

## Host- and Tissue-Specific Pathogenic Traits of *Staphylococcus aureus*

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**Comparative genomics were used to assess genetic differences between *Staphylococcus aureus* strains derived from infected animals versus colonized or infected humans. A total of 77 veterinary isolates were genetically characterized by high-throughput amplified fragment length polymorphism (AFLP). Bacterial genotypes were introduced in a large AFLP database containing similar information for 1,056 human *S. aureus* strains. All *S. aureus* strains isolated from animals in close contact with humans (e.g., pet animals) were predominantly classified in one of the five main clusters of the AFLP database (cluster I). In essence, mastitis-associated strains from animals were categorized separately (cluster IVa) and cosegregated with bacteremia-associated strains from humans. Distribution of only 2 out of 10 different virulence genes differed across the clusters. The gene encoding the toxic shock syndrome protein (*tst*) was more often encountered among veterinary strains ( $P < 0.0001$ ) and even more in the mastitis-related strains ( $P < 0.0001$ ) compared to human isolate results. The gene encoding the collagen binding protein (*cna*) was rarely detected among invasive human strains. The virulence potential, as indicated by the number of virulence genes per strain, did not differ significantly between the human- and animal-related strains. Our data show that invasive infections in pets and humans are usually due to *S. aureus* strains with the same genetic background. Mastitis-associated *S. aureus* isolated in diverse farm animal species form a distinct genetic cluster, characterized by an overrepresentation of the toxic shock syndrome toxin superantigen-encoding gene.**

*Staphylococcus aureus* can colonize and infect a variety of members of the animal kingdom, including mammals, reptiles, and birds. Infection models for this clinically highly relevant bacterial species in lower organisms, such as insects (*Drosophila melanogaster*) and worms (*Caenorhabditis elegans*), have been described as well (28, 40). Various studies address the molecular basis of the apparent host specificity or diversity of *S. aureus* strains. This is usually approached by determining genome polymorphism with multilocus enzyme electrophoresis (8, 17), pulsed-field gel electrophoresis (PFGE) (46), binary typing (45), or shotgun sequencing (12). In addition, animal studies often involve *S. aureus* strains with specific mutations in defined virulence factors (6). These studies have demonstrated that strains from humans can cross-infect domestic animals (42) and poultry (35) and vice versa (38). It has been determined that separate host-specific lineages of *S. aureus* do exist, but the question with regard to cross-species pathogenicity of these lineages still remains unanswered (17).

Genomic studies as mentioned above have revealed exten-

sive genetic variation in natural populations of *S. aureus*. Pathogenic strains may harbor complete pathogenicity islands (20) or specific “accessory” genes, such as the one encoding the Pantan-Valentine leucocidin (22). The evolutionary processes in *S. aureus* restricting or expanding invasiveness in different hosts are ill defined (9), although, for instance, numbers and combinations of certain virulence genes may be important contributors to pathogenic potential (31). We studied *S. aureus* host specificity and virulence by comparative genomics (high-throughput amplified fragment length polymorphism [ht-AFLP]). In addition, the distribution and number of virulence genes were compared between clinical and nonclinical strains isolated from different host species.

### MATERIALS AND METHODS

**Bacterial strains.** Two strain collections were used; the first strain collection comprised 77 *S. aureus* strains isolated from different infection sites in a variety of animal species, including dogs ( $n = 8$ ), monkeys and apes ( $n = 4$ ; different species), pigs ( $n = 6$ ), birds ( $n = 5$ ; different species), cats ( $n = 12$ ), sheep ( $n = 6$ ), seals ( $n = 4$ ), goats ( $n = 8$ ), rabbits ( $n = 3$ ), cows ( $n = 7$ ), and horses ( $n = 10$ ), a rat, a chinchilla, a guinea pig, and an iguana (one isolate each). The second strain collection consisted of 168 human *S. aureus* strains, which were classified as invasive ( $n = 56$ ) or colonizing ( $n = 112$ ). Strains were obtained and selected from a previous study (44). An invasive strain was derived from a patient with a positive nasal culture upon admission to the hospital while suffering from a

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manifest *S. aureus* infection by the same strain at a normally sterile site more than 2 days after admission. A colonizing strain was derived from a positive nasal culture from a matched control patient for whom no *S. aureus* infection was documented during hospitalization. For each invasive isolate, two matched controls were selected. The risk factors for both patient groups were matched as well (same hospital, ward, age, gender, and period of hospitalization) (44). Prior to genomic analysis, bacteria from glycerol stocks, stored at  $-80^{\circ}\text{C}$ , were inoculated on Columbia III agar supplemented with 5% sheep blood (Becton Dickinson, Etten-Leur, The Netherlands) and incubated at  $37^{\circ}\text{C}$  for 24 h. All strains were identified as *S. aureus* by accepted microbiological methods (2).

**DNA extraction.** Chromosomal DNA was extracted from bacterial cells with a MagnaPure LC DNA system (DNA isolation kit III; Roche, Almere, The Netherlands) according to the manufacturer's instructions, with the modification that 50  $\mu\text{g}/\text{ml}$  lysostaphine (Sigma, Zwijndrecht, The Netherlands) was added during the cell lysis step. The DNA concentration was measured by UV spectroscopy, samples were diluted with distilled water to a final concentration of 10  $\text{ng}/\mu\text{l}$ , and DNA was stored at  $-20^{\circ}\text{C}$  until use.

**ht-AFLP.** The genomes of the animal-associated *S. aureus* strains ( $n = 77$ ) were compared by ht-AFLP. Individual AFLP-PCRs were performed essentially as described before in the presence of radioactive nucleotides for the visualization of the fingerprints (25). Ht-AFLP was performed using the restriction enzyme combination MboI and Csp6I (New England Biolabs, Westburg, Leusden, The Netherlands). Each restriction site was filled in by ligation with specific linker oligonucleotide pairs (for MboI, 5'-CTCGTAGACTGCGTACC-3' and 5'-ATCGGTACGCAGTCTAC-3'; for Csp6I, 5'-ACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3'). Subsequently, a nonselective preamplification was performed using the MboI primer (5'-GTAGACTGCGTACCGATC-3') and Csp6I primer (5'-ACGATGAGTCCTGACTAC-3'). In the final amplification, a  $^{32}\text{P}$ -labeled MboI primer containing one selective nucleotide (either +C or +G) and a Csp6I primer containing two selective nucleotides (+TA) were used.

**AFLP data analysis.** Agglomerative hierarchical cluster analysis was used for two-dimensional data clustering of the AFLP patterns. The unweighted pair group arithmetic mean method was performed for cluster analysis. After inclusion of the AFLP patterns in the AFLP database, the Tanimoto method was used to calculate the similarity matrix. The resulting dendrogram was ordered by average value. Spotfire DecisionSite 7.2 software has been applied to perform statistical analyses.

**AFLP database.** This database consists of ht-AFLP fingerprints obtained for 1,056 *S. aureus* strains from human origin (25) and comprises 829 nonclinical carriage strains from healthy individuals, 74 strains from children with invasive *S. aureus* disease (bacteremia, arthritis, and abscess), 90 isolates from elderly ( $>55$  years) individuals with *S. aureus* bacteremia, 40 isolates obtained from lesions of children suffering from impetigo, 21 international epidemic methicillin-resistant *S. aureus* strains (26), and 2 reference strains (20).

**MLST.** A selection of 34 of the animal-associated strains were analyzed by the microarray-mediated multilocus sequence typing (MLST) protocol as described previously (43). Briefly, allele types of seven *S. aureus* housekeeping genes were defined by specific hybridization of amplified gene fragments to short oligonucleotide probes, synthesized on a Gene Chip array (Affymetrix, Santa Clara, Calif.; bioMérieux, Marcy l'Etoile, France). The resulting allelic sequences were matched with those from the MLST database ([www.MLST.net](http://www.MLST.net)), and an allelic profile with the corresponding sequence type (ST) and clonal complex (CC) could be determined.

**PCR of putative virulence factors.** For both strain collections, the genes coding for adhesins *fbaA*, *clfA*, *clfB*, *cna*, *sdrE*, and *ebpS* and for the exoprotein toxins *ist*, *eta*, *etb*, and *pvl* were amplified using the PCR primers and amplification conditions outlined in Table 1. For the confirmation of the PCR results, a second PCR, targeting a different domain of the same genes, was done. PCR amplifications were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). A positive control (Table 1) and a negative control (PCR mixture excluding DNA) were included in each PCR run.

**Statistical analysis.** The distribution of putative virulence factors across *S. aureus* strains originating from different hosts were compared using Fisher's exact test. The odds ratio was defined as the cross-product ratio of the numbers shown in a two-by-two contingency table. Confidence intervals of 95% were used throughout. *P* values less than 0.05 were considered significant.

## RESULTS

**Genotyping of *S. aureus* strains.** Ht-AFLP was used to determine the genomic variability within the veterinary *S. aureus*

strain collection. AFLP results are summarized in Fig. 1. Four clusters, I, IVa, II, and III (corresponding to the previously defined AFLP clusters for human strains included in the database (25), representing 54.5% ( $n = 42$ ), 28.6% ( $n = 22$ ), 9.1% ( $n = 7$ ), and 3.9% ( $n = 3$ ) of the veterinary collection, respectively, could be identified. Strains causing infections in pet animals (21 out of 24; cat, dog, guinea pig, and rabbit) were overrepresented in AFLP cluster I (Fisher's exact test;  $P < 0.0001$ ). AFLP cluster IVa mainly comprised geographically unrelated isolates obtained from farm animals (goats, sheep, and cows) and caused mastitis (14/22 versus 3/55, Fisher's exact test;  $P < 0.0001$ ), which suggested tissue specificity.

**Integration of the veterinary strains in the AFLP database.** The resulting AFLP patterns obtained for the animal-associated strains were introduced in the AFLP database (25), and the analysis is summarized in Fig. 2. The different AFLP clusters of the animal-related strains matched with the different AFLP clusters of the database. In essence, the strains causing mastitis in the farm animals formed a homogenous subcluster in IVa (see red bars in Fig. 2). In comparison to the human strains (63/1,056), the animal strains (22/77) were significantly overrepresented in AFLP cluster IVa (Fisher's exact test;  $P < 0.0001$ ) (Table 2). The human isolates in this cluster were also associated with bacteremia (Fisher's exact test;  $P = 0.0095$ ) (25). None of the animal-related strains were classified in AFLP cluster IVb. *S. aureus* strains (21 out of 24) isolated from pet animals were predominantly found in major AFLP cluster I (87.5%) but were scattered over its different subclusters (Fig. 2). Strains belonging to AFLP clusters II and III were significantly underrepresented among the animal strains (7/77 and 3/77, respectively) compared to the human strains (263/1,056 and 210/1,056;  $P = 0.0012$  and  $P = 0.0001$ , respectively) (Table 2).

**AFLP versus MLST.** MLST analysis was performed for a selection of the animal strains, representing each subcluster in Fig. 1. The animal-related strains classified in AFLP cluster I revealed five different sequence types (ST1, ST7, ST8, ST9, and ST15). In contrast, AFLP clusters II, III, and IVa were more homogenous, showing limited marker variability within each cluster (Fig. 2), and only harbored a single clonal complex (CC30, CC45, and CC22, respectively). Notably, the human-associated strains in cluster IVa are more genetically heterogeneous (25). MLST results agreed with the AFLP classification and were concordant with existing MLST sequence types within each AFLP cluster in the database as determined before (25).

**Comparison of the virulence gene distribution between human and veterinary *S. aureus* isolates.** The presence or absence of 10 genes associated with pathogenicity were measured for the animal ( $n = 77$ ) and human ( $n = 168$ ) strain collections. Results obtained with both PCR strategies were fully concordant. Virulence gene incidence among the animal-related strains was compared to those of the invasive ( $n = 56$ ) and colonizing ( $n = 112$ ) strains. Significant differences in distribution of virulence genes between the groups are outlined in Fig. 3. No significant variation in virulence gene distribution within the different subgroups of the animal strains (mastitis associated, pet animal, and rest group) was measured (data not shown). Most of the genes coding for adhesion proteins were evenly distributed among the human and veterinary strains.

TABLE 1. PCR primers and conditions for identification of potential virulence genes

PCR product	Oligonucleotide sequences (5'–3')	GenBank accession no.	Position in gene	Positive control	PCR conditions	Reference
<i>fmbA</i>	Fw, CAC AAC CAG CAA ATA TAG Rv, CTG TGT GGT AAT CAA TGT C	AJ629121	424–441 1785–1667	8325-4	1 min at 94°C, 1 min at 50°C, 2 min at 72°C	31
	Fw, GGT AAT CAT TCA TTC GAG Rv, TGG CAC ACT GTC GAA GTC		2355–2372 2561–2544	8325-4		41
<i>clfA</i>	Fw, GTA GGT ACG TTA ATC GGT T Rv, CTC ATC AGG TTG TTC AGG	Z18852	368–386 1951–1934	Newman	1 min at 94°C, 1 min at 45°C, 2 min at 72°C	31
	Fw, GAT TAA GCT TTA CGT TCA AC Rv, GAT TGG TAC CAT TTT TAG GTG		1805–1824 2948–2928	Newman		23
<i>clfB</i>	Fw, TGC AAG ATC AAA CTG TTC CT Rv, TCG GTC TGT AAA TAA AGG TA	AJ224764	425–444 1020–1001	Newman	1 min at 94°C, 1 min at 45°C, 2 min at 72°C	31
	Fw, AGG ACA ATC GAA CGA TAC AAC G Rv, ACT ACG TAC AGC TCT CGT TCT AAC ACT		162–183 618–592	Newman		32
<i>cna</i>	Fw, AGT GGT TAC TAA TAC TG Rv, CAG GAT AGA TTG GTT TA	M81736	1719–1735 3457–3441	Phillips	1 min at 94°C, 1 min at 55°C, 2 min at 72°C	31
	Fw, ATG GTA CCA AGA AGA TAC G Rv, TCT TGA TAC CAA GCT TGT G		688–705 1052–1034	Phillips		30
<i>sdrE</i>	Fw, CAG TAA ATG TGT CAA AAG A Rv, TTG ACT ACC AGC TAT ATC	AJ005647	650–668 1416–1399	Isolate 476	1 min at 94°C, 1 min at 45°C, 1 min at 72°C	31
	Fw, CTG AAA ACA CTA GTA CAG AAA ATG CA Rv, GGT ACT GTT AAA CCT GAA GAA AAG		158–182 1795–1818	Isolate 476		16
<i>ebpS</i>	Fw, CAA TCG ATA GAC ACA AAT TC Rv, CAG TTA CAT CAT GGT TA	U48826	40–59 565–546	Isolate 252	1 min at 94°C, 1 min at 50°C, 1 min at 72°C	31
	Fw, CGT CAA TCG ATA GAC ACA AAT Rv, CTG TAC CAG CAC CAA TT		37–57 638–621	Isolate 252		29
<i>tst</i>	Fw, AAG CCC TTT GTT GCT TGC G Rv, ATC GAA CTT TGG CCC ATA CTT T	AY074881	36–54 480–459	Mu 50	1 min at 94°C, 1 min at 55°C, 2 min at 72°C	3
	Fw, ACC CCT GTT CCC TTA TCA TC Rv, TTT TCA GTA TTT GTA ACG CC		88–107 394–375	Mu 50		24
<i>eta</i>	Fw, GCA GGT GTT GAT TTA GCA TT Rv, AGA TGT CCC TAT TTT TGC TG	M17357	719–738 811–792	Isolate D72	2 min at 94°C, 2 min at 57°C, 2 min at 72°C	24
	Fw, ACT GTA GGA GCT AGT GCA TTT GT Rv, TGG ATA CTT TTG TCT ATC TTT TTC ATC AAC		308–330 496–467	Isolate D72		15
<i>etb</i>	Fw, ACA AGC AAA AGA ATA CAG CG Rv, GTT TTT GGC TGC TTC TCT TG	M17348	509–528 734–719	Isolate I 128	2 min at 94°C, 2 min at 57°C, 2 min at 72°C	24
	Fw, CAG ATA AAG AGC TTT ATA CAC ACA TTA C Rv, AGT GAA CTT ATC TTT CTA TTG AAA AAC ACT C		574–661 1183–1153	Isolate I 128		15
<i>pvl (lukF)</i>	Fw, ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A Rv, GCA TCA AST GTA TTG GAT AGC AAA AGC	AB186917	579–609 2072–2046	Isolate D48	1 min at 94°C, 1 min at 55°C, 2 min at 72°C	33
<i>pvl (lukS)</i>	Fw, GCA AGG TTT TAT CAA TTC AAA GAC TAC TT Rv, GGG TCA TTT GTT TTG AGA CCA ATA T		234–262 344–320	Isolate D48		27

The gene encoding collagen binding protein, *cna*, was under-represented in the human invasive *S. aureus* strains (6/56) compared to the human colonizers (29/112, Fisher's exact test;  $P = 0.026$ ) and the animal strains (23/77, Fisher's exact test;  $P = 0.01$ ). This gene was not equally distributed among the different genetic lineages of *S. aureus*, since all animal strains from AFLP cluster II harbored the *cna* gene ( $P = 0.0001$ ; data not shown). The exotoxin gene, *tst*, was found significantly more often in the animal-associated strains (21/77 versus 11/168 in the human strains [ $P < 0.0001$ ] or versus 3/56 in the human-invasive strains [ $P = 0.0011$ ]). Strains causing mastitis in animals contribute strongly to *tst* overrepresentation. The *tst* gene was present in 13 out of the 17 mastitis-associated strains, which significantly differs from results observed among strains isolated from pets (1/24,  $P < 0.0001$ ) and among the rest group animals (7/39,  $P = 0.0001$ ) (data not shown). The bicomponent leucocidin gene, *pvl*, was only detected in two human-related strains and not at all in the animal isolates. The association between the number of virulence genes and the proportion of animal and human *S. aureus* strains is stated in Table 3. No difference in the number of virulence genes of the strains causing a variety of infections in the animal host ( $n = 77$ ) and in the human host ( $n = 168$ ) was found.

## DISCUSSION

**Host specificity of *S. aureus*.** Previous studies have compared host specificity of *S. aureus* strains isolated from humans and animals. Determinants such as antimicrobial resistance could not discriminate between human and veterinary isolates (5). Other studies identified a certain genetic schism between human and veterinary isolates of *S. aureus*, based on variability in PFGE (45, 46) or multilocus enzyme electrophoresis patterns (8, 17). In general, these studies compared bovine mastitis-associated isolates with strains of human origin. It was concluded that the overall genetic constitution of these *S. aureus* strains seemed to indicate that the majority of cases of bovine mastitis are caused by a few "specialized" clones (8, 10, 17, 37). This suggests the existence of bacterial host-specificity factors (12). The aim of the present study was to identify genetic polymorphism of *S. aureus* associated with host specificity. Moreover, the pathogenic potential, defined by the presence, absence, or number of selected bacterial virulence determinants, was determined.

**Genome comparison.** Comparative genomics of the strains was initially done by PFGE. This was not useful for effective determination of the population structure of veterinary *S. au-*

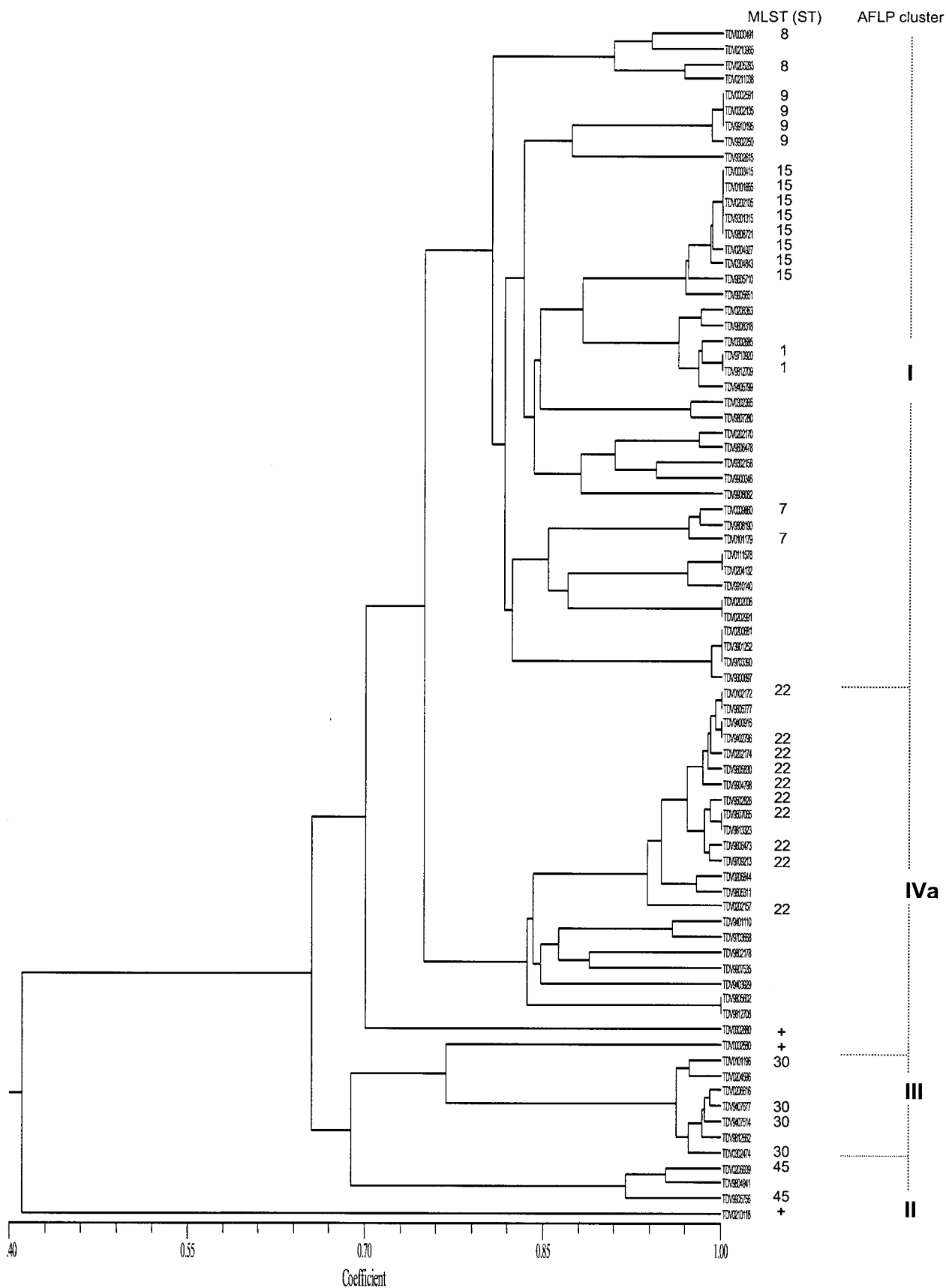


FIG. 1. The dendrogram shows the level of similarity, expressed by the similarity coefficient, between AFLP patterns from the animal strains ( $n = 77$ ). AFLP cluster identification is indicated on the right side of the figure. MLST was performed of a selection of 2, 4, 10, and 18 animal strains, representing each AFLP subcluster (defined by an arbitrarily chosen similarity-coefficient level  $\geq 0.8$ ) within AFLP clusters III, II, IVa, and I, respectively. Plus signs represent three unique AFLP patterns that were found for 0302880 (horse), 0002580 (ape [gorilla]), and 0210118 (seal).

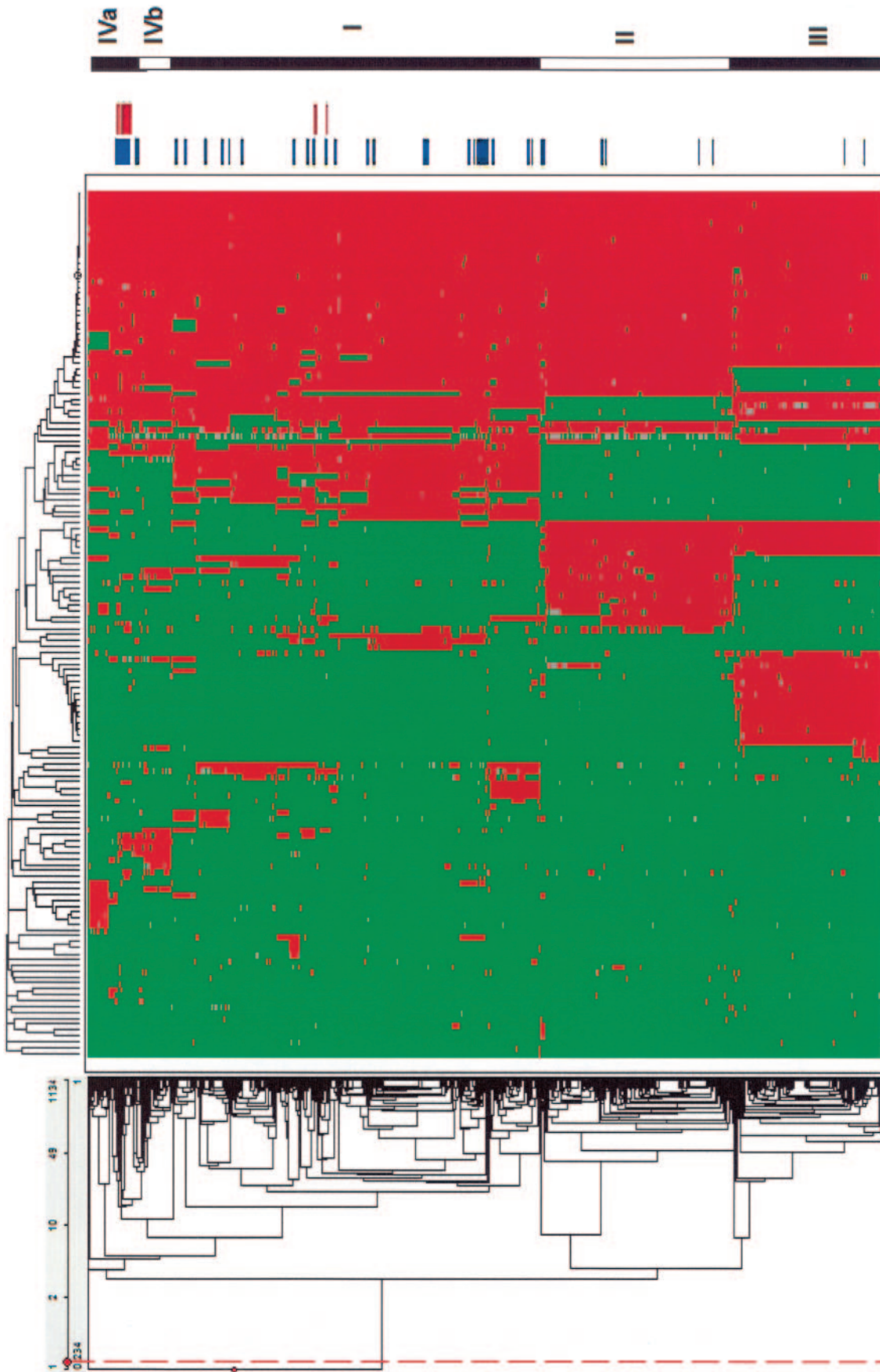


FIG. 2. Agglomerative two-dimensional clustering of strains ( $n = 1,133$ ) and AFLP markers. AFLP fingerprints of the animal strains ( $n = 77$ ) are included. The red fields in the figure represent the presence of AFLP markers; green indicates absence. Blue bars at the right side of the figure indicate the position of each animal strain in the analysis; the red bars represent the masfitis-associated strains. The AFLP database is divided in three main strain clusters (indicated as I, II, and III at the far right of the figure) and two minor clusters (indicated as IVa and IVb) (25).

TABLE 2. Distribution of lineages (AFLP clusters) across the human strains and the veterinary strains

AFLP cluster	No. (%) of animal strains	No. (%) of human strains	P	Odds ratio	95% Confidence interval
I	42 (54.5)	480 (45.5)	0.1256	1.44	0.9–2.3
II	7 (9.1)	263 (24.9)	0.0012	0.3	0.14–0.67
III	3 (3.9)	210 (19.9)	0.0001	0.16	0.05–0.52
IVa	25 (32.5)	63 (6.0)	<0.0001	7.3	4.28–12.6
IVb	0	40 (3.8)			
Total	77	1,056			

TABLE 3. Association of the number of virulence genes and the proportion of animal and human *S. aureus* strains

No. of virulence genes	No. of animal strains (n = 77)	No. of human strains (n = 168)	P	Odds ratio	95% Confidence interval
2	0	5	0.33	0.19	0.01–3.5
3	3	14	0.28	0.45	0.1–1.6
4	17	41	0.75	0.9	0.5–1.7
5	39	80	0.68	1.1	0.7–1.9
6	15	27	0.58	1.3	0.6–2.5
7	3	1	0.09	6.77	0.7–66.2

*reus* strains. No host specificity could be determined except for the apparent clonality of goat-specific strains. Ht-AFLP was the prime method used to compare the animal-associated *S. aureus* strains. The veterinary strains integrated conveniently

into the AFLP database, comprising strains isolated from humans. Animals in close contact with humans for reasons of care and treatment (pet animals and animals in contact with humans in a children’s farm, riding school, or seal sanctuary or

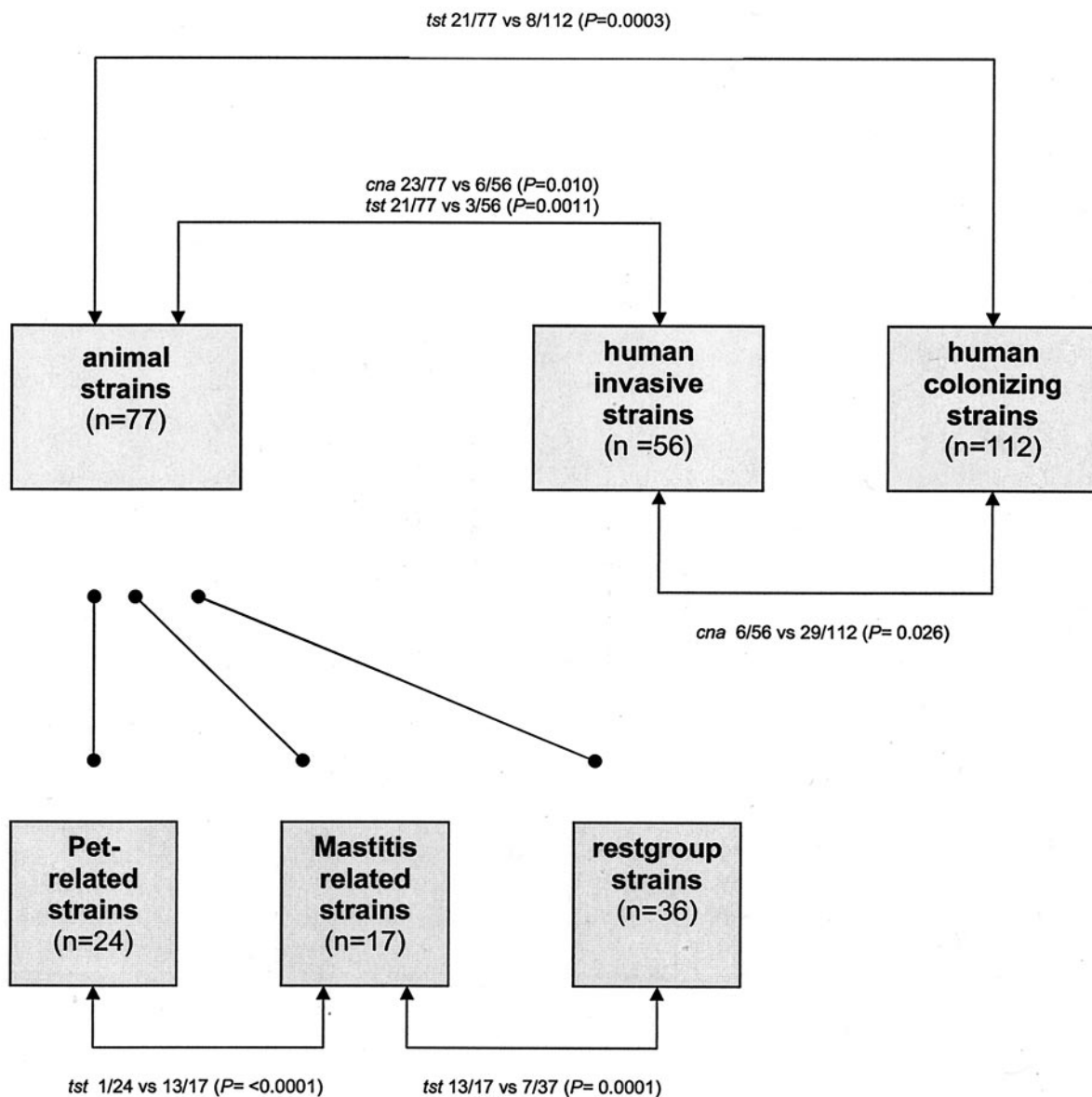


FIG. 3. A flow diagram of the virulence gene distribution in the four different strain clusters. Only significant differences are noted.

through caretakers in a zoo) were infected with these human-associated strains. Notably, strains isolated from cats and dogs were mainly classified in the heterogeneous AFLP cluster I, scattered over its subclusters that consist of human-related *S. aureus* strains. The mastitis-related strains, isolated from diverse host species (sheep, goat, and cow) were genetically clustered, signifying tissue specificity.

Cross-infection with *S. aureus* between humans and domestic animals in the household has been described previously (42). Cats were involved in an outbreak of an epidemic methicillin-resistant *S. aureus* in a geriatric ward (38). Dogs and children's farm cattle are potential reservoirs for the transmission of *S. aureus* strains to humans, causing diverse skin infections (34). Transmission of strains from humans to animals has also been observed (1, 35). We here clearly confirm these earlier findings on the basis of an integrated comparison between human and animal isolates and show that many of these veterinary isolates from various animal hosts fall within the same genomic classes.

**Host-specific virulence potential.** The presence of certain virulence determinants and subsequent pathogenicity in humans has been observed previously (3, 13, 19, 22, 31). Identical virulence determinants were found in *S. aureus* strains causing animal infections. Bovine mammary isolates harbor genes encoding superantigens, such as *tst* (18, 21), exfoliative toxins (7), and enterotoxins (4). These virulence factors were also identified in *S. aureus* strains causing pneumonia in horses (14, 36, 39) or in poultry (11). However, these studies concern small numbers of strains or single isolation sites. We analyzed 10 different virulence genes and determined their distribution in various *S. aureus* populations. The *tst* gene, encoding the exotoxin with superantigen activity, was found significantly more often in the mastitis-associated *S. aureus* strains. Whether these genes are all actively expressed and whether this signifies tissue specificity is not known but is considered quite likely. The *cna* gene was not evenly distributed between the different AFLP clusters of the animal-related strains. The prevalence of exotoxin-encoding genes (*pvl*, *eta*, and *etb*) in both strain collections is low. The numbers of virulence genes in the animal- and human-related *S. aureus* strains were similar on a per strain basis. Apparently, the nature of the virulence genes encountered in an *S. aureus* strain is primarily an important determinant for host specificity. It has to be noted, however, that the virulence genes selected for the present study were based on those found for human pathogens. Most likely, veterinary pathogens may contain other host-specific virulence genes that are currently unknown.

In conclusion, many *S. aureus* clones have disseminated widely among humans, colonizing more than 30% of the population and causing a wide variety of severe infections. These same clones have the potency to colonize and infect many different host species. On the other hand, we here identified a tissue-specific clone (udder) responsible for causing disease in diverse host species. The presence of (combinations of) virulence factors plays an important role in host or even tissue specificity in *S. aureus* infections.

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AFLP is a registered trademark of Keygene N.V., and the AFLP technology is subject to patents and patent applications owned by Keygene N.V.

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