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Staphylococcus aureus from Italian red deer carries prophages that harbour the bovine leukocidin lukM/lukF-P83

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Abstract

Staphylococcus (S.) aureus can harbour many virulence factors and these include leukocidins, which are bi-component toxins encoded by two co-expressed genes. Some of these toxin genes are localised on prophages and thus they are potentially transmissible. In many cases, they determine the host specificity of the strain they reside in. One set of leukocidin genes, lukM/lukF-P83, is known to occur in isolates from bovids, small ruminants and rodents. It has also been shown to occur in two lineages of S. aureus from red deer (Cervus elaphus) in Italy.

Three deer isolates from two lineages, clonal complexes (CC) 350 and 425, were subjected to genome sequencing applying nanopore technology. In addition, phages were induced by mitomycin C treatment, sequenced and imaged by transmission electron microscopy (TEM). The *lukM/lukF-P83* leukocidin genes were situated on a *Peeveelvirus* prophage that integrated into the A5IU43 = *yfkAB* gene and that was inducible by mitomycin C. The prophage sequences of CC350 and CC425 were nearly identical. They differed from other, previously published *lukM/lukF-P83*-prophage sequences with most of which, however, they shared the integration site. In addition, the CC350 isolate harboured another, apparently hybrid prophage which was not inducible. Both CC425 isolates also carried an inducible *Triavirus* prophage.

While various S. aureus lineages have been observed in deer, data on their virulence factors remain insufficient. Our observations should prompt further investigations on cervids, both wild or captive. Red deer in zoo or game park populations, together with post-mortem examinations of free-ranging deer found dead, culled for management or harvested by hunters, should provide ample opportunities to observe animals for skin and soft tissue infections including mastitis which are known to be associated with lukM/lukF-P83 in other animals.

Introduction

Staphylococcus (S.) aureus is a Gram-positive, facultatively anaerobic bacterium that acts as an opportunistic pathogen in humans as well as in animals. It can cause a wide range of diseases, from superficial skin and soft tissue infections to severe and potentially life-threatening conditions such as pneumonia, endocarditis, sepsis, and osteomyelitis [1, 2]. *S. aureus* exhibits remarkable genetic plasticity, which enables a rapid adaptation to environmental pressures and thus contributes to the global emergence of antibiotic-resistant strains, including methicillin-resistant *S. aureus* (MRSA; [3]).

S. aureus has a broad arsenal of virulence factors [4, 5], including chromosomal determinants such as protein A and haemolysins, as well as phage-encoded genes. The importance of bacteriophages is increasingly acknowledged because they can be spread via horizontal gene transfer between strains and even between different host species. One class of virulence factors is the leukocidins. These are bicomponent cytotoxins that primarily target host leukocytes. The two components are encoded by neighbouring genes. They are co-expressed and form polymeric pores in host membranes leading to cell death accompanied by a release of cytokines [6, 7]. Several leukocidin genes such as *lukA/B*, *lukF/S-hlg* or *lukD/E* are localized on the chromosome. Others are phage-borne. A prominent example is Panton-Valentine leukocidin (PVL, encoded by *lukF/S-PV*). It is associated with recurrent, chronic or severe skin and soft tissue infection as well as with rare but life-threatening necrotising pneumonia [8]. PVL has been known for almost a century but in recent years it became associated with several strains of community-acquired MRSA. While PVL is highly specific for human leukocytes [9], *S. aureus* strains from animals might carry other phage-borne leukocidin genes. Such differences raise questions about host adaptation, pathogenicity and zoonotic potential of these strains. Since many of these genes are located on prophages, it can be assumed that phage infections of *S. aureus* significantly influence the host specificity of individual strains and the clinical manifestations in different vertebrate hosts. Leukocidin genes *lukP/Q* have been described in isolates originating from horses [10, 11]. Another set of genes, *lukF/S-BV*, has been described from Eurasian beavers where they are also associated with abscess formation and life-threatening disease [12]. Finally, there are *lukM/lukF-P83* genes [13, 14]. They are known to target bovine neutrophils [15] and in a rodent model, this has been shown to promote skin and soft tissue infection [16]. Genes *lukM/lukF-P83* are particularly relevant because of its association with mastitis in cattle [17–20] and small ruminants [21, 22], an important animal-welfare and economic burden, literally in an order of magnitude of more than twenty billion dollars annually [23, 24]. These genes also have been implicated in an outbreak of exudative dermatitis in English squirrels [25–27], and they might occur in swine [28]. Genes *lukM/lukF-P83* are usually associated with clonal complexes CC97, CC479 and CC705 in cattle, with CC130, CC133 and CC522 in small ruminants and with CC49 in rodents, but can also occur in other, less known lineages (CC3572, CC3580, CC3591).

Recently, *lukM/lukF-P83* genes were identified by microarray in *S. aureus* from free-living red deer (*Cervus elaphus*) from the Stelvio National Park in the Central Italian Alps [29, 30]. A large increase in red deer density was observed over the past decades leading the Park to start a culling program in 2011, aiming on the reduction of the red deer density and mitigating its negative impact on agricultural activities, forest regeneration and biodiversity. Red deer were annually culled in winter, under the supervision of the Park Authority after authorization by the *Istituto Superiore per la Protezione e la Ricerca Ambientale* (ISPRA), the Italian Ministry of Environment (Prot. 48585/T-A25-Ispra), in the Lombardy sector of protected area.

Sampling for bacteriological investigations was carried out in two consecutive seasons (2016–2017 and 2017–2018) taking nasal and rectal swabs. *S. aureus* was frequently detected [29]. The isolates were obtained from deer that appeared clinically healthy and showed no gross lesions likely attributable to *S. aureus* infection. Prevalence rates of 90%, 26.2% and 10.7% of *S. aureus* were detected in nasal swabs, rectal swabs and faeces, respectively, from a total of 284 red deer. Seventy-nine representative isolates from thirty individual deer were genotyped by microarray. All bacteriological, prevalence, and genotyping data reported above have been previously described in detail [29]. In short, eight clonal complexes (CC), namely CC7, CC9, CC121, CC350, CC425, CC707, CC2328 and CC2671, were identified. CC425 was the most prevalent lineage (n = 48; 60.8%), followed by CC2671 (n = 13; 16.5%) and CC350 (n = 5; 6.3%). Thirty-three CC425 and three CC350 isolates were found to carry *lukM/lukF-P83* genes. This was an interesting finding, prompting the question whether i) these genes in red deer represent the same allele as the one known from other animals, and if ii) the *lukM/lukF-P83* prophage of red deer isolates was identical to the one in other animals. If that were the case, one might assume that a transmission not of the bacteria between wildlife and domestic animals occurred but of bacteriophages that infect different, host-specific strains of bacteria. To gather data to assess this issue, representative deer isolates of CC350 and CC425 were sequenced. In a One Health context, prophages are of particular interest because they can mediate the exchange of virulence

determinants across wildlife, livestock, and companion animals. Free-ranging red deer are relevant at the wildlife–livestock interface and may serve as sentinels or reservoirs, while zoo and game park populations offer additional opportunities for surveillance of carriage and disease.

Materials and Methods

Isolates

Isolates were obtained and characterised by microarray as part of earlier work [29, 30] which is summarised in the Introduction. They were characterised using a microarray-based approach, as previously described in detail [31–33]. From this strain collection, one *lukM/lukF-P83*-positive isolate assigned to CC350 as well as two *lukM/lukF-P83*-positive isolates belonging to CC425 were randomly selected for genome sequencing.

Whole-genome and phage DNA sequencing

Genomic DNA was sequenced using Oxford Nanopore Technologies (ONT, Oxford, UK) native barcoding chemistry that allows multiplex sequencing of up to 24 samples per run. Libraries were prepared using the native barcoding kit (SQK-NBD114.24) following the manufacturer's instructions with minor modifications.

Bacterial isolates stored in Microbank vials (Pro-Lab Diagnostics, Ontario, Canada) were streaked onto Columbia agar supplemented with 5% sheep blood (Becton Dickinson, Heidelberg, Germany) and incubated overnight at 37°C. One inoculation loop was suspended in 500 µL 1× phosphate-buffered saline (PBS, pH 7.4), centrifuged (5 min, 4,000×g), and resuspended in 100 µL buffer BE. DNA extraction was performed using the NucleoSpin DNA kit (Macherey-Nagel, Düren, Germany). Cell disruption was performed in a BeadBug homogenizer (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) for 12 min at maximum speed. Proteinase K was subsequently inactivated by incubation at 70°C for 5 min. After cooling, 4 µL RNase A (100 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) were added, followed by incubation for 5 min at 37°C. DNA was eluted in 50 µL nuclease-free water (Carl Roth, Karlsruhe, Germany).

DNA concentrations were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA clean-up and size selection to remove short fragments were performed using AMPure XP beads (Beckman Coulter) at a bead-to-sample ratio of 1:1. A total of 600 ng DNA per sample was used for library preparation. End-repair and dA-tailing were performed using the NEBNext Ultra II End Repair/dA-Tailing Module (NEB, Frankfurt a.M., Germany) (10 min at 20°C, followed by 10 min at 65°C). After bead clean-up, native barcodes were ligated using Blunt/TA ligase master mix (NEB) and incubated for 20 min at room temperature. Barcoded samples were purified, quantified, pooled equimolarly, and sequencing adapters were ligated using Quick T4 DNA ligase (NEB) and ONT adapter mix. The final library was purified according to ONT recommendations.

Libraries were loaded onto R10.4.1 flow cells and sequenced on a MinION device after standard flow cell priming. Runs were controlled using MinKNOW software (v25.02.16).

Reads were demultiplexed, adapter-trimmed, and quality-filtered prior to analysis.

Bioinformatics analysis

Raw POD5 files were base-called with Dorado (v1.0.2; ONT) using the super-accuracy model *dna_r10.4.1_e8.2_400bps_sup* with integrated barcode trimming. Quality-filtered reads were assembled using Flye (v2.9.1) to generate circular contigs. Assemblies were polished by four iterative rounds of Racon (v1.5.0; parameters: -c -m 8 -x -6 -g -8 -w 500), followed by a final polishing step with Medaka (v2.1.0) using the model *r1041_e82_400bps_sup_v5.2.0*.

Assembly quality, read coverage, and contig circularity were assessed for all genome and phage sequences prior to downstream analyses (Supplemental File 1a). Genome sequences of the study strains were submitted to GenBank and they are provided as Supplemental File 1b, 1c and 1d.

MLST and spa-typing

Sequences of the markers used for multi locus sequence typing and core genome multi locus sequence typing (MLST/cgMLST), as well as the *spa*-gene, were extracted from the whole-genome sequences and analysed publicly accessible software tools (https://pubmlst.org/bigsdb?db=pubmlst_saureus_seqdef&page=sequenceQuery for MLST/cgMLST [34] and https://spatyper.fortinbras.us/ for *spa*-typing)

Prophage induction and phage DNA isolation

Prophages were induced as previously described [11, 12]. For this purpose, bacterial cultures of Milano-03, -18 and – 38 were grown overnight in 2 × TY medium and subsequently cultivated at 37°C until they reached the mid-exponential growth phase (t = 3 h, OD = 0.42, 0.33 and 0.47 for Milano-03, -18 and – 38, respectively). For induction, mitomycin C (Roche, Basel, Switzerland) was added to a final concentration of 0.5 µg/mL, whereupon cultivation was continued at 30°C until a decrease in optical density (OD at 600 nm) was evident compared to the previous measurement (t = 6 h, ΔOD = 0.7 for Milano-03 and t = 5 h, ΔOD = 0.5 and 1.1 for Milano-18 and – 38, respectively).

The resulting lysate was centrifuged at 4°C and 3,000 × g for 12 min. The supernatant was neutralised with 0.1 N NaOH and filtered through a 0.20 µm cellulose acetate (CA) membrane filter (Sartorius, Göttingen, Germany). To isolate the phage DNA (p-DNA), the phage filtrate was centrifuged again at 4°C and 3,000 x g for 30 min. Subsequently, the supernatant was treated with 10 µg/mL DNase IA (NEB, Frankfurt/Main, Germany) and 10 µg/mL RNase (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for one hour at 37°C and 550 rpm. Then, 20 mM EDTA (Carl Roth GmbH + Co KG, Karlsruhe, Germany), 50

µg/mL Proteinase K (Roche, Basel, Switzerland) and 0.1% SDS (Carl Roth GmbH + Co KG, Karlsruhe, Germany) were added stepwise, and the mixture was incubated for another hour at 65°C and 550 rpm. Subsequently, a phenol-chloroform DNA extraction was performed according to a proven method [35]. The final DNA concentration was determined to 11.0 ng/µl, 3.5 ng/µl and 2.3 ng/µl for Milano-03, -18 and – 38, respectively, using a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Phage Detection by Transmission Electron Microscopy

For the negative staining procedure, 400-mesh copper grids filmed with formvar and coated with carbon were hydrophilised by glow discharge immediately prior to use. The phage preparations were vortexed and 30 µl-drops placed on dental wax plates. Copper grids were incubated on the drops for 30 min. After washing with distilled water, they were put on a drop of 1% uranyl acetate for 1 min for contrasting. Grids were examined in a transmission electron microscope (Tecnai 12, FEI Deutschland GmbH, Dreieich, Germany) and micrographs of at least twenty phage particles were taken with a digital camera (TEMCAM FX416, TVIPS, Gauting, Germany). Particle sizes were measured using the EM-Measure software (TVIPS).

Results

MLST, core genome MLST and spa-typing

Typing data and genotypic features of the study strains are summarised in Table 1 where they are also compared to reference sequences of their respective clonal complexes. In short, the findings for these isolates were fully consistent with those of previously described strains from the respective clonal complexes.

Table 1
Genotypic characteristics of the study strains and of reference genomes from their respective clonal complexes.

Isolate/ strain	GenBank	Host organism	Geo-graphic origin	CC	ST	cgMLST-type	*spa* type	*agr* group	Capsule type	GI-associated MSCRAMMs	Resistance genes	L g
Milano-18	*pending*	Red deer	Italy	CC350	ST350	cgST-28459 or cgST-39809, with 184 mismatches each	t3390 (99-20-69-31-70-13-13-17-16-16-16)	II	5	*cna*	-	*l* *F*
NCTC5663	LS483317	Horse	UK	CC350	ST350	cgST-28459 or cgST-39809	t10610 (99-20-69-31-70-13-17-16-16-16)	II	5	*cna, vwb3*	*cadA/D*	*l*
IMT37083	CP176566	Horse	Germany	CC350	ST1660	cgST-38513 with 3 mismatches	t3043 (04-20-69-31-70-13-17-16-16-16-16)	II	5	*cna, vwb3*	*blaZ, aacA-aphD, dfrA*	*l*
Milano-03	*pending*	Red deer	Italy	CC425	ST425	cgST-33203 or cgST-38835, with 217 mismatches each	t6832 (14-44-12-12-17)	II	5	*cna*	-	*l* *F*
Milano-38	*pending*	Red deer	Italy	CC425	ST425	cgST-33203 or cgST-38835, with 216 mismatches each	t6832 (14-44-12-12-17)	II	5	*cna*	-	*l* *F*
V40	CP097571	Badger	Germany	CC425	ST425 (1 mis-match)	cgST-28244 or cgST-39754	N/A (14-44-12-17-23-44-12-17-17-17-23-24)	II	5	*cna*	-	-
O55-B118	CP038268	Sheep	Brazil	CC425	ST425	cgST-27946 or cgST-39686	t3573 (14-44-12-12-23-18-17-17-17-23-24)	II	5	*cna, vwb3*	*blaZ*	-
LGA251	FR821779	Cow	UK	CC425	ST425	cgST-4414, cgST-19714, cgST-19750 or cgST-21473	t6300 (14-44-12-17-23-18-110-17-17-23-24)	II	5	*cna*	*mecC, blaZ-*SCC*mec*XI	-
NCTC13552	LR134084	Cow	UK	CC425	ST425	cgST-4414, cgST-19714, cgST-19750 or cgST-21473 with 140 mismatches	t6300 (14-44-12-17-23-18-110-17-17-23-24)	II	5	*cna*	*mecC, blaZ-*SCC*mec*XI	-

The lukF-P83 sequence

All *lukF-P83* sequences are highly conserved with study strains being no exceptions, as shown in Supplemental File 2. There were few single nucleotide polymorphisms (SNPs); among others an apparently CC479-specific SNP in position 221 as well as seven CC705-specific SNPs (positions 7, 51, 477, 548, 555, 560, 820), but there was no evidence for deer-specific SNPs or any other host-specific variation in that gene.

The lukM (lukS) sequence

The *lukM* sequences are also highly conserved, with CC705 being slightly different than the others (Supplemental File 3). Here, the three red deer isolates of both, CC350 and CC425, differ from the other sequences in two SNPs, with one of them (pos. 67 in the alignment) resulting in an amino acid change, from phenylalanine to leucine.

Analysis of prophage and of phage sequences

As part of a genomic analysis using Oxford Nanopore sequencing, the three *S. aureus* isolates from the cohort were examined for the presence of prophages. The sequence data showed the integration of two distinct prophage elements in the bacterial genome of all isolates (Milano-03, Milano-18 and Milano-38). Table 2 provides an overview of the isolates, prophages, and integration sites. Supplemental Files 4 and 5 a/b show the phage/prophage sequences and the individual genes detected within these sequences. Genes that occur only in *Peeveel-* or *Triavirus* phages (based on a GenBank search of all *S. aureus* phage sequences with a known taxonomic classification, January 2026) and that therefore have been used as markers for these genera are indicated in these Supplemental Files.

Table 2
Prophages in the study strains.

Isolate	Genus	Prophage integration site	Prophage length (first nt of integrase gene to first nt of chromosomal integr. site)	Notable genes	Inducible by mitomycin C treatment
CC350, Milano-18	*Peeveelvirus*	Within A5IU43 = *yfkAB*, cgMLST SAUR2054	43,449 nt	SAOV_1974-like site-specific integrase gene, *lukM/lukF-P83*	Yes (detected by TEM and by sequencing)
CC350, Milano-18	*Peeveel-/Triavirus*	Within *lip2 = geh*, cgMLST SAUR0317	43,073 nt	SACOL0318-like site-specific integrase gene, partial duplication, see text	No (no positive identification by TEM, non-circular fragment by sequencing)
CC425, Milano-03	*Peeveelvirus*	Within A5IU43 = *yfkAB*, cgMLST SAUR2054	43,687 nt	SAOV_1974-like site-specific integrase gene, *lukM/lukF-P83*	Yes (detected by TEM and by sequencing)
CC425, Milano-03	*Triavirus*	Within *lip2 = geh*, cgMLST SAUR0317	46,064 nt	SACOL0318-like site-specific integrase gene, *virE* (MW1408)	Yes (detected by TEM and by sequencing)
CC425, Milano-38	*Peeveelvirus*	Within A5IU43 = *yfkAB*, cgMLST SAUR2054	43,686 nt	SAOV_1974-like site-specific integrase gene, *lukM/lukF-P83*	Yes (detected by TEM and by sequencing)
CC425, Milano-38	*Triavirus*	Within *lip2 = geh*, cgMLST SAUR0317	46,064 nt	SACOL0318-like site-specific integrase gene, *virE* (MW1408)	Yes (detected by TEM and by sequencing)

All three strains carry a *Peeveelvirus* prophage integrated into the A5IU43 gene, which carried the leukocidin genes *lukM* and *lukF-P83* (see Supplemental File 4). The A5IU43-integrating prophage sequences were nearly identical, apart from a few apparently random SNPs. The prophage in the CC350 strain Milano-18 differed from those in the two CC425 strains by a replacement of a gene encoding a hypothetical protein (SACOL0353) with a putative variant/allele (SAOV_0303) and by the absence of a directly adjacent hypothetical protein gene (NC_024391:11961..12074). These phages were inducible from all three isolates by mitomycin C treatment. Sequencing of the resulting phage clearly revealed the presence of A5IU43-integrating *Peeveelviruses* carrying *lukM* and *lukF-P83* in phage preparations of all three strains whose sequences matched the corresponding prophages from the genome sequences, although some few sequencing artefacts, i.e., insertions of single nucleotides or short fragments into the phage sequences, were noted (Supplemental File 4).

In addition, all three strains also carried a prophage that integrated into the *lip2 = geh* gene (cgMLST: SAUR0317). Here, prophage sequences differed significantly.

The *lip2 = geh* integrating prophage from the CC350 isolate Milano-18 carried both, genes that were associated with *Triavirus* and genes associated with *Peeveelvirus* (see Supplemental File 5a). Upon closer inspection, a complex of 28 genes or roughly 22,000 bp was nearly identical to the corresponding region of the *lip2 = geh* integrating prophage of a CC350-ST1660 isolate from a horse that occupied the same position in the genome but that carried the horse-associated leukocidin *lukP/Q* (GenBank CP176566.1:[347458.. 369485]). A partial sequence obtained from the mitomycin C treatment resembled that prophage sequence, but it was not circular and it lacked nineteen genes (DUF1381 to MW1896) as well as a part of the twentieth one (encoding a tail tape measure protein, MW1895/SAS1878).

The sequences of the respective prophages as well as of the induced phages from mitomycin C from the two CC425 isolates were, based on sequences (see Supplemental File 5b) as well as on TEM (see below) assignable to the genus *Triavirus*. They carried the "*virE*" gene (GenBank AP009363.1 [15347..17794] or GenBank BA000033.2 [1558299..1560746], locus tag MW1408), a gene encoding a "virulence-associated protein E" or a DNA helicase associated with *Triaviridae*, which should not be confused with a pathogenicity island associated gene "*virE*", GenBank PQ594946 [36]. The former gene, MW1408, is one of the genetic markers used for the identification of *Triavirus*.

Phage Detection by Transmission Electron Microscopy (TEM)

In the mitomycin C-induced phage preparation of the CC350 isolate Milano-18 (Table 3, Fig. 1), many "*Siphovirus*"-like phage particles were present. Isometric particles with head diameters of around 60 nm were most frequent. They had icosahedral heads, thin, moderately long, straight or slightly curved tails and moderate-sized baseplates. Sometimes, the heads appeared to be slightly stunted. One small isometric particle comparable was also observed (which resembled the small-headed particles described for Milano-03, see below).

Table 3
Frequency and measurements of phage particles found in the Milano-18 preparation (AV, average value; STD, standard deviation).

Type	n	Head length in nm		Head diameter in nm		Tail diameter in nm		Tail length in nm		Baseplate in nm
		AV ± STD	Range	AV ± STD	Range	AV ± STD	Range	AV ± STD	Range	Range
Peeveelvirus, isometric, large	21	58 ± 3	53–63	59 ± 3	55–65	10 ± 1	9–12	236 ± 12	221–262	11–28 × 14–29
Peeveelvirus, isometric, small	1	53	---	57	---	9	---	233	---	13 × 14

In the preparation of the CC425 isolate Milano-03 (Table 4, Fig. 2), many phage particles with morphological characteristics of *Siphoviridae* were observed. Isometric particles comparable in morphology with the 60 nm particles in Milano-18 were most frequent. Some isometric particles had head diameters of around 54 nm. A tail was detected in one of these small-headed particles only. In addition, prolate particles were present. They had thin angular or thicker oval heads, thin, long, curved tails and moderate-sized baseplates. Based on the data from the literature (https://phagedive.dsmz.de/strain/473, accessed at 2026, July 29), on previous observations [37] and on the sequencing data from strains and phage preparations (see above), they were interpreted as *Triavirus*.

Table 4
Frequency and measurements of phage particles found in the Milano-03 preparation (AV, average value; STD, standard deviation).

Type	n	Head length in nm		Head diameter in nm		Tail diameter in nm		Tail length in nm		Baseplate in nm
		AV ± STD	Range	AV ± STD	Range	AV ± STD	Range	AV ± STD	Range	Range
Peeveelvirus, isometric, large	51	59 ± 3	52–66	58 ± 3	53–65	10 ± 1	9–14	230 ± 20	185–265	12–25 × 16–22
Peeveelvirus, isometric, small	10	53 ± 1	51–54	54 ± 2	50–57	11 ± 1	9–13	225 ± 57	132–276	17 × 23
Triavirus, prolate	15	97 ± 7	80–110	46 ± 7	38–58	10 ± 1	8–10	298 ± 19	276–320	29 × 29

In the phage preparation of the CC425 isolate Milano-38 (Table 5, Fig. 3), large isometric particles comparable in morphology with the 60 nm particles in Milano-18 and – 03 were frequently observed. Isometric particles with head diameters of around 53 nm and no tails were detected less frequently. In addition, prolate particles as described for Milano-03 and interpreted as *Triavirus* were observed. They were more numerous than the isometric particles.

Table 5
Frequency and measurements of phage particles found in the Milano-38 preparation (AV, average value; STD, standard deviation).

Type	n	Head length in nm		Head diameter in nm		Tail diameter in nm		Tail length in nm		Baseplate in nm
		AV ± STD	Range	AV ± STD	Range	AV ± STD	Range	AV ± STD	Range	Range
Peeveelvirus, isometric, large	36	59 ± 2	56–64	59 ± 2	56–64	10	---	148	---	19 × 26
Peeveelvirus, isometric, small	12	53 ± 2	50–55	53 ± 2	50–55	---	---	---	---	---
Triavirus, prolate	52	91 ± 8	72–115	48 ± 6	39–64	10 ± 1	9–11	294 ± 14	277–311	17–22 × 17–22

In conclusion, TEM imaging was largely consistent with sequencing of phage preparations. In all three cases, large-headed *Peeveelvirus*-like particles were observed that might correspond to the *lukM/lukF-P83* carrying prophages in the sequences of the three isolates. The less-frequent small-headed *Peeveelvirus*-like particles in all three preparations did not correspond to a separate prophage. Since method-related TEM artifacts (such as dehydration errors or irregular negative staining) can be ruled out due to the uniform sample preparation and the consistency of the background structures, and since a presence of Staphylococcal Pathogenicity Islands (SaPIs) can be excluded based on genome sequencing, the different phenotypes are most likely attributable to processes of viral morphogenesis, *i.e*, to a presence of particles at different stages of the phage head maturation and/or to defects in their assembly, see below.

There was no visual finding that might fit to the putative hybrid prophage from the CC350 isolate; and the presence of non-circular fragments of it in the phage preparation might be explained by fragments of prophage DNA from bacterial detritus rather than of complete phage particles.

The prolate *Triavirus* particles observed confirm the identification of the *lip2 = geh* integrating prophages in the CC425 genomes as *Triavirus.* Here again, morphological differences affecting head shapes were attributed to different stages of head maturation and/or to defects in assembly.

Discussion

The issue of phenotypic variability in prophages

Large- and small-headed *Peeveelvirus*-like particles were observed in phage preparations of all three isolates, and *Triavirus*-like particles from the CC425 isolates had thin angular or thicker oval heads. Much of the observed heterogeneity can be explained by the dynamics of phage maturation. The morphogenesis of double-stranded DNA phages is not synchronized, which is why different developmental stages typically coexist in a lysed sample. The smaller particles (as seen in Fig. 2B) presumably represent the so-called procapsid stage. In this stage, the capsid is still empty and associated with internal scaffolding protein. The transition to the mature phage head is a highly complex mechanical process: As the viral genome is pumped into the head by the ATP-dependent terminase complex, a conformational rearrangement of the major capsid proteins occurs. This leads to an expansion of the head and the capsid assumes its final, icosahedral, and mechanically stable geometry. Such a mechanism of capsid expansion and the associated structural maturation have been elucidated at the atomic level for the bacteriophage P22 using high-resolution cryo-electron microscopy [38].

In addition to normal maturation stages, the observed abnormal morphologies, such as incomplete capsid walls or stunted head structures, are consistent with errors during viral *de novo* synthesis. The assembly of a phage head requires a precise stoichiometric interaction between scaffold, coat, and portal proteins. Spontaneous mutations, limitations in the cellular translation rate, or cellular stress in the host cell can disrupt this balance.

In particular, the formation of elongated, tubular capsid structures is a well-documented phenomenon in cases of assembly defects. Classic studies on bacteriophage T4 have shown that the absence or malfunction of the scaffold protein or the portal protein complex causes the major capsid proteins to polymerize unhindered into long cylinders instead of adopting the energetically more favourable icosahedral shape [39]. Variations in the degree of genomic packaging (over- or under-packing) also lead to changes in the physical pressures within the capsid, which may explain the morphological deformations observed in TEM.

Review of lukM/lukF-P83 prophages

Observations from this study are consistent with earlier work showing that *lukM/lukF-P83* is carried on temperate prophages and that, as observed for bovine isolates, mitomycin C can induce lysis of, and phage release from, most *lukM/lukF-P83*-positive strains, supporting horizontal transmission of the locus via phages [14]. The *lukM/lukF-P83* prophages from the three study isolates were compared to 25 *lukM/lukF*-P83 prophage sequences from previously published high-quality and long-read *S. aureus* genomes of various clonal complexes. The original phiPV83 virus of which there are two GenBank entries (AB044554, NC_002486) was also included (Supplemental File 6a**)**.

As already mentioned (see above and Supplemental Files 4 and 5), the sequences of the leukocidin genes *lukM* and *lukF-P83* were highly conserved with no major differences beyond a few, apparently random, SNPs. There was no indication for CC- or host-specific variation in these genes apart from some SNPs unique to CC705 (see above).

However, when analysing the entire thirty prophage/phage sequences, it became clear that there is a high grade of diversity, and that very different types of prophages/phages might carry these leukocidin genes. There is a group of truncated phage remnants and two sequences could be assigned to two different *Biseptimaviruses* that differ in gene content and integration site. The other sequences are *Peeveelviruses*, which, however, are also not homogenous as discussed below.

First, the group of truncated phage remnants includes CC705 sequences, among them the arguably longest known genome sequence of a *lukM/lukF-P83*-positive strain, GenBank AJ938182, strain RF122. Other examples are CP134620, strain Sa90; CP155057, strain Jena-124984; CP186897, strain A-SAU74; CP186899, strain A-SAU80; CP186904, strain A-SAU90; LS48330 and strain NCTC7485. In these sequences, the prophage is mutilated, consisting of a truncated integrase gene (SAB0779c) and another mere eight genes out of which two are the leukocidin genes *lukM* (SAB0782) and *lukF-P83* (SAB0783). This phage remnant integrates between *sufB* (SAB0778, cgMLST: SAUR0895) and a nameless gene corresponding cgMLST: SAUR0896.

Second, a *Biseptimavirus* occupies the same position in a CC3580/ST7619 sequence, GenBank CP095119, strain IVB6154 from a camel in Marsabit, Kenya. This prophage consists of 75 genes including *lukM/lukF-P83* and the enterotoxin A gene, *sea.*

Third, another unique prophage is present in the ST3572 sequence of IVB6171, GenBank CP095109 which originates from a Somali camel. This phage integrates between *rpmF* (locus tag=MUA24_07705, cgMLST: SAUR1124) and *isdB* (locus tag=MUA24_07370, cgMLST: SAUR1125) and could also be assigned to *Biseptimavirus.*

Fourth, the CC49-ST1957 strain 3036-IOW744N4, GenBank CP069351 has its *lukM/lukF-P83*-prophages integrated between the downstream end of the genomic island harbouring leukocidin genes *lukD/E* and a cluster of tRNA genes. Here again, markers of *Peeveelvirus* are present.

Fifth, a majority *of lukM/lukF-P83*-prophages integrates into the A5IU43 = *yfkAB* gene, cgMLST: SAUR2054. These twenty prophages all have *Peeveelvirus*-like sequences and they have identical site-specific integrase genes corresponding to GenBank CP001996 (2017417..2018463), locus tag SAOV_1974. Based on gene content, these prophage sequences can be grouped into eleven clades (Supplemental File 6b**)**. One comprises two CC49 prophage sequences (from CP065355 and CP136585). A second one consists of two CC133 sequences (from CP001996 and CP034102) and a third one of two other CC133 prophage sequences (LR134271, LR134305). A fourth clade includes CC479 prophages (from CP155058 and CP155061). Two CC30-ST433 (CP019117, CP028190)

prophage sequences and the original phiPV83 virus (AB044554/NC_002486) form a fifth clade. Another one comprises the three *lukM/lukF-P83* prophages of the CC350 and CC425 strains studied herein (with the CC350 sequence differing only slightly from the CC425 sequence as described above). In addition, there are five "singletons" including one distinct prophage sequence each (CC1, CP160024; CC97, AP018376; CC130, CP024649 and CP025395; CC3591, CP063802).

Finally, there is the curious case of the CC133 strain Jena-124622, GenBank CP155059, in which *lukM* and *lukF-P83* are associated with a haemolysin-beta-integrating prophage. However, the observation that there were two holin (locus tags ABC967_09480 and _09615) and two major tail protein genes (locus tags ABC967_09535 and _09650), up- and downstream of *lukM* and *lukF-P83* (ABC967_09580 and _585) suggests that here a fragment of a *lukM/lukF-P83* prophage was integrated into the haemolysin-beta-integrating prophage. The gene normally serving as integration site, A5IU43 = *yfkAB*, see above, was complete and of normal length (1152 bp) which rather ruled out an assembly/sequencing artifact.

The mosaic structure and diversity of these prophages are in line with previous reports on *S. aureus* prophages, which have been shown to undergo frequent genetic exchange and rearrangement, resulting in highly variable phage backbones carrying accessory virulence genes [40]. In conclusion, available data indicate that the *lukM/lukF-P83* locus is conserved at a level of gene sequences while it is highly promiscuous with respect to the prophages/phages it resides in. The dissemination of this leukocidin is obviously neither tied to a single successful bacterial clone nor to one pandemic phage. It may occur through a transmission of the bacteria themselves, through an exchange of prophages/phages among different *S. aureus* lineages across different host species and through recombination, i.e., by shuffling genes across different genera of prophages/phages. In the case discussed herein, CC350 and CC425, which are typical "wildlife and ungulate" associated lineages (see below) acquired *lukM/lukF-P83* leukocidin genes with *A5IU43 = yfkAB*-integrating *Peeveelviruses*. This appears to be the largest group of *A5IU43 = yfkAB*-prophages/phages. Although no identical prophage/phage sequence was identified among published sequences, CC350 and CC425 prophage sequences are typical representatives of this group. All their genes or gene clusters can also be detected, in varying combinations, in other published sequences of *A5IU43 = yfkAB*-integrating *Peeveelviruses* (Supplemental File 6b).

CC350, CC425 and S. aureus genotypes in cervids

Regarding the CCs involved, CC350 has been identified in horses and humans [11]. Horse-associated strains can carry the "horse leukocidin" *lukP/Q*, but neither PVL nor *lukM/lukF-P83* have been observed in this lineage before. CC425 was previously found in domestic rabbits [41] as well as in wild lagomorphs from Spain [42]. It was identified in genets from Spain [43] and in foxes and badgers from Germany [44] with the latter carrying the rare Enterotoxin E gene [45] as did isolates from boars [46]. Ibexes from Spain have been shown to carry *S. aureus* CC425, among other clonal complexes [43, 47]. European cervids including roe deer, red deer, fallow deer, feral sika deer, moose and reindeer have been examined for *S. aureus* carriage [29, 30, 44, 47–51] yielding a number of CCs including ones common among humans and animals (CC1, CC7, CC9, CC15, CC30, CC45), CCs known to be associated with animals such as CC425 (but also others: CC97, CC130, CC133, CC398, CC522) as well as poorly known lineages (CC2328, ST2671/ST3237, ST2691). CC425 also evolved *mecC*/SCC*mec* XI MRSA that have been found in livestock [52] and in hedgehogs [53, 54]. However, the studies on Italian deer, from which the isolates sequenced herein were derived [29, 30] were the only ones that reported *lukM/lukF-P83* positive CC425 isolates; in all other studies, these leukocidin genes were either absent or isolates were not tested for them. Taken together with the frequent detection of *lukM/lukF-P83* in cattle and small ruminants, the occurrence of this locus in red deer supports its relevance at the wildlife–livestock interface and in a One Health context.

While *lukM/lukF-P83* is known to be associated with mastitis in domestic ruminants and dermatitis in rodents, there is no information a possible role in pathogenesis in red deer or other cervids. The animals examined in this study were culled for management purposes rather than because of suspected disease, and no gross lesions likely attributable to *S. aureus* infection were observed. Our observation should prompt further investigations on cervids, wild or captive. The widespread increase and expansion of red deer populations across much of Europe [55] will likely provide increasing opportunities to investigate the pathogenic role and molecular epidemiology of *S. aureus* in this host. Zoo or game park populations, together with *post-mortem* examinations of free-ranging animals found dead, culled for wildlife management or harvested by hunters, should provide ample opportunities to observe animals for skin and soft tissue infections including mastitis and to culture their pathogens for subsequent molecular study.

Declarations

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Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Authors contributions

RP, SL, MN and CL provided isolates and background data used for the present study; SBV, SDB, CD, EM, MR and ELT performed experiments and initial analyses; SM analysed the sequences, SBV and SM wrote the draft manuscript whereas CD and ELT wrote individual chapters of the text; RE reviewed the draft; RP, CL and RE acquired funding. All authors read and approved the final version of the manuscript.

Data Availability

All relevant information, including genome sequences, is present in the manuscript and in the Supplemental files. The complete sequences of the isolates have been submitted to GenBank: Bioproject PRJNA1518041; BioSamples SAMN62677023, SAMN62677024, SAMN62677025; accession numbers pending.

Ethics

Not applicable. Animal isolates were collected during previous studies where the issues of hunting permits and ethics clearances were already described in detail [29, 30].

Consent to participate and consent for publication

Not applicable.

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Figures

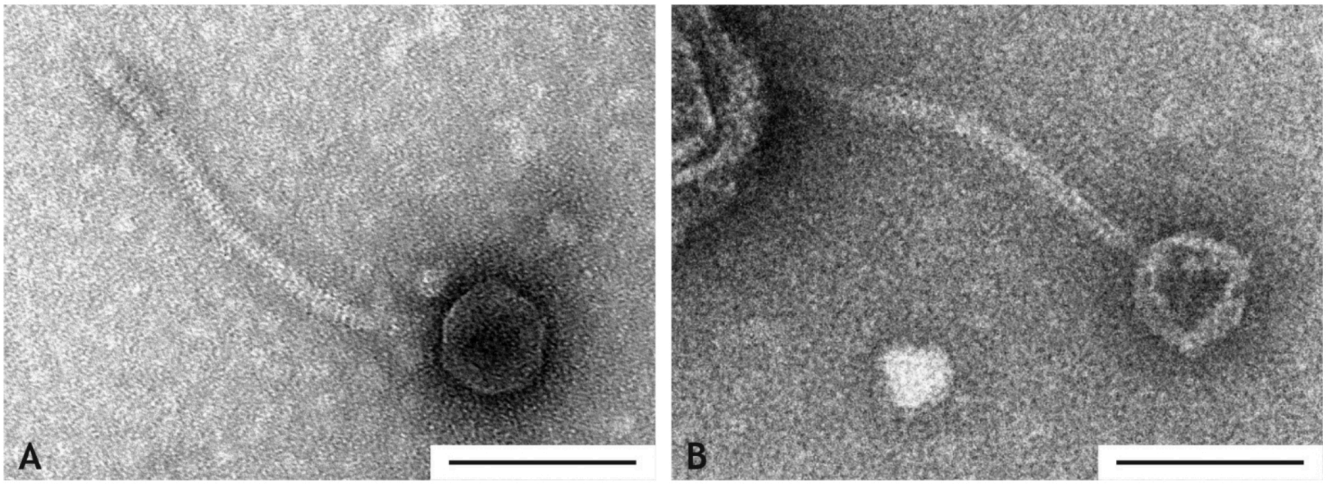


Figure 1

Phages from the preparation of the CC350 isolate Milano-18 including one large-headed isometric *Peeveelvirus* particle (A) and one stunted *Peeveelvirus* particle (B). Scale bar = 100 nm.

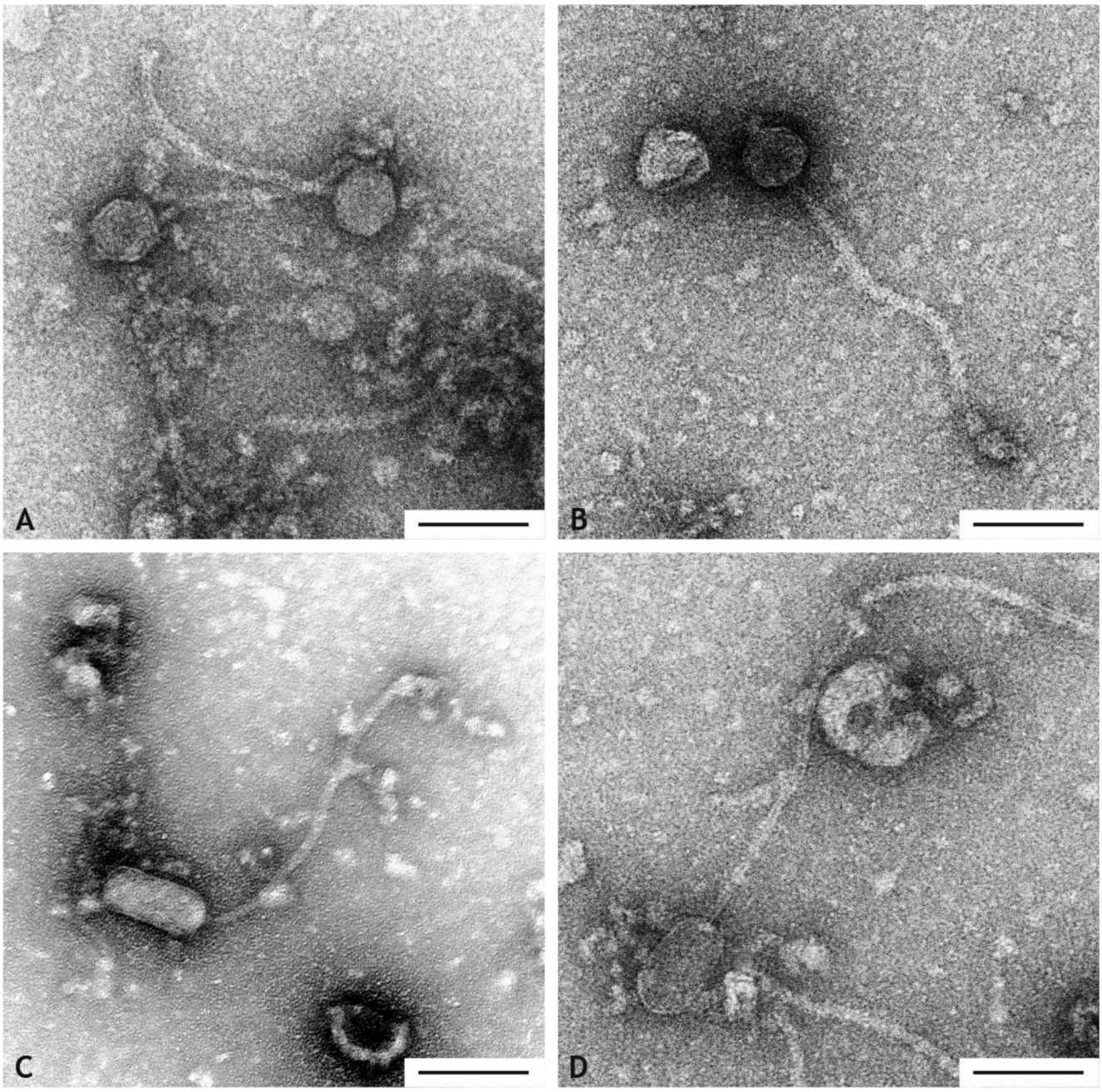


Figure 2

Phages from the preparation of the CC425 isolate Milano-03, including large-headed *Peeveelvirus*-like phages (A), the tailed small-headed *Peeveelvirus*-like phage particle (B) and *Triavirus*-like particles with slightly different head morphologies (C, D). Scale bar = 100 nm.

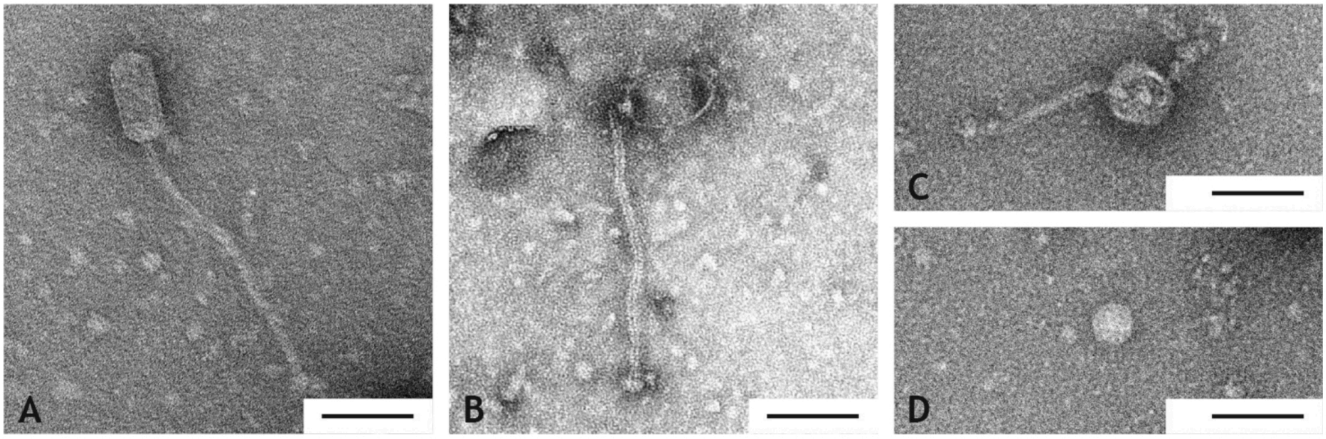


Figure 3

Phages from the preparation of the CC425 isolate Milano-38 including *Triavirus*-like particles with slightly different head morphologies (A, B), a large-headed *Peeveelvirus*-like phage (C) and a tail-less, small-headed *Peeveelvirus*-like phage particle (D). Scale bar = 100 nm.

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