AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Characterization of a Novel Composite Staphylococcal Cassette Chromosome *mec* in Methicillin-Resistant *Staphylococcus pseudintermedius* from Thailand

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A novel staphylococcal cassette chromosome *mec* (SCC*mec*) composite island (SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI) was identified in *Staphylococcus pseudintermedius*. Four integration site sequences for SCC subdivided the 60,734-bp island into 41,232-bp SCC*mec*$_{AI16}$, 19,400-bp SCC*czr*$_{AI16}$, and 102-bp SCC-like$_{AI16}$ elements. SCC*mec*$_{AI16}$ represents a new combination of *ccrA1B3* genes with a class A *mec* complex. SCC*czr*$_{AI16}$ contains *ccrA1B6* and genes related to restriction modification and heavy metal resistance. SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI was found in methicillin-resistant *S. pseudintermedius* sequence type 112 (ST112) and ST111 isolated from dogs and veterinarians in Thailand.

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has been associated with clinical manifestations in companion animals and occasionally causes diseases in humans (1, 2). MRSP contains the staphylococcal cassette chromosome *mec* (SCC*mec*) element, which is defined by the presence of the *mec* gene that mediates β-lactam resistance and by the cassette chromosome recombinase (*ccr*) gene(s) responsible for site-specific integration/excision of the element (3). Composite SCC structures containing two *ccr* gene complexes likely resulting from multiple element integrations have been increasingly reported in *Staphylococcus aureus* and in coagulase-negative staphylococci where such structures frequently carry heavy metal resistance gene clusters (3–9). So far, only five SCC*mec* elements have been completely characterized in MRSP, including SCC*mec* II-III, SCC*mec* VII-241, SCC*mec* V, ΨSCC*mec*$_{57395}$, and SCC*mec* IV, and are mainly associated with predominant clones of the following sequence types (ST): ST71, ST93, ST68, ST233, ST45, and ST261 (10–14). Recently, MRSP strains of ST112 and ST111 (a single locus variant of ST112) isolated from dogs and veterinarians in Thailand were found to contain an unusual combination of the class A *mec* gene complex with the type I *ccr* gene complex, suggesting a new SCC*mec* type (15). We therefore aimed to characterize this novel SCC*mec* element and to determine whether it is conserved within other MRSP isolates of the same clonal lineage.

Identification of the SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$ composite element (CI). MRSP strains of ST111 and ST112 were obtained from a previous study and are listed in Table 1 (15). Phenotypic and genotypic characteristics, including those determined by antimicrobial susceptibility testing and antibiotic resistance gene detection, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST), were determined previously (15). In this study, the strains were additionally tested for *mec*-associated direct repeat unit (*dru*) and *spa* types (16, 17), for the presence of the novel SCC*mec* element, and for resistance to heavy metals. The genomic DNA of all strains was extracted using the peqGOLD bacterial DNA kit (Peqlab Biotechnologie GmbH, Jena, Germany), and that of strain AI16 was sequenced using Ion Torrent semiconductor (Life Technologies, Carlsbad, CA) and Illumina MiSeq (Illumina, San Diego, CA) technologies. Sequence reads from the Ion Torrent were assembled *de novo* using MIRA v3.4.1.1, generating 64 contigs with an N_{50} (length weighted median) of 121,469 bp and a contig sum of 2,829,899 bp (mean read length, 265 bp; average coverage, >100-fold). The sequences were corrected by read mapping with MiSeq reads using Geneious version R8 (Biomatters, Auckland, New Zealand) (18), and unresolved discrepancies were verified by Sanger sequencing (ABI Prism 3100 genetic analyzer; Applied Biosystems, Foster City, CA). Nucleotide analysis searching for SCC-associated components (*orfX* gene, *mec* gene complexes, *ccr* gene complexes, and integration site sequence [ISS] for SCC [19]) using BLAST (http://blast.ncbi.nlm.nih.gov/) enabled the identification of a 60,734-bp SCC composite element within a 100,011-bp contig. Open reading frames (ORF) were defined using Prodigal software for locating genes in prokaryotes (34) and were annotated manually using BLASTn and BLASTp algorithms. The element was integrated at the 3′ end of the chromosomal *orfX* gene and contained a *mec* gene complex, two *ccr* gene complexes, and four ISSs. The ISSs contained direct repeats (DRs) that flanked the composite SCC element and that also divided it into three subunits, including SCC*mec*$_{AI16}$, SCC*czr*$_{AI16}$, and SCC-like$_{AI16}$ according to the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) (4) (Fig. 1). ISSs were also linked to imperfect inverted repeats (IRs) at all

Received 17 September 2015 **Returned for modification** 7 October 2015
Accepted 19 November 2015

Accepted manuscript posted online 7 December 2015

Citation Chanchaithong P, Prapasarakul N, Perreten V, Schwendener S. 2016. Characterization of a novel composite staphylococcal cassette chromosome *mec* in methicillin-resistant *Staphylococcus pseudintermedius* from Thailand. Antimicrob Agents Chemother 60:1153–1157. doi:10.1128/AAC.02268-15.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02268-15.

TABLE 1 Origin and molecular characteristics of the six methicillin-resistant *Staphylococcus pseudintermedius* strains used in this study

Strain	Source	Site	Sequence type[a]	PFGE result[a]	SCC*mec*[b]	*spa* type[c]	*dru* type[d]	Antimicrobial resistance profile [resistance genes][e]
AI16	Dog	Groin with crusty exudate	ST112	A	SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI	t05	11y	OXA-PEN-TET-GEN-KAN-STR-ERY-iCLI-SMX-TMP [*mecA*, *blaZ*, *tet*(M), *aac(6′)-Ie*, *ant(6′)-Ia*, *aph(2′)-Ia*, *aph(3′)-III*, *erm*(B), *sat4* and *dfrG*]
AJ1	Dog	Perineal carriage	ST112	A	SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI with 1,868-bp insertion	t05	11cv	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-SMX-TMP-MUP [*mecA*, *blaZ*, *tet*(K), *tet*(M), *aac(6′)-Ie*, *ant(6′)-Ia*, *aph(2′)-Ia*, *aph(3′)-III*, *erm*(A), *erm*(B), *sat4*, *dfrG* and *mupA*]
AK5	Dog	Nasal carriage	ST112	A	SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI	t05	11y	OXA-PEN-TET-GEN-KAN-STR-ERY-iCLI-SMX-TMP [*mecA*, *blaZ*, *tet*(K), *tet*(M), *aac(6′)-Ie*, *ant(6′)-Ia*, *aph(2′)-Ia*, *aph(3′)-III*, *erm*(B), *sat4* and *dfrG*]
AM33	Dog	Perineal carriage	ST111	C	SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI	Negative	11y	OXA-PEN-TET-GEN-KAN-STR-ERY-iCLI-SMX-TMP [*mecA*, *blaZ*, *tet*(K), *aac(6′)-Ie*, *ant(6′)-Ia*, *aph(2′)-Ia*, *aph(3′)-III*, *erm*(B), *sat4* and *dfrG*]
VA26	Human	Nasal carriage	ST112	A	SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI with 1,868-bp insertion	t06	11y	OXA-PEN-TET-GEN-KAN-STR-ERY-iCLI-SMX-TMP-CIP [*mecA*, *blaZ*, *tet*(M), *aac(6′)-Ie*, *ant(6′)-Ia*, *aph(2′)-Ia*, *aph(3′)-III*, *erm*(B), *sat4* and *dfrG*]
VB16	Human	Nasal carriage	ST112	A	SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI	t06	11y	OXA-PEN-TET-GEN-KAN-STR-ERY-CLI-SMX-TMP-MUP-CIP [*mecA*, *blaZ*, *tet*(M), *aac(6′)-Ie*, *ant(6′)-Ia*, *aph(2′)-Ia*, *aph(3′)-III*, *erm*(B), *sat4*, *dfrG* and *mupA*]

[a] MLST and PFGE analyses were performed as a part of the previous study (15).
[b] SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI was identified in all strains by long-range PCR and restriction analysis. AJ1 and VA26 contained a 1,868-bp insertion.
[c] The *spa* typing resulted in two *spa* types, t05 (r01-r02-r03-r03-r03-r03-r06-r05) and t06 (r01-r02-r03-r03-r06-r05), and one negative strain with no *spa* amplification by PCR.
[d] The *dru* types presented *dru* repeats as follows: 11y, 5a-2d-4a-1b-2d-5b-3a-2g-3b-4e-3e; 11cv, 5a-2d-4a-1b-2d-6f-3a-2g-3b-4e-3e.
[e] Antimicrobial resistance phenotypes and detection of resistance genes were determined in the previous study using broth dilution methods and microarray (15). Abbreviation of antimicrobials are as follows: OXA, oxacillin; PEN, penicillin; TET, tetracycline; GEN, gentamicin; KAN, kanamycin; STR, streptomycin; ERY, erythromycin; CLI, clindamycin; iCLI, inducible resistance to clindamycin; SMX, sulfamethoxazole; TMP, trimethoprim; MUP, mupirocin; CIP, ciprofloxacin. Antibiotic resistance genes and functions are as follows: *mecA*, penicillin-binding protein 2a; *blaZ*, β-lactamase; *tet*(K), tetracycline efflux protein; *tet*(M), ribosomal protective protein; *aac(6′)-Ie*, aminoglycoside acetyltransferase; *ant(6′)-Ia*, aminoglycoside nucleotidyltransferase; *aph(2′)-Ia* and *aph(3′)-III*, aminoglycoside phosphotransferases; *erm*(B) and *erm*(C), erythromycin resistance methylase; *sat4*, streptothricin acetyltransferase; *dfrG*, dihydrofolate reductase; *mupA*, isoleucyl-tRNA synthetase.

subunit boundaries, forming parts of the SCC attachment sites (*att*) (19) (Fig. 1).

SCC*mec*$_{AI16}$. SCC*mec*$_{AI16}$ consisted of a classical SCC*mec* element, as it contained the *mec* gene as well as the *ccr* genes and was flanked by two DRs (DR1 and DR2). It was located downstream of *orfX*, had a size of 41,232 bp, and contained 44 ORFs. The class A *mec* gene complex was located directly downstream of *orfX* and was only separated by a small 108-bp noncoding joining region (Fig. 1). The second joining region of SCC*mec*$_{AI16}$ spanning the region between the *mec* gene complex and the *ccr* gene complex contained several genes coding for hypothetical protein, cadmium resistance (*cadCAD*), transposase B and C of transposon Tn*554*, and a putative cyclopentanol dehydrogenase (*cpnA*). The entire 5′ region of SCC*mec*$_{AI16}$, including the *mec* gene complex up to *cpnA*, was highly identical (99%) to the nucleotide sequence of SCC*mec* VII-241 (without *cpnA*) and to a hybrid SCC*mec-mecC* region of *Staphylococcus sciuri* GVGS2 (including *cpnA*) (10, 21). However, SCC*mec*$_{AI16}$ differed from those two elements by the presence of a different type of *ccr* gene complex and a different 3′ end-joining region that encoded various hypothetical proteins, some of them already identified in other SCC*mec* elements (see Table S1 in the supplemental material). The *ccr* complex of SCC*mec*$_{AI16}$ contained *ccrA1* and *ccrB3* genes and shared the closest identity to those of *S. aureus* JCSC6945 and *S. sciuri* MCS24 with 92% and 89% nucleotide identity, respectively (20, 22) (see Fig. S1 in the supplemental material). *ccrA1B3* was assigned as a type 8 *ccr* complex, which was originally found in SCC*mec* XI together with a class E *mec* complex in *S. aureus* M10/0061 (23). However, the *ccrA1* of SCC*mec*$_{AI16}$ shared only 81% nucleotide identity with the *ccrA1* of SCC*mec* XI but shared up to 90% sequence similarity with the *ccrA1* in SCC*mec* IX and X in livestock-associated methicillin-resistant *S. aureus* (LA-MRSA) in Thailand (20, 24). The combination of the type 8 *ccr* complex and the class A *mec* complex of SCC*mec*$_{AI16}$ has not been previously reported and represents a new SCC*mec* type in *S. pseudintermedius*.

SCC*czr*$_{AI16}$ and SCC-like$_{AI16}$. The SCC*czr*$_{AI16}$ element contained recombinase genes but no *mec* gene. It was flanked by DR2 and DR3, arranged in tandem with the SCC*mec*$_{AI16}$, and followed by a 102-bp noncoding SCC-like$_{AI16}$ fragment demarcated by DR3 and DR4. SCC*czr*$_{AI16}$ was 19 kb in length, contained 18 ORFs (see Table S1 in the supplemental material), and consisted of three main segments displaying different functions. The first segment contained genes coding for proteins (HsdR, HsdM, and HsdS) associated with a type I restriction-modification system. HsdR and HsdM showed 99% and 98% amino acid (aa) identity with those encoded on SCC*fusC*, respectively (25), and were combined with a novel HsdS that shared 61% aa identity to the closest HsdS (see Table S1), indicating a new restriction specificity in this

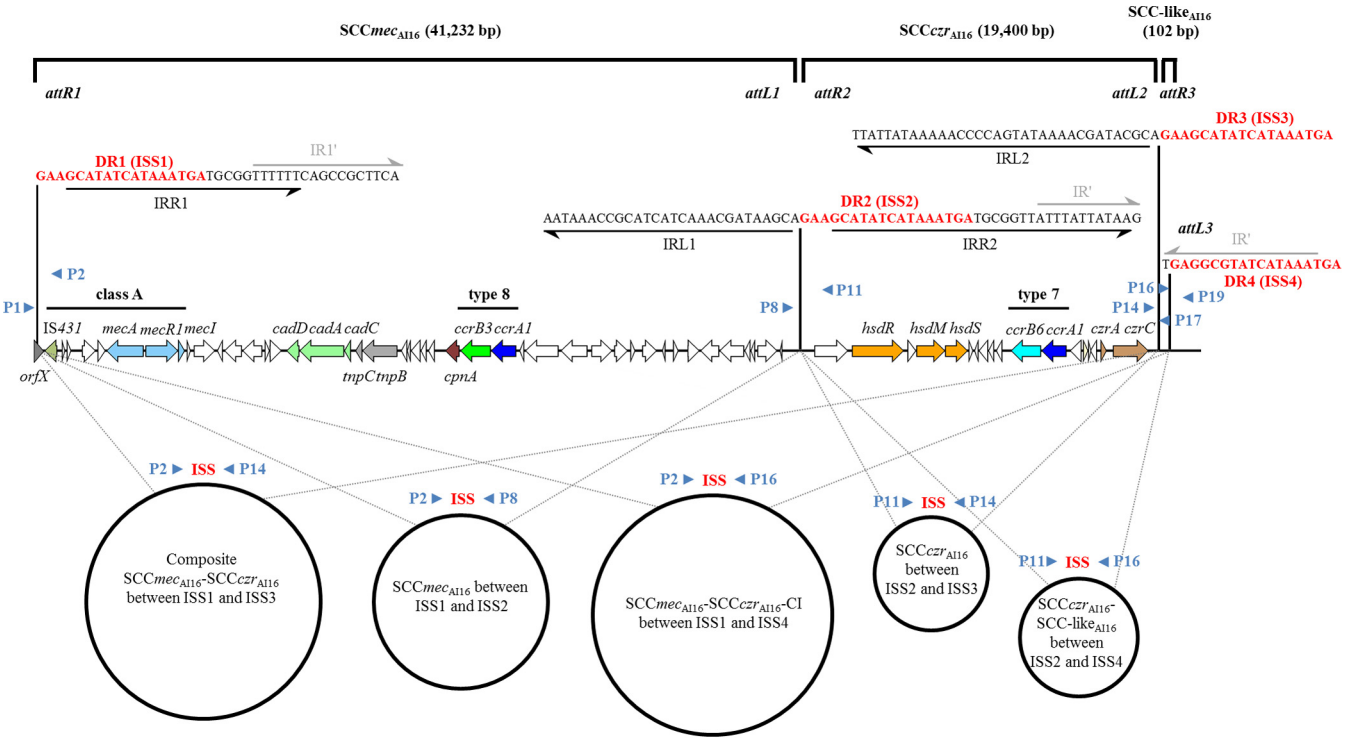

FIG 1 Genetic organization of the novel composite SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI element of *Staphylococcus pseudintermedius* strain AI16 and formation of circular intermediates. The single units of the SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI are flanked by direct repeats (DR) containing the integration site sequence (ISS) (red) and by inverted repeats (IR) (harpoon arrows) forming part of the attachment sites (*att*). White arrows represent sequences encoding hypothetical proteins, and other genes are color coded as follows: *orfX*, gray; IS*431*, pale green; *mec* operon, sky blue; transposases of Tn*554*, light gray; cyclopentanol dehydrogenase, brick red; type I restriction-modification system, orange; and *czr* operon, brown. The *ccrA1*, *ccrB3*, and *ccrB6* recombinase genes are shown in blue, green, and cyan, respectively. Small blue arrows indicate the primers used for PCR, identifying circularization and excision of the SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI subunits (see Tables S2 and S3 in the supplemental material). The genetic map of SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI was drawn using the Easyfig software (32).

MRSP lineage (26). The second segment contained a type 7 *ccr* complex, with *ccrA1* and *ccrB6* sharing 99% and 89% identity to those of SCC*mec* X from LA-MRSA ST398 (JCSC6945) isolated from a human in Thailand (20) (see Fig. S1 in the supplemental material). This *ccr* complex was preceded by three ORFs that also displayed high similarity (96%, 100%, and 99%) to those found upstream of the *ccr* genes of SCC*mec* X. The third segment situated at the 3′ region of SCC*czr*$_{AI16}$ showed 99% DNA identity to the 3′ region of SCC$_{SH32}$ identified in *Staphylococcus haemolyticus* SH32 isolated from a Chinese patient (27) and to a fragment of the SCC*mec* V(5C2&5)c found in *S. aureus* (28) and *S. haemolyticus* (9). It carried six ORFs, including *czrC*, which has been shown to confer resistance to zinc and cadmium in *S. aureus* (29). While resistance to cadmium increased up to 4-fold in *S. pseudintermedius* strains containing *cadA* compared to that in strains which lack *cadA* or carry only *czrC*, we did not observe reduced susceptibility to zinc in any of the tested strains, including those containing *czrC*, using broth microdilution assays after 20-h and 48-h (data not shown) incubations at 37°C (30) (Table 2). The same MIC of zinc for strains with and without *czrC* indicates that this gene does not confer measurable zinc resistance in *S. pseudintermedius*. Additionally, decreased susceptibility to other heavy metals was not observed (Table 2). The presence of *cadA* and *czrC* genes was confirmed by PCR using the primers listed in Table S2 in the supplemental material.

Excision and circularization of SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI subunits. Ccr recombinases recognize the *att* sites of SCC and catalyze DNA cleavage, strand exchange, and recombination to integrate an element or to release a nonreplicative circular element. Sequencing PCR products obtained using primer pairs reading inwards and outwards from the *att* sites identified circular forms containing one copy of ISS as a joining region as well as chromosomal segments remaining after element excision. Such circular forms were detected for the individual SCC*mec*$_{AI16}$ and SCC*czr*$_{AI16}$ and for the composites SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$ and SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI (Fig. 1; see also Table S3 in the supplemental material). This indicated CcrAB activity in the strain AI16 and possible mobilization of the individual SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI circular subunits. Notably, ISS3 was preferred over ISS4 for recombination, which may be caused by the presence of a divergent IR in the ISS4-containing *att* site (Fig. 1; see also Table S3).

Presence of SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI in additional MRSP isolates. Long-range PCR amplification followed by restriction analysis (see Tables S2 and S4 in the supplemental material) confirmed the overall structure of SCC*mec*$_{AI16}$-SCC*czr*$_{AI16}$-CI in MRSP strain AI16 and allowed detection of similar composite elements in five additional clonally related MRSP strains of ST111 and ST112 isolated from dogs and humans (Table 1). Two strains, AJ1 (ST112) and VA26 (ST112), showed a minor variation in the restriction analysis caused by a 1,868-bp insertion between *czrC*

TABLE 2 Heavy metal susceptibility of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* strains as determined using broth microdilution test

Strain	Metal resistance gene(s)	MIC (mM) of metal						Source
		$CuSO_4$	$CdCl_2$	$ZnSO_4$	$AsNaO_2$	$Pb(CH_3COO)_2$	$HgCl_2$	
S. aureus								
RN4220		4	≤0.015	0.5	0.03	≤0.125	≤0.015	33
S. pseudintermedius								
CCUG 49543 (=LMG 22219)		16	0.25	1	2	1	≤0.015	Culture Collection, University of Göteborg, Sweden
Methicillin-resistant *S. pseudintermedius*								
E120		16	0.25	1	2	1	≤0.015	1
KM241	*cadA*	16	1	1	2	1	≤0.015	10
196511	*czrC*	16	0.25	1	2	1	≤0.015	D. Elad, Kimron Veterinary Institute, Bet Dagan, Israel
AI16	*cadA*, *czrC*	16	1	1	2	1	≤0.015	This study, 15

and ISS3 as confirmed by primer walking and Sanger sequencing. In addition, the SCC*mec* element of AJ1 displayed an alternative hypervariable repeat region (*dru* 11cv) that was different from that of the other strains (*dru* 11y), suggesting different evolutionary processes within SCC of the same clonal lineage.

Characterization of the novel SCCmec_{AI16}-SCCczr_{AI16}-CI element in *S. pseudintermedius* revealed regions with homology to other SCC*mec* elements. The 5′ region of SCCmec_{AI16} obviously shared a common ancestral origin with SCC*mec* elements found in *S. pseudintermedius* KM241 and in *S. sciuri* GVGS2 but displayed a substituted 3′ region resulting in a new combination of the class A *mec* complex with *ccrA1B3* genes. The presence of SCCczr_{AI16} harboring a second *ccrAB* complex illustrates the challenge for PCR-based SCC*mec* typing. Furthermore, the location of two related *ccrAB* gene complexes aligned in proximity offer a source for homologous recombination-mediated deletion, a mechanism observed previously with *ccrC* sequences (28, 31). The presence of this composite element in other strains of the same lineage rather supported a spread of this cassette with a clone as opposed to an autonomous transfer between strains. However, the ability of the SCCmec_{AI16}-SCCczr_{AI16}-CI subunits to circularize independently may play a role in the mobilization and in the further evolutionary diversification of this composite SCC*mec* structure.

Nucleotide sequence accession numbers. The nucleotide sequences of the SCCmec_{AI16}-SCCczr_{AI16}-CI of *S. pseudintermedius* AI16 and the 1,868-bp insertion in *S. pseudintermedius* VA26 have been deposited in GenBank under accession numbers LN864705 and LN874217, respectively.

ACKNOWLEDGMENTS

We thank Matt Riley (University of Tennessee, Knoxville, TN) for advice with sequence analysis, Arshnee Moodley (University of Copenhagen, Copenhagen, Denmark) for help with *spa* typing, Richard V. Goering (Creighton University School of Medicine, Omaha, NE) for help with *dru* typing, Daniel Elad (Kimron Veterinary Institute, Bet Dagan, Israel) for providing strain 196511, and Alexandra Collaud (Institute of Veterinary Bacteriology, University of Bern) for technical assistance.

FUNDING INFORMATION

Institute of Veterinary Bacteriology, University of Bern provided funding to Vincent Perreten and Sybille Schwendener under grant number 35-539. Ratchadaphiseksomphot Endowment Fund, Chulalongkorn University provided funding to Pattrarat Chanchaithong under grant number GDNS 57-045-31-006.

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