis of all 20 sequenced *Staphylococcus* species isolates, previously identified as *S. hyicus* by MALDI-TOF, indicated that there were 754 core genes (i.e., genes found in 99% to 100% of strains), 3 soft core genes (95% to 99% of strains), 3,429 shell genes (15% to 95% of strains), and 2,024 cloud genes (0% to 15% of strains). The smaller number of core genes and the high number of cloud genes indicates that the 20 sequences of isolates initially identified as *S. hyicus* might actually be different *Staphylococcus* species, rather than strains within a species. A pangenome analysis for all sequenced isolates is presented in Fig. 1.

To confirm the sequence isolate identity, corrected paired Illumina reads from each isolate sequenced were mapped against the genomes of reference strains of *S. hyicus*, *S. agnetis*, *S. chromogenes* and *M. sciuri*. Of the 21 sequenced isolates, 80.5% to 90.32% of paired Illumina reads from 16 isolates were mapped with *S. agnetis*, while <30% and <10% of the reads from these 16 isolates were mapped with reference genomes of *S. hyicus* and *S. chromogenes*, respectively. From the remaining isolates, 78 to 82% of the reads from four isolates mapped with *S. hyicus*, but <30% and <10% of the reads from these four isolates mapped with reference genomes of *S. agnetis* and *S. chromogenes*, respectively. The 89.5% reads from the *M. sciuri* isolate obtained from this study, mapped exactly with the reference genome of *M. sciuri*.

The argument for reclassifying 16 of the *Staphylococcus* isolates was further strengthened when a pangenome analysis of these 16 suspected *S. agnetis* isolates resulted in 1,981 core genes, 0 soft core genes, 806 shell genes, and 896 cloud

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### TABLE 2 Details of bacterial genome assembly for isolates sequenced in this study

| Isolate ID | Assembly status | Genome size (bp) | No. of contigs | Longest contig length (bp) | No. of: | Accession no. |
|------------|-----------------|-----------------|---------------|---------------------------|----------|---------------|
| BR2786_aL  | Draft           | 2,481,539       | 89            | 304,262                   | 2,402    | JALGOP000000000 |
| BR2787_aL  | Draft           | 2,482,413       | 112           | 304,101                   | 2,403    | JALGOO000000000 |
| BR2795_aL  | Complete        | 2,449,123       | 1             | NA                        | 2,465    | JALGON000000000 |
| BR2832_aL  | Draft           | 2,533,020       | 90            | 304,049                   | 2,470    | JALGOM000000000 |
| BR2844_aL  | Draft           | 2,440,290       | 60            | 225,370                   | 2,358    | JALGOL000000000 |
| BR2845_aL  | Complete        | 2,437,387       | 1             | NA                        | 2,371    | JALGOK000000000 |
| BR2847_aL  | Complete        | 2,414,453       | 1             | NA                        | 2,423    | JALGOU000000000 |
| BR2855_aL  | Complete        | 2,481,102       | 1             | NA                        | 2,465    | JALGOV000000000 |
| BR2858_aL  | Draft           | 2,404,324       | 53            | 318,300                   | 2,334    | JALGOH000000000 |
| BR2859_aL  | Draft           | 2,472,985       | 69            | 194,377                   | 2,402    | JALGOG000000000 |
| BR2862_aL  | Draft           | 2,434,216       | 76            | 229,021                   | 2,359    | JALGOF000000000 |
| BR2864_aL  | Complete        | 2,438,730       | 1             | NA                        | 2,373    | JALGOE000000000 |
| BR2816_aL  | Complete        | 2,506,912       | 1             | NA                        | 2,517    | JALGOD000000000 |
| BR2804_aB  | Complete        | 2,462,691       | 1             | NA                        | 2,539    | JALGOC000000000 |
| BR2814_aB  | Draft           | 2,492,807       | 88            | 303,910                   | 2,429    | JALGOB000000000 |
| BR2828_aB  | Draft           | 2,485,945       | 88            | 181,960                   | 2,426    | JALGOA000000000 |
| BR2821_hL  | Complete        | 2,434,473       | 1             | NA                        | 2,390    | JALGZ000000000 |
| BR2823_hL  | Draft           | 2,597,767       | 38            | 489,936                   | 2,556    | JALGNY000000000 |
| BR2829_hL  | Complete        | 2,452,563       | 1             | NA                        | 2,369    | JALGNX000000000 |
| BR2831_hN  | Draft           | 2,519,724       | 76            | 270,422                   | 2,448    | JALGNW000000000 |
| BR2822_sN  | Draft           | 2,784,109       | 135           | 251,211                   | 2,760    | JALGNV000000000 |

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*The suffixes aL, aB, sN, hL, and hN indicate *S. agnetis* from lesions, *S. agnetis* from buffalo flies, *M. sciuri* from normal skin, *S. hyicus* from lesions, and *S. hyicus* from normal skin, respectively.

*NA, not applicable.*
genes. This finding indicated that these 16 isolates were *S. agnetis*. Pangenome analysis of the four confirmed *S. hyicus* isolates resulted in the identification of 810 core genes, 0 soft core genes, 4,211 shell genes, and 0 cloud genes, indicating more strain variation among these isolates.

The identity of 21 sequenced isolates was confirmed by a multilocus sequence phylogenetic analysis based on four housekeeping genes (*tuf*, *rpoA*, *rpoB*, and *recN*) (Fig. 2). This showed that all 16 suspected *S. agnetis* isolates clustered (97% branch threshold of homology) with the reference strain of *S. agnetis* (DSM23656) and a bovine mastitis isolate of *S. agnetis* (1379). This confirmed that these 16 isolates belonged to the species *S. agnetis*. Four additional *S. hyicus* isolates clustered strongly (99% branch threshold of homology) with the reference strain of *S. hyicus* (NCTC10350), while one *M. sciuri* isolate from the current study clustered with the reference strain of *M. sciuri* (NCTC12103) (Fig. 2).

**Staphylococcal VF identification.** We identified 13 genes that belonged to eight virulence factors (VF) of the adherence category, including those encoding autolysin (*atl*), clumping factors (*clfA* and *clfB*), collagen adhesion (*cna*), fibrinogen binding proteins (*efb*), fibronectin-binding proteins (*fnbA* and *fnbB*), intracellular adhesin (*icaA*, *icaB*, and *icaC*), Ser-Asp-rich fibrinogen binding proteins (*sdrD* and *sdrF*), and staphylococcal protein A (*spa*). The distribution and percentage similarities for the staphylococcal VF genes identified are provided in Fig. 3. The distribution of the VF varied between the species isolated as well as between strains within species. The autolysin gene (*atl*) was the only gene identified in all the *Staphylococcus* isolates for which genome sequencing was undertaken. The VF genes responsible for adherence identified in the genome sequences of all the *S. agnetis* isolates were almost the same, except that *clfA*, *clfB*, *sdrD*, and *sdrF* were present in 87.5%, 93.75%, 75%, and 62.5% of the isolates, respectively. Similarly, VF genes responsible for adherence identified in the genome sequences of the *S. hyicus* isolates were also the same, except that *clfA*, *efb*, and *sdrD* were absent in the single isolate obtained from a normal skin sample. The staphylococcal protein A gene (*spa*) was found only in the *S. hyicus* isolates, whereas *clfA*, *clfB*, *cna*, *efb*, *fnbA*, *fnbB*, *sdrD*, and *sdrF* were not identified in the *M. sciuri* isolate, which instead contained *icaA*, *icaB*, and *icaC*.

**FIG 1** (A) The pangenome analysis indicates a similar and unique gene group among all the sequenced isolates with a core genome phylogenetic tree. (B) Single representative nucleotide sequence inferred for each gene of the pangenome. (C) Presence (orange) or absence (white) of blocks relative to the genes and contigs in the pan-genome. For isolate designations, the suffixes aL, aB, sN, hL, and hN indicate *S. agnetis* from lesions, *S. agnetis* from buffalo flies, *M. sciuri* from normal skin, *S. hyicus* from lesions, and *S. hyicus* from normal skin, respectively.
Among the exoenzymes examined, we identified nine VF, including aureolysin (aur), adenosine synthase A (adsA), cysteine protease (sspB), lipases (geh and lip), serine V8 protease (sspA), staphylokinase (sak), thermonuclease (nuc) and von Willebrand factor-binding protein (vWbp). Of these, aur and nuc were the only genes identified in all the sequenced Staphylococcus isolates. Most of the exoenzyme genes identified within the S. agnetis isolate were similar, except for the gene geh, which was found in 68.75% of the isolates. Similarly, VF genes for exoenzymes were mainly similar in the S. hyicus isolates, except for the genes geh, lip, and sak, which were present in 75%, 75%, and 50% of the isolates, respectively. The gene sspA was present in only the M. sciuri isolate.

The VF category involved in host immune evasion consists of genes for capsule formation (capA, capB, capC, capD, capE, capF, capG, capH, capI, capJ, capK, capL, capM, capN, capO, and capP) and the staphylococcal binder of immunoglobulin (sbi). The gene sbi was identified in the genome sequences of all isolates except the M. sciuri isolate. All the capsule-forming genes classified in the host immune evasion VF category were identified in the genome sequence of all the S. agnetis isolates characterized. The gene capJ, however,
The genes for exotoxin (eta and seln) were identified only in S. hyicus isolates. The beta-hemolysin S. agnetis and M. sciuri. The genes for enterotoxin (sell and seln) were identified in 50% of S. hyicus and 18.75% S. agnetis isolates but absent in M. sciuri. All the isolates of S. agnetis and S. hyicus were found to have exfoliative toxin A and C genes (eta and etc), while the M. sciuri isolate had only the etc gene. The genes for exotoxin (set26 and set30) were identified in all the S. agnetis isolates, while the genes set15 and set16 were present in only 81.25% of the normal skin, respectively.
and 87.5% of the isolates, respectively. All S. hyicus isolates carried set6, set15, and set16 genes for exotoxins, while set26 and set30 were found in 75% and 25% of the isolates. The gene for phenol-soluble modulins (PSM4) was identified in all S. agnetis and S. hyicus isolates, but no exotoxin or phenol-soluble-modulin genes were observed in M. sciuri.

**Nonstaphylococcal VF identification.** We also identified some VF in our isolates that had ≥50% amino acid homology with VF of nonstaphylococcal species in the VF core data set (VFCD) (Fig. 4). Among these, 22 genes of the VF enzyme category were detected, including those encoding urease (ureA, ureB, and ureG), 6-phosphogluconate dehydrogenase (gnd), catalase (katA), adenylylsulfate kinase (cysC1), ATP-dependent Clp protease proteolytic subunit (clpP), capsule biosynthesis protein (capC), chaperonin (groEL), endopeptidase Clp ATP-binding chain (clpC), flagellum-related 3-oxoacyl-ACP (acyl carrier protein) reductase (flmA), glutamate-1-semialdehyde-2,1-aminomutase (hemL), molecular chaperone (ct396), autolysin (aut), nitrate reductase (narH), nucleoside diphosphate kinase (ndk), pantoate–beta-alanine ligase (panC), UTP–glucose-1-phosphate uridylyltransferase (bpsC), undecaprenyl diphosphate synthase (uppS), phosphopyruvate hydratase (eno), prolipoprotein diacylglycerol transferase (lgt), and protein disaggregation chaperone (clpB). All isolates had almost all the above-mentioned VF genes except the aut gene, which was absent in three S. agnetis, all S. hyicus, and M. sciuri isolates. The gene cysC1 was also absent in all S. hyicus and M. sciuri isolates. All isolates had three additional genes for iron uptake and metabolism, including fagC, vctC, and cpsJ, whereas the fourth gene, iraT, was found only in three S. hyicus isolates from lesions. Three genes for putative proteins (plr/gapa, hpt, and lpa) were identified in all S. agnetis isolates, while S. hyicus and M. sciuri exhibited only plr/gapa and lpa.

**Exfoliative toxin analysis.** Exfoliative toxin A (eta) identified from all S. agnetis isolates had 89.21% to 89.54% and 77.77% to 78.10% amino acid homology with exhA from S. hyicus and eta from different strains of S. aureus, respectively. The eta gene from all S. hyicus isolates had 94.12% to 95.08% and 78.75% to 79.73% homology with
etA from *S. hyicus* and *eta* from different strains of *S. aureus*, respectively. The *eta* gene was not identified in the *M. sciuri* isolate from normal skin.

Evolutionary tree analysis for the *eta* gene showed that all the *eta* sequences from the current study occurred in the same clade as *S. hyicus* etHA, but on different branches (Fig. 5A). Exfoliative toxin C (*etc*) identified from all the isolates had 87.09% to 87.61% amino acid homology with *etc* from *S. aureus*. The *etc* gene from all the isolates had <1% similarity with *S. hyicus* and *M. sciuri*. Evolutionary tree analysis of *etc* gene showed all the *etc* sequences from the current study occurred in the same clade as *S. aureus*, but on different branches (Fig. 5B).

**PCR based identification.** As MALDI-TOF was unable to differentiate *S. agnetis* from *S. hyicus*, an *aroD* gene-based species-specific PCR amplifying 295 bp for *S. agnetis* and 425 bp for *S. hyicus* was used for reidentification of all isolates. In initial identification by MALDI-TOF, 43 isolates from 2020 were identified as *S. hyicus*, and one normal skin isolate was identified as *M. sciuri*. However, reidentification by *aroD* gene-based PCR identified 35/43 isolates as *S. agnetis* and only 8/43 as *S. hyicus*. From 2021, 11/19 isolates were identified as *S. hyicus* and 8/19 as *S. agnetis*. All the lesion and BF isolates collected from H1 in 2020 and 2021 were identified as *S. agnetis*. From H2, seven lesions and three normal skin isolates were identified as *S. hyicus*, whereas three lesion isolates were identified as *S. agnetis*. Two lesion isolates from the year 2021 were also identified as *S. hyicus*. All 20 lesion isolates from H3 were confirmed as *S. agnetis* with species-specific PCR. From H4, five lesion isolates, three BF isolates (two from homogenized BFs and one from washings) and one normal-skin isolate were identified as *S. hyicus*, whereas three lesion isolates were confirmed as *S. agnetis*. The details of isolation, source, and identification of bacterial isolates are provided in Table 1.

**DISCUSSION**

In the past, the development of *Haematobia*-associated lesions has generally been attributed to the effects of infection with *Stephanofilaria* spp. nematodes transmitted by BFs and HFs, but in a number of studies, the failure to find nematodes in all lesions...
has suggested that other causal factors, including bacteria, may also be involved (10, 12). In this study, we identified *S. agnetis* and *S. hyicus* from BFs and BF lesions from different North Australian beef herds using whole-genome sequencing and conducted a subsequent comprehensive VF identification, which indicated a potential role of these bacterial species in lesion pathogenesis.

All BF lesions sampled in this study were found to be infected with either *S. agnetis* or *S. hyicus*, and the bacteria were isolated in pure cultures from unscabbed lesions. *Staphylococcus hyicus* has been reported as a causative agent of skin lesions and intramammary infection in cattle (12, 14, 23) and exudative epidermitis in pigs (19). Devriese and Derycke suggested that *S. hyicus* skin infection in cattle occurred secondary to parasitic infestation (14) but Hazarika et al. reproduced the skin lesions in rabbits by experimental inoculation of *S. hyicus* isolated from cattle skin lesions (15). However, it is necessary to clarify here that all of these studies reporting *S. hyicus* as the cause of cattle skin lesions and intramammary infection used phenotypic methods for species-level identification of isolates. As these methods were not able to differentiate between *S. hyicus* and *S. agnetis*, there is the possibility that some or all of the *S. hyicus* isolates reported in these studies are in fact *S. agnetis*. Al-Rubaye et al. indicated the potential of *S. agnetis* to cause skin lesions following the identification of exfoliative toxin genes similar to *S. hyicus* isolates of swine origin and *S. aureus* of the scalded-skin syndrome in humans (24). Both *S. agnetis* and *S. hyicus* have been isolated as causative agents of bovine mastitis and intramammary infections (12, 22, 25).

We isolated *S. agnetis* in pure culture from surface-sterilized homogenized BFs from four cattle, and a single colony of *S. agnetis* was also isolated from BF exocuticle washings from one animal. Similarly, *S. hyicus* were isolated in pure cultures from BFs from two animals with lesions, whereas one BF washing sample yielded two colonies of *S. hyicus*. Horn flies have also been reported to vector *S. aureus* and transmit bacteria into the teat skin, resulting in the development of abscesses and lesions (9, 11). Anderson et al. isolated *S. aureus* from 55.8% of the HFs collected from three herds with *S. aureus* intramammary infection (26), and Owens et al. found that *S. aureus* can remain active in infected HF without a significant change in the bacterial count for up to 4 days (10). In a 16S rRNA gene-based pyrosequencing microbiome study of HFs, Palavesam et al. identified *S. hyicus* in adult male HFs and HF eggs (13). This 2012 study (13) did not identify any *S. agnetis* in HFs, although this could be because *S. agnetis* was first described as a separate species from *S. hyicus* in 2012 (25).

The 43 *S. agnetis* isolates in our study included 23 different strain types, and the 19 *S. hyicus* isolates were of 12 different strain types. Four strains of *S. agnetis* were isolated from multiple animals, and one of these strains was isolated from lesion samples from two separate herds. Two strains of *S. hyicus* were also identified from BF lesions from multiple animals. Hazarika et al. collected 47 *S. hyicus* isolates from cattle skin lesions which were separated into 10 different strains (15). However, that study used biochemical characterization for strain typing, which was later found to be less efficient than modern methods. Wegener et al. also reported that the pigs with exudative epidermitis were infected with multiple strains of *S. hyicus* (19). Similarly, 42 *S. agnetis* isolates from a mastitis study of a dairy herd showed 23 different banding patterns with pulsed-field gel electrophoresis (PFGE) strain typing (22). Isolation of multiple strains of *S. agnetis* and *S. hyicus* from BF lesions, in combination with previous reports (15, 19, 22) of multiple strain involvement of these species in skin and intramammary infections, suggests that there might be multiple strains of these two species involved in the pathogenesis of BF lesions.

The genotypic patterns of 75% *S. agnetis* isolates (three of 4 isolates) from BFs collected from animals with lesions were identical to those of lesion isolates from two herds, whereas of three *S. hyicus* isolates from BF, only one (33.33%) showed similarity with two lesion isolates. In a study of mastitis in three herds, Anderson et al. identified eight different genotypic patterns from 244 isolates of *S. aureus* from teats, milk/colos- trum, and HFs (26). Of the *S. aureus* isolates from HFs, 82.7% belonged to a single
genotypic group and 51.6% had a genotypic pattern identical to that of the mastitis isolates. Similarly, Gillespie et al. noted that the *S. aureus* isolates from HF isolates from udder/teat infections in heifers (12), whereas in our study, 50% of BF isolates had genotypic patterns identical to those of the BF lesion isolates. None of the *S. hyicus* isolates from the normal skin had a genotypic pattern similar to that of the lesion isolates from the respective animals except in one instance, and no *S. agnetis* organisms were isolated from the normal skin samples in our study. The isolation of pure cultures of *S. agnetis* and *S. hyicus* suggests that BFs might play an important role in the transmission of these bacteria.

The inability of MALDI-TOF to differentiate between *S. hyicus* and *S. agnetis* species in our study has also been noted previously (27, 28). In our study, this was due to the lack of *S. agnetis* in the MALDI-TOF database used, which meant that a lower percentage of isolates was correctly identified despite a MALDI-TOF score of ≥2.0 when this species was not in the database. MALDI-TOF reference libraries are based on 16S gene sequencing, which evaluates proteins and might not be useful for differentiating these species, as 16S rRNA gene sequences of *S. agnetis* isolates showed 99.87 to 99.92% similarity with *S. hyicus* NCTC10350, higher than the recommended cutoff value of 98.7% similarity for differentiating species (29). Taponen et al. also reported 99.7% similarity of 16S rRNA gene sequences between *S. agnetis* isolates and *S. hyicus* ATCC 11249 (25). The complete sequencing of the β subunit of the RNA polymerase (*rpoB*) gene and the elongation factor Tu (*tuf*) gene have been used previously to differentiate *S. hyicus* and *S. agnetis* at significantly higher similarity cutoff values (≥97% and ≥98%, respectively) (22, 30). However, from a cross-species gene similarity comparison, we determined that the DNA repair protein gene (*recN*) of *S. agnetis* isolates has 99.34 to 99.76% and 82.32 to 82.44% similarity with *S. agnetis* DSM23656 and *S. hyicus* NCTC10350, respectively, while *recN* gene of *S. hyicus* isolates has 98.75 to 99.10% and 82.14 to 82.44% similarity with *S. hyicus* NCTC10350 and *S. agnetis* DSM23656, respectively. These apparent differences in the *recN* gene sequence similarities of these two species indicate that the *recN* gene can also be used as a potential marker to differentiate these two species.

The 78 different virulence factor genes identified in sequenced isolates in this study are known virulence factors from the genus *Staphylococcus*, and 28 genes had been identified in bacterial species other than *Staphylococcus*. The genes for adherence are similar in both *S. agnetis* and *S. hyicus* isolates, except for the staphylococcal protein A gene (*spa*), which was present only in *S. hyicus* in this study. This finding was consistent with the work of Naushad et al., who reported *spa* genes in all three bovine mastitis *S. hyicus* isolates but not in 13 *S. agnetis* isolates (31). The presence of clumping factor B gene (*clfB*) in all our *S. agnetis* and *S. hyicus* isolates was the only difference between the adherence genes identified in this study and those from the bovine mastitis study of Naushad et al., where only 15% of bovine mastitis *S. agnetis* isolates had this gene (31). The intracellular adhesion genes and biofilm-producing genes (*icaA, icaB, and icaC*) were identified only in the *M. sciuiri* isolate in our study, which is also consistent with the results of previous studies (31, 32).

After adherence to the host surface, bacterial pathogens produce different enzymes which help neutralize the host immune response and promote tissue degradation (33). Our study identified 10 different exoenzyme genes potentially involved in host immune system neutralization, and most of them were common to *S. hyicus* and *S. agnetis* isolated in the current study. The exoenzyme gene profile of isolates from our study was very similar to those previously reported from bovine mastitis isolates of these species (31, 34), except that we identified the gene for lipase enzyme (*geh*) in 11 *S. agnetis* and three *S. hyicus* isolates. Our study also identified three genes responsible for urease activity (*ureA, ureB, and ureG*) in all isolates except *M. sciuiri*, and this is in line with the study by Åvall-Jääskeläinen et al. (34).

Pathogenic bacteria also use encapsulation to evade the host immune system, and staphylococci are well equipped with encapsulation genes, enabling their protection
against phagocytosis and enhancing persistence of infection (35, 36). The S. agnetis isolates from our study had all of the previously identified encapsulation genes (capA to capP), except that the capJ gene was absent in all isolates and capL and capN were absent in 50% of the S. hyicus isolates. Similarly, Naushad et al. reported the absence of capN gene in all isolates of S. agnetis and S. hyicus, and capJ in S. hyicus isolates (31). In addition, Ávall-Jääskeläinen et al. did not find capH-capK genes in S. agnetis isolates from bovine mastitis in Finland (34).

Bacterial pathogens require iron for replication and to maintain infection, and pathogenic bacteria have various iron acquisition mechanisms (37–39). The profile of iron uptake genes for S. agnetis and S. hyicus isolates from our study was similar to that in previous reports (31, 34), with four type VII secretion system (T7SS) genes in all S. hyicus isolates, but none of the S. agnetis isolates had these genes, which was the only virulence factor difference we observed between these two species. The T7SS genes encode a protein secretion pathway, considered important for the virulence of Gram-positive bacteria (40, 41). This finding is similar to that of Ávall-Jääskeläinen et al., who also found lack of T7SS genes in their S. agnetis isolates (34). In contrast, Naushad et al. reported six different T7SS genes in 31% and 67% of the S. agnetis and S. hyicus isolates, respectively (31). Our study also identified the beta hemolysin gene (hlb) in all sequenced isolates except M. sciuri, which supports the findings of Ávall-Jääskeläinen et al. (34) and Naushad et al. (31).

Identification of exfoliative toxin A (eta) and C (etc) genes from S. agnetis and S. hyicus in this study was a major finding in relation to skin lesion development. These toxins are also known as epidermolytic toxins and are serine proteases, able to digest skin desmoglein-1, resulting in the deterioration of desmosomal cell adhesions and epidermal damage (42). The presence of the eta gene in S. agnetis and S. hyicus isolates is in accordance with previous mastitis isolate studies (24, 31, 34), but none of these studies found the etc gene in their isolates. Our study also identified the etc gene in M. sciuri from normal skin, which is contrary to findings of Naushad et al. (31). The close amino acid homology of exfoliative toxins A and C from this study with exfoliative toxins A and C from S. hyicus isolated from exudative epidermitis of pigs and S. aureus from skin infections in horses indicates that these toxins might play an important role in the epidermal damage in BF lesions. This suggestion is further strengthened by the observations from histological studies of BF lesions, which indicated epidermal damage in all bacterially infected lesions (our unpublished observation). Our study also indicated similarity between the VF profiles of BF and BF lesion isolates, which further suggests that the BF isolates where the genotype is not similar to that of the lesion isolate are equally pathogenic and may be involved in lesion pathogenesis when transmitted during BF feeding. The close similarity between the VF profiles of S. agnetis and S. hyicus indicate that both of these species could potentially be involved in the pathogenesis of BF lesions.

**Conclusion.** The findings from this study indicate that the bacteria S. agnetis and S. hyicus, vectored by BF, are likely to be significant factors in the pathogenesis of BF lesions. This suggests that the role of bacteria should be a consideration in the development of optimal treatment and control strategies for BF lesions.

**MATERIALS AND METHODS**

**Sample collection.** Samples were collected from cattle (n = 34) with ulcerated to partially scabbed BF lesions from four different herds. Lesions around the eye and on the shoulder, dewlap, and belly of cattle were swabbed using Amies agar gel transport swabs (Copan, Murrieta, CA, USA). Multiple lesion swabs were collected if the animal had more than one lesion. The cattle sampled included five Brahman heifers from herd 1 (H1) and six Brangus steers from H2, kept at the University of Queensland Pinjarra Hills Research Precinct (−27.53, 152.91) in spatially separated paddocks sampled in May 2020. Eight Brahman heifers from a commercial cattle property in north Queensland (H3) (−20.50, 146.0) were sampled in August 2020, and eight Droughtmaster cattle kept at Darbalara farm (−27.59, 152.38) (H4) were sampled in January 2021. One swab was also collected from visually normal skin from each animal (at least 15 cm away from lesions). Lesion swabs from five heifers from H1 and two steers from H2 were sampled again in August and September 2021, respectively, and skin swabs were also collected from

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15 cm down the median caudus from three steers without any lesions from H2. This study was approved by the University of Queensland Animal Ethics committee (approval no. 2021/AE000054).

Buffalo flies were also collected from the back of each swabbed animal with lesions in H1, H2, and H4 and the three steers without lesions from H2 using an insect collection net. The insect net was disinfected with 80% ethanol between collections, and BFs from each collection were transferred to a sterile plastic zip bag, labeled with the animal ID, and transported to the laboratory on ice.

**Bacterial isolation.** Each swab was used to streak 5% (vol/vol) sheep blood agar and MacConkey agar within 24 h of collection. Buffalo flies (n = 110) were streaked following the method described previously (43). Briefly, five BFs from each animal were washed three times by dipping into 250 μL of sterile normal saline, and the saline washing solution from each group was streaked onto blood agar and MacConkey agar. After washing, the flies were surface disinfected by immersion in 10% sodium hypochlorite (NaClO) followed by 70% ethanol for at least 10 min each. Flies were then rinsed in normal saline, air-dried, homogenized in 100 μL of sterile normal saline, and plated on blood agar and MacConkey agar plates. Plates were incubated at 37°C for 24 h. Bacterial colonies were distinguished as Gram-positive or negative by the potassium hydroxide (KOH) test (44).

**Species identification by MALDI-TOF.** Pure cultures of all isolates (n = 44) from the 2020 sampling were submitted to the Biosecurity Queensland Veterinary Laboratories (Department of Agriculture and Fisheries, Coopers Plains, Queensland, Australia; −27.55, 153.04) for initial species identification by MALDI-TOF mass spectrometry (MALDI-TOF MS) (manufactured by Bruker Daltonics, Germany). A MALDI-TOF score of ≥2.0 was set as the cutoff point for species identification.

**DNA extraction.** DNA was extracted using the Qiagen DNeasy blood and tissue extraction kit (Qiagen Pty. Ltd, Hilden, Germany) according to the manufacturer’s protocol. Briefly, extraction involved suspending a loop full of the bacterial isolate from a fresh overnight blood agar culture into lysis buffer. The suspension was incubated for 1.5 h at 56°C. The remainder of the protocol was as recommended by the manufacturer. DNA concentration was measured using a NanoDrop spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, MA, USA).

**Genotyping by rep-PCR.** rep-PCR was performed as described by Versalovic et al., using primers REP 1R-IDt and REP 2-IDt (REP_1R-IDt, 5'-NNNNCGNCGCATCNGGC-3’, and REP_2-IDt, 5’-NCNGCT TATCNGGCTACT-3’) (45). Briefly, the PCR was performed in a total volume of 25 μL containing 5× GoTaq buffer (Promega, Madison, WI, USA), 2.5 mM MgCl2, a 6.25 mM concentration of each deoxynucleoside triphosphate (dNTP), a 50 μM concentration of each primer, 2U of GoTaq DNA polymerase (Promega, Madison, WI, USA) and 100 ng of DNA template. The cycling conditions included initial denaturation at 95°C for 7 min, annealing at 42°C for 60 s, and Taq polymerase activation at 65°C for 8 min, followed by 33 cycles of annealing and extension at 94°C for 60 s, 42°C for 30 s, and 65°C for 8 min, respectively, with a final extension at 65°C for 8 min. The reaction was conducted in an Eppendorf Mastercycler Pro thermal cycler (AG 22331; Eppendorf, Hamburg, Germany). The PCR products were run on a 2% TAE buffer (40 mM Tris, 20 mM acetate, and 1 mM EDTA, pH 8.5) agarose gel containing 0.05 μg/mL of ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA) for 3.5 h at 70 V.

The rep-PCR profiles of 44 isolates (from 2020) were compared using BioNumerics software (version 4.50; Applied Maths, Inc., Saint-Martens-Latem, Belgium). Genotypic profiles were compared using band matching tolerance and optimization of 0.5%. For cluster analysis of DNA fingerprinting data, the similarities were calculated using the Dice similarity coefficient (46). A comparison dendrogram was also developed using the unpaired group method with arithmetic average (UPGMA). The genotyping of 19 isolates from 2021 was compared with that of 21 isolates (one representative isolate from each cluster) collected in 2020, using BioNumerics software with the same band matching tolerance and optimization of 0.5%.

**Selection of isolates for sequencing.** The 44 *Staphylococcus* isolates from 2020 were grouped into 21 clusters using a cutoff threshold of 90% similarity in rep-PCR-based genotyping. A total of 21 isolates were selected (one representative from each cluster) for whole-genome sequencing using the Illumina NovaSeq 6000 (Illumina, San Diego, CA, USA) platform. The selected isolates included three isolates from BFs, two isolates from normal cattle skin, and 16 isolates from BF lesions. Nine of the 21 isolates sequenced with the Illumina platform, including eight isolates from lesions and one from BFs, were also sequenced using the Oxford Nanopore Technologies (ONT) platform to generate complete genomes. These nine isolates were selected at a cutoff threshold of 70% similarity of the genotypic patterns.

**DNA extraction and quality testing for sequencing.** Selected bacterial isolates were subcultured from storage (−80°C) by inoculation onto 5% (vol/vol) sheep blood agar. The cultures were incubated for 24 h at 37°C. For genome sequencing, DNA was extracted using Gentra Puregene core kit A (Qiagen Pty Ltd, Hilden, Germany) with some modifications of the manufacturer’s protocol. A standard suspension from the blood agar (optical density at 600 nm [OD600] = 1.85, or 1.74 × 10⁸ bacteria) was prepared in sterile phosphate buffer saline (PBS), and an aliquot of 300 μL was transferred to a 2-mL tube. For efficient lysis, 50 μL of lysozyme (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 2.9 mg/mL and 50 μL of lysisphastin at a final concentration of 0.14 mg/mL (Sigma-Aldrich, St. Louis, MO, USA) were also added to each tube and incubated at 37°C for 3 h in a Thermomixer C (Eppendorf, Hamburg, Germany). After 3 h of incubation, 500 μL of cell lysis solution was added to each tube and incubated for 1 h at 36°C. To maximize the DNA yields, the tubes were incubated for 5 min at 80°C. The remaining protocol was followed as recommended by the manufacturer. DNA for sequencing was quantified with a Qubit 4 fluorometer (Thermo Scientific, Waltham, MA, USA) using a Qubit double-stranded-DNA (dsDNA) BR assay kit (Invitrogen, Waltham, MA, USA).

Before library preparation, DNA quality was tested by Pippin pulsed-field gel electrophoresis. For this, 500 ng of the extracted DNA samples was run on a 0.75% SeaKem Gold agarose gel (Lonza Bioscience, Basel, Switzerland) for 16 h on a Pippin Pulse electrophoresis power supply system (Sage
Illumina library preparation and sequencing. DNA extracted from 21 isolates were sequenced at the Australian Centre for Eucogenomics (ACE), The University of Queensland (St. Lucia, Queensland, Australia). Briefly, DNA libraries were prepared according to the manufacturer’s protocol using the Nextera DNA Flex library preparation kit (Illumina, San Diego, CA, USA). Library preparation and bead cleanup were undertaken with the Mantis liquid handler (Formulatrix, Bedford, MA, USA) and EpMotion (Eppendorf, Hamburg, Germany) automated platform. These programs cover “Tagment Genomic DNA” to “Amplify DNA” in the protocol (Nextera DNA Flex library prep protocol; Illumina, San Diego, CA, USA) and “Clean Up Libraries” in the protocol (EpMotion-library cleanup protocol). On completion, each library was quantified, and quality control performed using the Quant-iT dsDNA HS assay kit (Invitrogen, Waltham, MA, USA) and Agilent D1000 HS tapes (Agilent Technologies, Santa Clara, CA, USA) on the TapeStation 4200 (Agilent Technologies, Santa Clara, CA, USA), as per the manufacturer’s protocol.

Nextera DNA Flex libraries were pooled at equimolar amounts of 2 nM per library to create a sequencing pool. The library pool was quantified in triplicate using the Qubit dsDNA HS assay kit (Invitrogen, Waltham, MA, USA). Library quality control was performed using the Agilent D1000 HS tapes on the TapeStation 4200 as well as per the manufacturer’s protocol. The library was prepared for sequencing on the NovaSeq 6000 (Illumina, San Diego, CA, USA) using NovaSeq 6000 SP kit v1.5 and 2 × 150-bp paired-end chemistry, according to the manufacturer’s protocol.

ONT library preparation. For ONT sequencing, a MinION sequencing library was prepared using the Nanopore Ligation Sequencing kit (Oxford Nanopore Technologies, Oxford, UK) as per the manufacturer’s protocol with starting DNA amount of 6 μg (185 fmol). A final amount of 650 ng (20 fmol) of the prepared library was loaded on a MinION Spot-On flow cell (Oxford Nanopore Technologies; version FLO-MIN106D R9.4.1) using a flow cell priming kit (Oxford Nanopore Technologies) as instructed by the manufacturer. The library was sequenced using a MinION device (MK1C, MC110367) for 5 to 6 h with default instrument settings. Primary acquisition of data and real-time base calling was carried out using the graphical user interface MinKNOW (version 20.10.6; Oxford Nanopore Technologies) and Guppy base caller (v4.5.2; Oxford Nanopore Technologies).

Sequence analysis, assembly, and annotation. All data analysis in this study was performed using programs on Galaxy Australia (https://usegalaxy.org.au/). For each isolate sequenced with the Illumina 6000 NovaSeq platform, 1 Gbp of data (400× coverage) was acquired in FASTQ format. Initial read quality was determined by FastQC (Galaxy version, 0.72 + Galaxy!) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Low-quality reads were removed using Trimmomatic (Galaxy version 0.36.6) (47) and trimmed from the start (leading) and at the quality score below 30. A sliding-window trimming was done if the average quality of four bases dropped below 20, and all unpaired reads and reads below 30 bp were removed. Paired reads were de novo assembled using the Shovil assembler (Galaxy version 1.10 + galaxy0) (with the settings "Estimated genome size": 2.5 Mbps; "Minimum contig length": 500; "Assembler": SPAdes) (https://github.com/tseemann/spades).

For the isolated sequences in duplicate with ONT, 2.0 Gbp data (800× coverage) was acquired in FASTQ format. Reads were concatenated using the tool "Concatenate (cat) tail to head" (version 0.10 + Galaxy) (https://github.com/bgruening/galaxytools). Reads were filtered for length and average Q score by FiltLong (Galaxy version 0.20 + galaxy!) (https://github.com/rwicke/FiltLong). Reads shorter than 5,000 to 15,000 were removed, depending upon the initial read length N_i, histogram (base-called base) report generated by MK1C for an individual isolate. For removal of low-quality reads, a threshold of Q score ≥7.0 (for 100% reads) and a Q score of ≥12 (for more than 80% reads) was used. The filtered ONT reads along with respective corrected Illumina reads of the same isolate were used to generate hybrid de novo assemblies with Unicycler (Galaxy version 0.4.8.0) with normal "Bringing mode" and “Pilon” option enabled for assembling polishing (48). The de novo assembled genome sequences were annotated with the prokaryotic genome annotation tool Prokka (Galaxy version 1.14.6 + galaxy0) (49).

Pangenome analysis, read mapping, and multilocus sequence phylogeny. To identify the extent of genomic diversity in the sequenced isolates, a pangenome analysis was performed using Roary (Galaxy version 3.13.0 + galaxy1) (50). Output from Roary was uploaded on an online web-based platform for interactive visualization of genome phylogenies. Phandango (51), to visualize the presence and absence of a gene and genomic similarity between isolates.

To confirm the identification of the sequenced isolates, corrected paired Illumina reads from all the sequenced isolates were mapped against genomes of reference strains of S. agnetis (DSM23656), S. hyicus (NCTC10350), S. chromogenes (NCTC10530), and M. sciuri (NCTC12103) using Bovtiew2 (Galaxy version 2.4.2 + galaxy0) (52). Accession numbers and the strain type for the genome sequences used for reads mapping are listed in Table 3.

A multilocus sequence phylogeny was constructed using nucleotide sequences of four housekeeping genes (tuf, rpoA, rpoB, and recN) from all the annotated genomes from this study and each of the reference strains of S. agnetis, S. hyicus, S. chromogenes, M. sciuri, S. aureus, S. caprae, S. epidermidis, S. intermedius, S. argenteus, and Bacillus subtilis. The nucleotide sequences for these four housekeeping genes were concatenated (in the order tuf, rpoA, rpoB, recN) and aligned for each genome using Geneious software (version 2021.1.1; Biomatters, Ltd., Auckland, New Zealand). Accession numbers and the strain types for the genome sequences used for phylogenetic analysis are listed in Table 3.

Before phylogenetic analysis, a model test was performed in Mega X (53) with the aligned nucleotide sequences of the above-mentioned genes to select the best suitable model for phylogeny. The model with the lowest Bayesian information criterion (BIC) scores was selected for further analysis. A phylogenetic tree based on concatenated sequences of tuf, rpoA, rpoB, and recN was inferred using the
maximum-likelihood method and general time-reversible model (54) using Mega X (53). Initial trees for the heuristic search were obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum-composite-likelihood (MCL) approach. The topology with the superior log likelihood value was then selected. A bootstrap consensus tree of 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed (55). Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates were collapsed. This analysis involved 32 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There was a total of 7,359 nucleotide positions in the data set.

### Phylogenetic Analysis

To select the best suitable model for phylogenetic analysis of eta and etc genes, a model test was performed in Mega X (53) with amino acid sequences from both genes, and the model with the lowest BIC scores was chosen for further analysis. Phylogenetic trees were inferred for eta and etc, respectively, using the maximum-likelihood method and general reversible chloroplast model (65) in Mega X. Initial trees for the heuristic search were obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model. The topology with a superior log likelihood value was then selected. The bootstrap consensus tree inferred from 1,000 replicates was taken to represent the evolutionary history of the taxa analyzed (55). Branches corresponding to partitions reproduced in less than 70% of the bootstrap replicates were collapsed. For the eta and etc proteins, 27 and 25 amino acid sequences and 306 and 280 amino acid positions were involved, respectively.

### PCR based Identification of Isolates

To identify the isolates from 2021 sampling and to confirm the identity of isolates from 2020 which were not utilized for genome sequencing, DNA samples extracted from each of the isolates were tested with an aroD-based multiplex PCR using the primers.
reported by Adkins et al. for species-specific identification of S. hyicus (aroD_hyF, S'-TATGGTG TGCCACATGAGGCT-3', and aroD_hyR, S'-ACCTATAGGCCGGTACTTT-3') and S. agnetis (aroD_agnetis, S'-CGGATGAGAGACCAATACGCT-3', and aroD_agnetisR, S'-TAGGAGTATAAGGGTG-3') (22). Briefly, the PCR was performed in a total volume of 20 μL containing 10 μL of 2X Phusion Hot Start II high-fidelity PCR master mix (Thermo Scientific, Waltham, MA, USA), a 10 μM concentration of each forward and reverse primer, and 3 ng of DNA template. The cycling conditions included an initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation, annealing, and extension at 98°C for 10 s, 60°C for 30 s, and 72°C for 30 s, respectively, and a final extension at 72°C for 10 m. The reaction was set up in an Eppendorf Mastercycler Pro thermal cycler. For visualization, the amplification products were run on 2% TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8) agarose gel for 75 m at 80 V using a Genefuiler 100-bp Plus DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA).

Data availability. The sequence data generated and analyzed in the current study have been deposited in the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome) under BioProject accession number PRJNA809943.

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