Insights into Alpha-Hemolysin (Hla) Evolution and Expression among *Staphylococcus aureus* Clones with Hospital and Community Origin

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Abstract

Background: Alpha-hemolysin (Hla) is a major virulence factor in the pathogenesis of *Staphylococcus aureus* infection, being active against a wide range of host cells. Although *hla* is ubiquitous in *S. aureus*, its genetic diversity and variation in expression in different genetic backgrounds is not known. We evaluated nucleotide sequence variation and gene expression profiles of *hla* among representatives of hospital (HA) and community-associated (CA) *S. aureus* clones.

Methods: 51 methicillin-resistant *S. aureus* and 22 methicillin-susceptible *S. aureus* were characterized by PFGE, *spa* typing, MLST and SCC*mec* typing. The internal regions of *hla* and the *hla* promoter were sequenced and gene expression was assessed by RT-PCR.

Results: Alpha-hemolysin encoding- and promoter sequences were diverse, with 12 and 23 different alleles, respectively. Based on phylogenetic analysis, we suggest that *hla* may have evolved together with the *S. aureus* genetic background, except for ST22, ST121, ST59 and ST93. Conversely, the promoter region showed lack of co-evolution with the genetic backgrounds. Four non-synonymous amino acid changes were identified close to important regions of *hla* activity. Amino acid changes in the RNAIII binding site were not associated to *hla* expression. Although expression rates of *hla* were in general strain-specific, we observed CA clones showed significantly higher *hla* expression (p = 0.003) when compared with HA clones.

Conclusion: We propose that the *hla* gene has evolved together with the genetic background. Overall, CA genetic backgrounds showed higher levels of *hla* expression than HA, and a high strain-to-strain variation of gene expression was detected in closely related strains.

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Introduction

Staphylococcus aureus is a human opportunistic pathogen responsible for a wide range of infections that can vary in its clinical presentation and severity. Methicillin-resistant *S. aureus* (MRSA) emerged in 1960 in the United Kingdom and has been a major problem in hospitals (HA-MRSA) worldwide during the last 40 years; however since the late 1990s, MRSA has been emerging as a leading cause of severe infection also in the community, in individuals without recent health-care contact (CA-MRSA) [1,2].

CA-MRSA present distinct genetic backgrounds from their hospital counterparts, are more susceptible to antibiotics other than beta-lactams, carry the smallest staphylococcal cassette chromosome *mec* types (SCC*mec* IV or V), and have higher virulence capacity [1,2,3]. The underlying reasons behind the enhanced virulence of CA-MRSA appear to be multiple including a different capacity to overcome host cell response [4], different distribution of mobile genetic elements carrying virulence determinants [5], allelic variation in virulence determinants located in the core genome and in mobile genetic elements [6], and different levels of expression and protein production of virulence determinants (alpha-hemolysin, collagen adhesin, staphylokinase, coagu-

lase, lipase, enterotoxins C3 and Q, V8 protease and cysteine protease) [7,8,9].

The alpha-hemolysin or α-toxin (Hla), is one of the major virulence determinants implicated in the pathogenesis of *S. aureus*, associated to severe skin and soft tissue infections (SSTI), necrotizing pneumonia and even sepsis [10]. Hla is the most prominent *S. aureus* cytotoxin that can act against a wide range of host cells including erythrocytes, epithelial cells, endothelial cells, T cells, monocytes and macrophages [10,11,12]. The gene encoding Hla is located in the core genome and is expressed as a water-soluble monomer (33.2 kD) that assembles to form a membrane-bound heptameric β-barrel pore (232.4 kD) on susceptible cells leading to cell death and lysis [11]. The overall structure is mushroom-like, divided into three domains: 1) Cap domain: largely hydrophobic, defining the entry of the pore; 2) Rim domain: underside of the Cap, in close proximity to membrane bilayer; 3) Stem domain: part of the transmembrane channel, forming the membrane-perforating β-barrel pore (Figure 1) [10,11]. Hla expression is mainly controlled by the global toxin accessory gene regulator (*agr*), via the regulatory effector molecule RNAIII [13]. While *agr* provides the first and most important mechanism of up-regulation of *hla*, expression can also be modulated by other regulators, such as SaeR, SarZ, ArlS [14,15,16] (up-regulators) and Rot, SarT [17](down-regulators).

Although polymorphisms in the *hla* promoter region have been described [18], the range of genetic diversity and evolution of this toxin has never been assessed in a large representative *S. aureus* collection. Furthermore, although differences in *hla* expression have been described between community- and hospital-associated MRSA, these studies have been performed with a limited number of CA-MRSA epidemic clones [9], or almost exclusively with representatives of the USA300 clone [19,20,21]. To better understand the evolutionary history of *hla* and its importance as a virulence factor for CA-MRSA, in this study we compared the *hla* nucleotide sequence and expression among the major epidemic and minor CA and HA clones, including both MRSA and MSSA strains.

Materials and Methods

Ethics Statement

Isolates were obtained from routine diagnostic and were analyzed anonymously and only the isolates, not humans, were studied. All data was collected according to the European Parliament and Council decision for the epidemiological surveillance and control of communicable disease in the European community. Ethical approval and informed consent were for that reason not required.

Bacterial collection

A total of 73 *S. aureus*, including 51 MRSA and 22 MSSA were analyzed in this study. Strains were collected in 13 different countries (Belgium, Bulgaria, Czech Republic, Denmark, Greece, Netherlands, Portugal, Romania, Spain, Sweden, United Kingdom, USA and Brazil), between 1961 and 2009 from both community (n = 46) and hospital (n = 27). The strains comprised a total of 52 *spa* types and 23 sequence types (STs) (see Table S1).

Strains were defined as belonging to CA or HA clones if they contained the same or related genetic backgrounds as the reference CA-MRSA and HA-MRSA epidemic control strains, based on ST, *spa* type and SCC*mec* (in case of MRSA).

Media and bacterial growth conditions

Before RT-PCR analysis, strains were grown overnight at 37°C on tryptic soy agar plates (TSA). Bacterial growth experiments were performed by growing bacteria in tryptic Soy Broth (TSB) at 37°C with shake and measuring OD (600 nm) each hour in the follow up automatic incubator Bioscreen C (Oy Growth Curves AB, Helsinki, Finland). Plates of 100-well honeycomb (Oy Growth Curves AB, Helsinki, Finland) were filled with 300 µl/well of
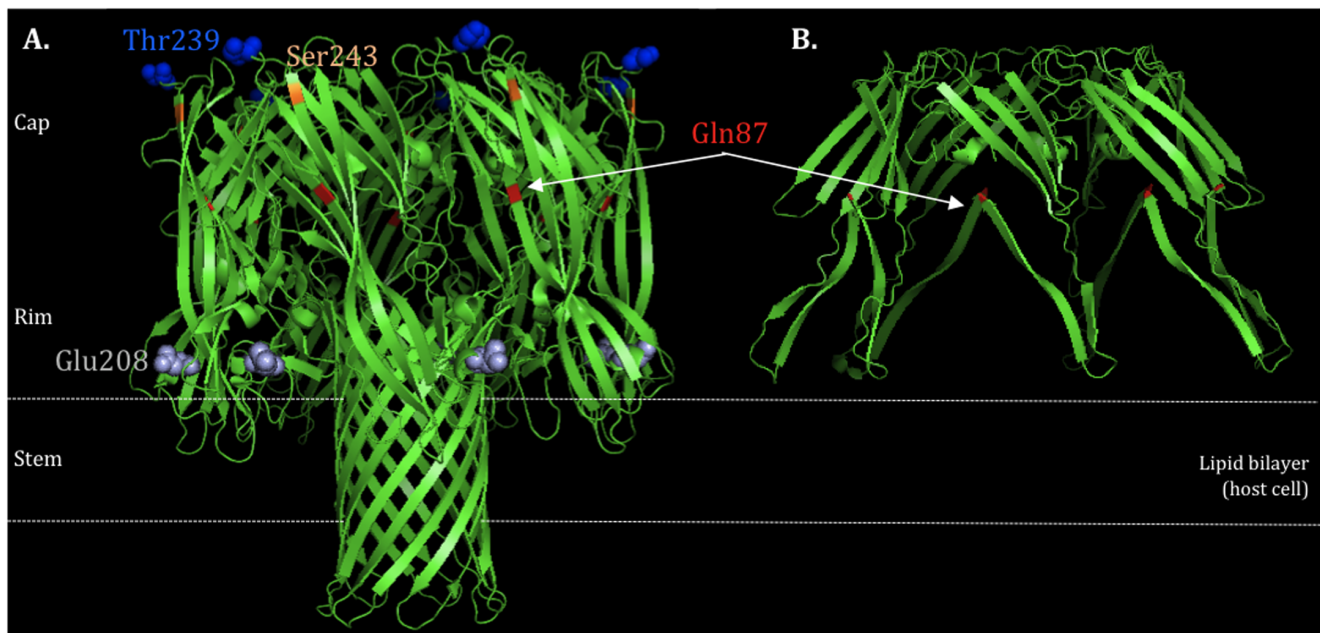

Figure 1. HLA protein structure. A) wildtype (highlighted the non-synonymous mutations Gln87, Glu208, Thr239 and Ser243) and B) truncated protein due to a stop codon at Gln87. Structure generated by the program PyMOL v.1.6.
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overnight culture diluted to $OD_{600} = 0.05$ in TSB growth medium. Three individual growth experiments (SetC, SetD and SetE) were performed for each strain and named accordingly e.g. HLZ6C, HLZ6D and HLZ6E (see Figure S2.I to III).

Nucleotide sequence of *hla* and promoter region

Chromosomal DNA was extracted from overnight cultures, using the boiling method (100°C for 10 min followed by centrifuged at 13.000 g for 5 minutes). Two sets of primers were designed to span the most polymorphic regions within the *hla* gene and *hla* promoter (considered as the region located −600 bp from *hla* starting codon), after alignment of sequences available on NCBI for *S. aureus*. One set of primers (Forward: hla-F_CGAA-AGGTACCATTGCTGGT; Reverse: hla-R_CCAATCGATTT-TATATCTTTC) amplified an internal fragment of the *hla* gene (nt 1170419–1170982, CP000730.1) and the other set (Forward: hlaPro-F_CACTATATTAAAAATACATAC; Reverse: hlaPro-R_GTTGTTACTGAGCTGAC) amplified an internal fragment of the *hla* promoter region (nt 1171289–1171773, CP000730.1) (Figure S3). PCR products were sequenced (Macrogen Europe, Amsterdam, The Netherlands) and sequences were analyzed using SeqMan (DNAstar, Lasergene v9, Madison, WI, USA). To each unique *hla* promoter (P) and gene sequence (*hla*) - allotype - a single Arabic number was attributed (e.g. P1, P2; *hla*1, *hla*2). Gene and promoter sequences were deposited in GenBank (accession numbers KM019547–KM019606; KM019607–KM019674).

Phylogenetic analysis

Phylogenetic relatedness was analyzed using the MEGA5 v5.05 software (http://www.megasoftware.net/) for gene, promoter region and concatenated sequences obtained from 1) gene with promoter region and 2) seven MLST alleles from the 23 representative STs within the collection. Phylogenetic trees were constructed using the Neighbor-Joining clustering method, and 1000 bootstrap replicates, which assigns confidence values for the groupings in the tree.

Moreover, nucleotide diversity (ND) between the two clusters was calculated based on the estimation of the average evolutionary divergence over sequence pairs within the two groups, where the number of base substitutions per site from averaging over all sequence pairs within each group are compared using the maximum composite likelihood model [22].

Detection of recombination

Alignments from the *hla* gene, *hla* promoter and internal fragments of each of the seven MLST gene were screened for the occurrence of putative recombination events using Recombination Detection Program version 4 (RDP4) (http://web.cbio.uct.ac.za/) with the default settings (with highest acceptable probability value of 0.05). Identification of recombinant sequences recombination breakpoints and major parent was determined using simultaneously nine recombination detection methods (RDP, BOOT-SCAN, GENECONV, MAXCHI, CHIMAERA, SISCAN, PhylPro, LARD and 3SEQ. The "minor parent" is considered a sequence closely related to that from which sequences in the proposed recombinant region may have been derived (the presumed donor). The "major parent" was considered as a sequence closely related to that from which the greater part of the recombinant's sequence may have been derived.

RT-PCR analysis

Culture growth was stopped at late exponential phase, when alpha-toxin is described to have maximal activity [23], corre-

sponding to the time-points 1) 3 hours 30 min in one group (65 strains) and 2) 4 hours 30 min in another (8 strains). Total RNA was extracted from three biological replicates. Cells were mechanically disrupted with FastPrep-24 Instrument (MP Biomedicals, Solon, OH, USA) and RNA was protected using RNA Protect (Qiagen, Valencia, USA). RNA was extracted automatically using the QIAsymphony platforms (Qiagen, Valencia, USA) with QIAsymphony RNA kit (Qiagen, Valencia, USA).

The RT-PCR assay was performed on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following primers and TaqMan probes: Hla RT_F: TAATGAATCCTG-TCGCTAATGCC; HlaRT_R: CACCTGTTTTTACTGTAG-TATTGCTTCC; Hla RT Probe: 6FAM-AAACCGGTACTA-CAGATAT-MGBNFQ. The RT-PCR reaction was performed using the EZ RT-PCR Core Reagents (Applied Biosystems, Foster City, USA), in which RNA is reverse transcribed and amplified in a single reaction. The following PCR protocol was used: 50°C for 2 min, 60°C for 30 min, 95°C for 5 min, followed by 42 cycles of 95°C for 20 sec and 62°C for 1 min. The 16S gene was used as internal or reference control. The primers used for 16S RNA amplification were those previously described [24].

RT-PCR data analysis

The relative *hla* gene expression was calculated based on the C_t (RT-PCR output) of the gene of interest (C_t hla) as compared to the C_t of the internal control (C_t 16S) as follows: Delta $C_t = C_t$ hla-C_t 16S. The lower the Delta C_t the higher is the amount of *hla* mRNA and the more the gene is expressed. The reproducibility of the assay was evaluated by the calculation of the arithmetic mean of the relative expression of the three biological replicates (Mean Delta $C_{t1-3} =$ Average (Delta C_{t1}; Delta C_{t2}; Delta C_{t3}). The reproducibility of RT-PCR reaction was evaluated by the calculation of the standard deviation (STDEV) of Delta C_t obtained for each biological replica (Delta C_{t1}; Delta C_{t2}; Delta C_{t3}). Values were considered valid when at least two C_t readings exist with STDEV<2.

Protein structure visualization (pyMOL)

The protein structure was modeled using PyMOL v.1.6 (http://www.pymol.org/) if a nucleotide mutation gave rise to a stop codon.

Statistical analysis

The statistical analysis was performed using the Graphpad Prism 6 (http://www.graphpad.com/scientific-software/prism/), with the two-tailed Student's t-test to determine whether the differences of mean expression rates (MSSA *versus* MSSA; HA backgrounds *versus* CA backgrounds) were statistically significant (p≤0.05).

Regression tree analysis was used to explore which variables could be related with the *hla* expression [25]. Trees explain the variation of a single response variable (in this study the *hla* mRNA expression) by repeatedly splitting the data into more homogeneous groups, using combinations of explanatory variables (in our case, the ST, *spa* type, MRSA, MSSA and the type of SCC*mec*).

Results

Analysis of polymorphisms in the *hla* gene and *hla* promoter

The sequence analysis of the internal region of *hla* and the *hla* promoter region among the 73 strains identified a total of 12 *hla* and 23 promoter region different sequences (allotypes) (Table 1). We obtained no amplification products for *hla* and *hla* promoter

Table 1. Summary of molecular characterization, sequence variation and relative expression rates of *S. aureus* strains collection.

Nº	Isolate ID	SCC*mec*	*spa* type	MLST	Branch[1]	Promotor Allotype	Gene Allotype	Nonsynonymous Mutation	Hla Expression (Mean Delta C_t)[2]	Stddev Delta C_t[3]	Expression (High/Low)
1	HLZ6	II	t002	ST5	L	P4	hla1	D208E	8.69	2*	Low
2	BK2464	II	t002	ST5	L	nt	hla1	D208E	5.37	1	**High**
3	HBR73	II	t067	ST5	L	P5	hla1	D208E	8.75	1	Low
4	C013	VI	t002	ST5	L	P3	hla1	D208E	6.84	1	Low
5	HDES26	VI	t062	ST5	L	P3	hla1	D208E	8.01	1	Low
6	HDE288	VI	t311	ST5	L	P3	hla1	D208E	6.67	1	Low
7	HSA29	–	t002	ST5	L	P3	hla1	D208E	Not Valid	–	Not Valid
8	HDE461	IV	t022	ST22	H	P10	hla12	S239T; T243S	6.60	1	Low
9	HAR22	IV	t022	ST22	H	P11	hla13	S239T; T243S	6.43	1	Low
10	HSMB280	IV	t032	ST22	H	P10	hla12	S239T; T243S	4.71	1	**High**
11	LBM12	IV	t747	ST1806	H	nt	hla12	S239T; T243S	9.28	1	Low
12	HSMB184	–	t5951	ST1806	H	P10	hla12	S239T; T243S	6.74	1	Low
13	HPH2	II	t018	ST36	H	P7	hla8	D208E; S239T; stop codon	8.02	2*	Low
14	HAR24	II	t018	ST36	H	nt	hla8	D208E; S239T; stop codon	9.62	2*	Low
15	DEN4415	II	t021	ST36	H	P7	hla8	D208E; S239T; stop codon	8.95	2*	Low
16	C563	IV	t015	ST45	H	nt	hla10	S239T	7.02	1	Low
17	C036	V	t015	ST45	H	nt	hla10	S239T	6.24	0	Low
18	HAR38	IV	t004	ST45	H	P7	hla10	S239T	10.38	1	Low
19	HFX77	III	t037	ST239	L	P1	hla4	–	8.74	2*	Low
20	HUC343	IIIA	t037	ST239	L	P1	hla4	–	8.27	0	Low
21	HU25	IIIA	t138	ST239	L	P1	hla4	–	8.17	1	Low
22	BK1953	IA	t051	ST247	L	P1	hla4	–	7.71	1	Low
23	HPV107	IA	t051	ST247	L	P1	hla4	–	7.56	0	Low
24	HSJ419	IA	t725	ST247	L	P1	hla4	–	8.23	1	Low
27	E2125	I	t051	ST247	L	P1	hla4	–	7.29	0	Low
25	10395	–	t008	ST250	L	P2	hla4	–	8.15	1	Low
26	COL	I	t008	ST250	L	P1	hla4	–	8.01	1	Low
28	HFX74	IV	t008	ST8	L	P1	hla4	–	6.46	1	Low
29	USA300	IV	t008	ST8	L	P1	hla4	–	6.19	3*	Low
30	C438	IV	t024	ST8	L	P1	hla4	–	6.07	1	Low
31	C574B	IV	t1257	ST612	L	P1	hla4	–	Not Valid	–	Not Valid
32	LBM27	–	t024	ST8	L	P1	hla4	–	8.12	0	Low
33	LBM74	–	t008	ST8	L	P1	hla4	–	5.87	1	Low
34	C270	IV	t1381	ST1	L	P17	hla2	–	8.81	1	Low
35	USA400	IV	t127	ST1	L	P17	hla2	–	6.01	2*	Low

Table 1. Cont.

Nº	Isolate ID	SCC*mec*	*spa* type	MLST	Branch[1]	Promotor Allotype	Gene Allotype	Nonsynonymous Mutation	Hla Expression (Mean Delta C_t)[2]	Stddev Delta C_t[3]	Expression (High/Low)
36	LBM36	–	t127	ST1	L	P18	hla2	–	11.09	1	Low
37	C577	IV	t216	ST59	L	P20	hla5	–	5.35	0	High
38	C583	IV	t437	ST59	L	P19	hla5	–	5.31	1	High
39	C434	V	t437	ST59	L	P19	hla5	–	9.14	1	Low
40	C018	IV	t1819	ST93	L	nt	hla7	–	5.16	1	High
41	C491	IV	t202	ST93	L	P21	hla7	–	5.45	0	High
42	LBM54	IV	t011	ST398	H	P12	hla11	–	4.46	2*	High
43	C482	IV	t011	ST398	H	P13	hla11	–	3.25	1	High
44	C496	VII	t108	ST398	H	nt	hla11	–	2.85	1	High
45	LBM40	–	t034	ST398	H	P12	hla11	–	5.37	1	High
46	C017	IV	t019	ST30	H	nt	hla9	D208E; S239T	4.53	0	High
47	C385	IV	t019	ST30	H	P7	hla9	D208E; S239T	7.25	1	Low
48	C479	IV	t019	ST30	H	nt	hla9	D208E; S239T	8.10	1	Low
71	HUC585	–	t342	ST30	H	P7	hla9	D208E; S239T	5.14	1	High
69	HFF204	–	t318	ST30	H	P9	hla9	D208E; S239T	6.23	1	Low
70	HFA30	–	t012	ST30	H	P8	hla8	D208E; S239T; stop codon	7.94	1	Low
49	HSJO7	IV	t148	ST72	L	P14	hla1	D208E	6.56	1	Low
50	USA700	IV	t148	ST72	L	P14	hla1	D208E	5.76	0	Low
51	COO3	IV	t791	ST72	L	P15	hla1	D208E	6.28	1	Low
52	SAMS1024	IV	t1346	ST1810	L	P14	hla1	D208E	4.78	1	High
53	HUC594	–	t148	ST72	L	P14	hla1	D208E	8.36	1	Low
54	HFA28	–	t126	ST72	L	P14	hla1	D208E	4.56	2*	High
55	C238	–	t3682	ST72	L	P14	hla1	D208E	4.64	1	High
56	C168	IV	t044	ST80	L	P16	hla1	D208E	8.20	0	Low
57	C485	IV	t044	ST80	L	P16	hla1	D208E	5.72	1	High
58	C014	IV	t131	ST80	L	P16	hla1	D208E	4.87	0	High
59	LBM25	–	t1509	ST15	L	P2	hla1	D208E	6.69	0	Low
60	C157	–	t084	ST15	L	P2	hla1	D208E	4.86	1	High
61	C230	–	t346	ST15	L	P2	hla1	D208E	9.03	2*	Low
62	HBA33	–	t258	ST25	L	P6	hla1	D208E	5.73	1	High
63	C095	–	t2909	ST25	L	P6	hla1	D208E	4.16	1	High
64	C141	–	t081	ST25	L	P6	hla1	D208E	4.50	2*	High
65	HBA34	IV	t308	ST121	L	nt	hla6	–	5.62	1	High
66	HUC574	–	t435	ST121	L	P1	hla6	–	5.19	1	High
67	HUC587	–	t159	ST121	L	P2	hla6	–	5.09	1	High

Table 1. Cont.

Nº	Isolate ID	SCC*mec*	*spa* type	MLST	Branch[1]	Promotor Allotype	Gene Allotype	Nonsynonymous Mutation	Hla Expression (Mean Delta C_t)[2]	Stddev Delta C_t[3]	Expression (High/Low)
68	HUC578	–	t284	ST121	L	P1	hla6	–	7.10	2*	Low
72	LBM23	–	t100	ST9	L	P22	hla1	D208E	5.48	2*	High
73	HFX84	–	t267	ST97	L	P23	hla3	–	9.03	1	Low

[1]H: High polymorphism; L: Low polymorphism;
[2]Mean Delta C_{t1-3} = Average (Delta C_{t1}; Delta C_{t2}; Delta C_{t3}), Delta $C_t = C_t$ hla–C_t 16S; Not valid: only one C_t reading;
[3]*low reproducibility between three C_T values (Stddv≤2). nt: non typable; Stddv: standard deviation.
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region in one and 13 strains, respectively, which probably result from mispairing of the primers used.

From the 12 *hla* (*hla1–12*), we observed that only a single *hla*-allotype was found among representatives of a specific ST, except for ST22 (*hla12*; *hla13*) and ST30 (*hla8*; *hla9*) where two different alleles were identified. On the other hand, the most frequent alleles, *hla1* (33.3%, n = 24) and *hla4* (20.8%, n = 15), were identified in more than one ST.

Regarding the nucleotide changes identified in the *hla*, some correspond to non-synonymous mutations (E208, T239 and S243) and, in one particular case, to a stop codon (Table 1 and 2). The substitutions observed did not correspond to any difference in the charge or polarity of the amino acid (aa). However, changes in molecular weight were observed: i) changes from aa D208 to aa E208 (D208E) and from aa S239 to T239 (S239T) gave rise to a higher molecular weight aa; and ii) change from aa T243 to S243 (T243S) resulted in a lower molecular weight aa; of note all changes occurred in the Rim domain of the protein. In a particular case, the aa change gave rise to a stop codon located in the CAP domain, in strains of ST36. Protein structure modeling showed that a protein of about one third of its real size is produced, truncated at the Gln87 (Figure 1, A and B). The truncation is in the outside part of the domain, suggesting that this will affect the capacity of the Hla to form cell wall pores, and ultimately to induce hemolysis.

A high number of sequence variations were identified in the *hla* promoter region, (n = 23) (P1–23) (Table 1 and 2). Although we found that some STs were associated to a specific promoter allotype, and some promoters were identified in a single ST, we also identified cases where single STs were associated to different promoters (8 out of 23) and examples in which a single promoter allotype was associated to different STs (5 out of 23). This is the case of the most frequent promoter (P1) that was found in about one third of the strains analyzed (25.4%, n = 16), including several different STs.

A particular highly polymorphic region corresponding to nt −22 to −24 from the start codon, was found in the majority (16 out of 23) of the promoter allotypes (exceptions P1, P6, P13, P14, P15, P18 and P23). These polymorphisms are located in the vicinity of RNAIII binding site [26]; however, we could not find a direct correlation between a particular nucleotide sequence and a specific expression pattern (high or low expression). For example, the sequence T**T**T, observed in two strains belonging to ST398 that have a high level expression, was also observed in strains with low expression belonging to other genetic backgrounds (ST8, ST239, ST247, ST250, ST36, ST45 and ST22).

Alpha-hemolysin evolutionary history

In order to better understand the evolution of *hla* gene within the *S. aureus* population, we constructed phylogenetic trees from the *hla* and *hla* promoter sequences, separately or concatenated (Figure 2, A) and compared it with the tree constructed from the concatenated sequences of the seven housekeeping genes used in MLST, including all the STs represented in the strain collection described here (Figure 2, B).

The phylogenetic tree constructed for the *hla* gene showed two distinct major clusters with different evolutionary clocks that differed in their nucleotide diversity (ND, see Materials and Methods): cluster (L) with lower diversity (ND = 0.005), and cluster H with higher diversity (ND = 0.019). Cluster L included more than 70% of strains (71.2%, n = 52), and five sub-clusters; Cluster H contained about 29% of the strains (28.8%, n = 21), and comprised four minor sub-clusters including *hla8–hla12* alleles,

Table 2. Strains data distribution based on promoter allotypes.

		Promotor allotype	Gene allotype	Non Synonymous Mutation	Isolates Molecular Characterization	Expression Category
CA backgrounds	ST398	P13	hla11	–	ST398-IV, t011	**High expression**
		P12			ST398, t034	**High expression**
		NT			ST398-VII, t108	**High expression**
		P12			ST398-IV, t011	**High expression***
	ST25	P6	hla1	D208E	ST25, t258	**High expression**
					ST25, t081	**High expression***
					ST25, t2909	**High expression**
	ST9	P22	hla1	D208E	ST9, t100	**High expression***
	ST93	P21	hla7	–	ST93-IV, t202	**High expression**
		NT			ST93-IV, t1819	**High expression**
	ST121	P2	hla6	–	ST121, t159	**High expression**
		P1			ST121, t435	**High expression**
		NT			ST121-IV, t308	**High expression**
		P1			ST121, t284	Low expression*
	ST72	P14	hla1	D208E	ST72-IV, t148	**High expression**
		P14			ST72, t3682	**High expression**
		P14			ST1810-IV, t1346	**High expression**
		P14			ST72, t126	**High expression***
		P15			ST72-IV, t791	Low expression
		P14			ST72-IV, t148	Low expression
		P14			ST72, t148	Low expression
	ST80	P16	hla1	D208E	ST80-IcV, t131	**High expression**
					ST80-IV, t044	**High expression**
					ST80-IV, t044	Low expression
	ST30	P7	hla9	D208E; S239T	ST30, t342	**High expression**
		NT			ST30-IV, t019	**High expression**
		P7			ST30-IV, t019	Low expression
		P9			ST30, t318	Low expression
		NT			ST30-IV, t019	Low expression
		P8	hla8	D208E; S239T; stop codon	ST30, t012	Low expression
	ST15	P2	hla1	D208E	ST15, t084	**High expression**
					ST15, t346	Low expression*
					ST15, t1509	Low expression
	ST59	P20	hla5	–	ST59-IV, t216	**High expression**
		P19			ST59-IV, t437	Low expression
		P19			ST59-V, t437	Low expression
	ST1	P17	hla2	–	ST1-IV, t1381	Low expression
		P17			ST1-IV, t127	Low expression*
		P18			ST1, t127	Low expression
	ST8	P1	hla4	–	ST8-IV, t008	Low expression
					ST8-IV, t024	Low expression
					ST8-IV, t008	Low expression*
					ST8, t008	Low expression
					ST612-IV, t1257	Not valid**
					ST8, t024	Low expression
	ST97	P23	hla3	–	ST97, t267	Low expression
HA backgrounds	ST22	P10	hla13	S239T; T243S	ST22-IV, t032	**High expression**
		P10	hla12		ST22-IV, t022	Low expression
		P11			ST22-IV, t022	Low expression

Table 2. Cont.

	Promotor allotype	Gene allotype	Non Synonymous Mutation	Isolates Molecular Characterization	Expression Category
	P10			ST1806, t5951	Low expression
	NT			ST1806-IV, t747	Low expression
ST5	NT	hla1	D208E	ST5-II, t002	**High expression**
	P3			ST5-VI, t002	Low expression
	P3			ST5-VI, t062	Low expression
	P3			ST5-VI, t311	Low expression
	P4			ST5-II, t002	Low expression*
	P3			ST5, t002,	Not valid**
	P5			ST5-II, t067	Low expression
ST36	P7	hla8	D208E; S239T; stop codon	ST36-II, t018	Low expression*
	P7			ST36-II, t021	Low expression*
	NT			ST36-II, t01	Low expression*
ST45	NT	hla10	S239T	ST45-IV, t015	Low expression
	NT			ST45-V, t015	Low expression
	P7			ST45-IV, t004	Low expression
ST239	P1	hla4	–	ST239-IIIA, t037	Low expression
				ST239-III, t037	Low expression*
			–	ST239-IIIA, t138	Low expression
ST247	P1	hla4	–	ST247-I, t051	Low expression
				ST247-IA, 051	Low expression
				ST247-IA, t051	Low expression
				ST247-IA, t725	Low expression
ST250	P1	hla4	–	ST250-I, t008	Low expression
	P2			ST250-I, t008	Low expression

(*)(**) relative expression values not valid (SDV≤2 or only one C_T reading).
doi:10.1371/journal.pone.0098634.t002

which were found in strains of ST30, ST36, ST45, ST398 and ST22.

As opposed to the phylogenetic tree constructed from *hla* gene, the one constructed from the promoter region did not show two distinct evolutionary branches (Figure S1). Moreover, dissimilar subgroup clustering was noticed in the tree constructed from the promoter gene sequence. For example, ST45, ST30 and ST36 backgrounds were clustered together in the promoter sequence-based tree whereas in the *hla* sequence-based tree ST45 was placed separately from ST30 and ST36 cluster (branch H). The same type of observations can be drawn for most of STs. Overall the promoter region showed to be more diverse than the *hla* gene sequence among the different backgrounds.

On the other hand, when we compared the phylogenetic tree constructed with the *hla* gene with that constructed from MLST concatenated genes, the same type of division into two distinct main clusters was observed (Figure 2). Moreover, the majority of STs were equally distributed between the two clusters in the two trees. The only exceptions were ST22, ST121, ST59 and ST93 that in the two trees have exchanged their positions from one cluster to the other (Figure 2, B-blue arrows).

Detection of recombination in *hla* gene, h*la* promoter and MLST genes

To understand if recombination could explain the incongruence found between the trees constructed from *hla* and MLST

concatenated genes, we screened the *hla* gene, *hla* promoter and each MLST gene for recombination events using the RDP4 software.

The SiScan and 3Seq methods detected one recombination event in the *hla* gene. This event corresponded to a fragment ending in positions 385–410 of the *hla* alignment, however the beginning breakpoint was not possible to determine. In the collection analyzed this event was detected in five isolates belonging to ST22 or related STs (HSMB280, HDE461, HAR22 and LBM12 (TLV ST22) and HSMB184 (TLV ST22)) and four isolates of ST398 (LBM54, LBM40, C496, C482_ST398). The ST30 HFF204 strain was identified as the minor parent (97.8% identity with ST22 strains and −99.3% identity with ST398 strains) and ST121 strain HUC587 was identified as the major parent (with 100% identity to ST398 strains and 93.5–95.2% identity with ST22 strains) of the recombining fragment. A trace signal of recombination of this same event was also identified among ST45 isolates; however this signal was not statistically significant. Interestingly all the recombination events were detected in strains belonging to the high genetic diversity cluster in the tree constructed from *hla* gene. In the *hla* promoter region no recombination events were detected.

We have performed the same type of analysis using the internal sequences of each of the seven housekeeping used in MLST scheme, including the alleles present in all STs identified in this
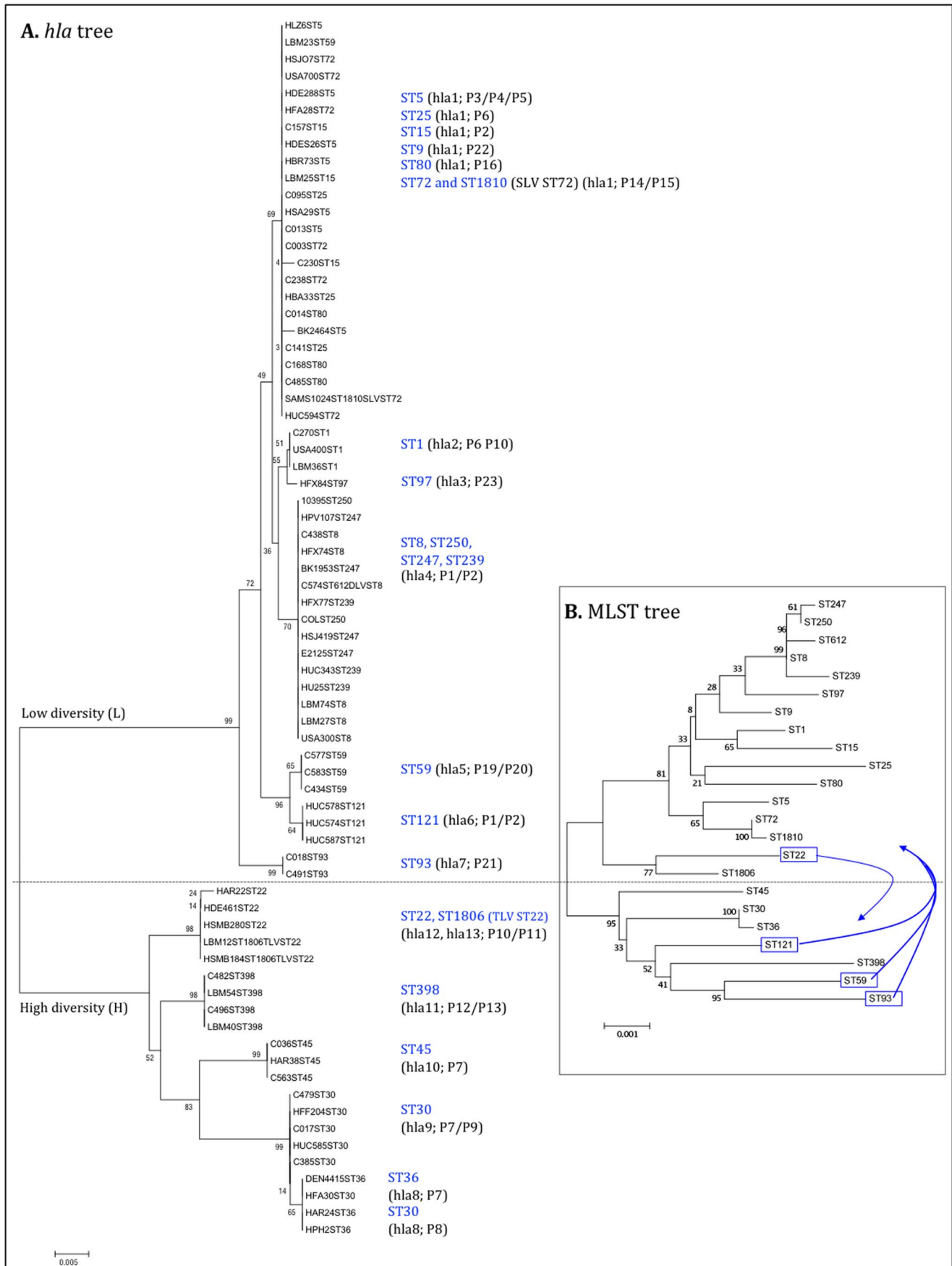
Figure 2. Phylogenetic trees of *hla* gene (A) and concatenated sequences of MLST alleles (B) from 23 STs representatives of the strains collection. The tree was constructed using MEGA 5 with Neighbour-joining method and bootstrap values provided as percents over 1000 replications. Branch length values are indicated and the percentage of replicate trees (bootstrap test) are shown next to the branches. The dashed line indicates the separation of the two evolutionary branches.
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study, however no recombination events were detected in any of the genes.

Altogether the data gathered suggest that for the majority of strains *hla* gene evolved together with the genetic background. The different clustering of ST22 and ST121 strains, in the trees constructed from MLST concatenated genes and *hla* gene, may derive from recombination events occurring in the *hla* gene. Similarly these type of events might explain the genetic diversity observed in cluster H in the *hla* tree in strains belonging to ST22, ST398, ST45, ST30 and ST36 (H cluster of *hla* tree).

Expression of alpha-hemolysin

The expression of alpha-hemolysin in the 73 strains was assessed by RT-PCR, in three biological replicates. Fifteen of the 73 strains (20.5%) were excluded from the final analysis, either because a single valid determination for Delta C_t (N = 2) was obtained or because C_T obtained from the different biological replicates were not reproducible (N = 13).

The analysis of the regression tree split the response variable into two distinct groups, according to the *spa* type of the strains. There was a group of strains with mean Delta $C_{t1-3} \leq 5.73$, that was classified as a high expression group and a second group with a mean Delta $C_{t1-3} > 5.73$ classified as a low expression group (Table 1, Table 2 and Figure 3). Overall the regression tree explained 60% of the variance in the data. This is mostly because there were strains expressing a low or high mean Delta C_t that were classified in the same *spa* type; those were the cases of *spa* types t002, t019, t044 and t437.

Furthermore, we explored in each of the *spa* types what other explanatory variables (ST, MRSA, MSSA and type of SCC*mec*) could differentiate the inclusion of some strains in the low or high expression group, but we found no associations with the variables we measured in the study.

We observed that the *hla* expression level varied within strains of the same ST (Figure 3; Table 1 and 2). In fact, in some cases the same ST comprised strains with both high and low levels of expression (ST5, ST15, ST22, ST30, ST59, ST72 and ST80). Moreover, we found that the expression rates did not differ significantly (P = 0.665) between MRSA and MSSA strains. However, we did find a correlation between the *hla* expression and the origin of the genetic backgrounds. Actually, strains of CA genetic backgrounds showed, in general, higher mean expression rates than strains of HA backgrounds (p = 0.003) (Figure 4). Among the 21 strains (36.2%, 21 out of 58) with high expression level, only two (9.5%) belonged to HA backgrounds (ST22-IVh, t032 and ST5-II, t002) whereas the majority (90.5%, n = 19) were represented by CA backgrounds (Table 1 and Table 2). Moreover, two additional CA strains, ST72-IVa-t148 and ST8-MSSA-t008, showed expression rates near the cutoff value (5.73), with 5.76 and 5.87, respectively. These were considered as belonging to the low-level expression group.

The three strains with the highest expression rate were ST398-VII-t108 (2.85), ST398-IVa-t011 (3.25) and ST25-MSSA-t2909 (4.16) and strains with the lowest rate were ST1806 (TLV ST22)-IVh-t747 (9.28), ST45-IVa-t004 (10.38) and ST1-MSSA-t127 (11.09).

We observed that some promoters and gene alleles (P6, P12/P13, P21; and *hla7*, *hla9*, *hla11*) were exclusively associated to a high expression level profile, while others (P3/P4/P5, P7, P8/P9, P11, P15, P17/P18, P23; and *hla4*, *hla8*, *hla10*) were exclusively associated to a low expression level (Table 1 and 2). But we also found promoter and gene allotypes that were associated to both high and low expression levels.

Discussion

Although Hla is one of the most important *S. aureus* virulence factors [10], to the best of our knowledge, this is the first study in which the variation in *hla* nucleotide sequence and gene expression was assessed in such a large and representative collection.

We found that the nucleotide sequence of *hla* was highly diverse. The high degree of diversity found within *hla* is in accordance to results obtained for other exotoxins, which are generally highly polymorphic [27]. Four non-synonymous substitutions (Q87 stop codon, D208E, S239T and T243S) were identified, that are located in two structural protein domains which are essential for Hla oligomerization and pore formation (Rim and Cap) [11,28,29]. The impact of these amino acid (aa) changes on *hla* activity is uncertain. If by one hand, the aa changes described implicate differences in the molecular weight of the aa, that can have influence in the three dimensional structure stability and activity of the protein; on the other hand these aa changes did not match any of the aa previously described to be essential for Hla pore formation.

Furthermore, Walker and Bayley showed that multiple mutations in this same region (residues spanning Hla235–250) did not alter Hla activity in terms of binding, oligomerization or lysis. Thus, it would not be expected that S239T or T243S had significant biological impact in terms of toxin function. The unique mutation with an identified role in Hla function is the stop codon found in the ST36 and ST30 strains that was previously described by DeLeo and co-authors [30] to hinder toxin production and to originate a less virulent strain in a murine infection model. The true effect of the non-synonymous substitutions identified in our study in the activity of the protein would have to be tested by the construction of site directed mutagenesis mutants and by performing binding, oligomerization, hemolysis and *in vivo* models assays.

The construction of phylogenetic trees from the *hla* defined the existence of two clusters with different levels of genetic diversity suggesting that *hla* is evolving at different rates in different genetic backgrounds. Interestingly, the most diverse cluster included the clonal types which are presently more disseminated or that emerged recently (like ST398). This might be related to the fact that these clones still need to evolve to evade the human immune system and not enough time as elapsed for the most adapted allele to have been selected [31]. On the other hand the recombination events detected in the *hla* gene in this study were all in strains belonging to the high genetic diversity cluster, suggesting that this mechanism might have been important in the most recent *hla* evolution and diversification.

Interestingly, the phylogenetic tree constructed from the *hla* gene was similar to that constructed from MLST genes, in the sense that both trees distributed the different STs similarly in two main clusters. This observation suggests that *hla* gene has evolved
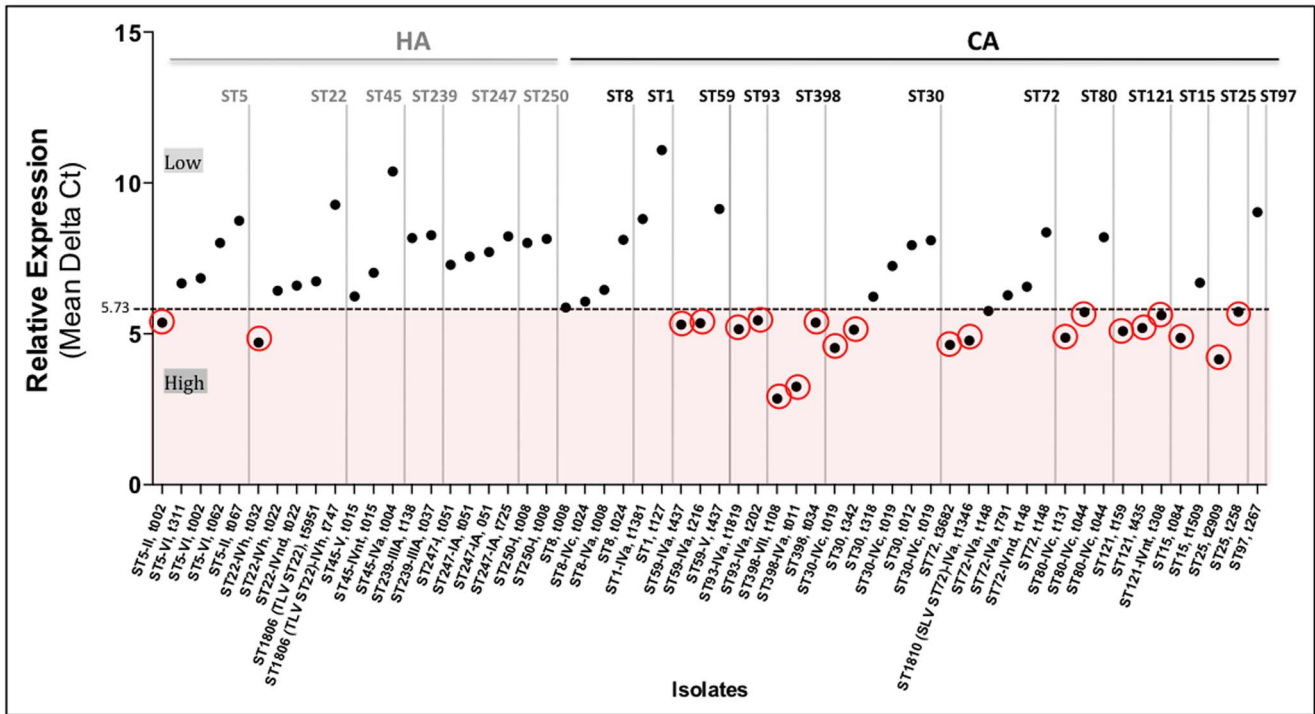
Figure 3. HA and CA strains relative expression distribution. Mean of expression rates from three biological replicates. Dashed line corresponding to the mean Ct value 5.73 results from the regression tree analysis which split strains in two distinct groups, at *spa* type level: a) high expression group - corresponding to strains with Mean Delta Ct≤5.73 and b) low expression group- corresponding to strains with Mean Delta Ct> 5.73). Highlighted in red are the high expressing strains.
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together with the *S. aureus* genetic background. A similar type of correlation with the genetic background was previously described for adhesins, either located in the core genome (*clfA*, *clfB*, *fnbA*, *map*, *sdrC*, and *spa*) or accessory genome (*ebpS*, *fnbB*, *sdrD*, and *sdrE*) [32]. Although this was the case for the great majority of

STs, we observed that four STs (ST22, ST121, ST59, ST93) were located in different clusters in the *hla* and MLST trees. Our results suggest that recombination occurring at the *hla* level, might explain the different clustering of strains belonging to ST22 and ST121. No recombination events were, however, detected in
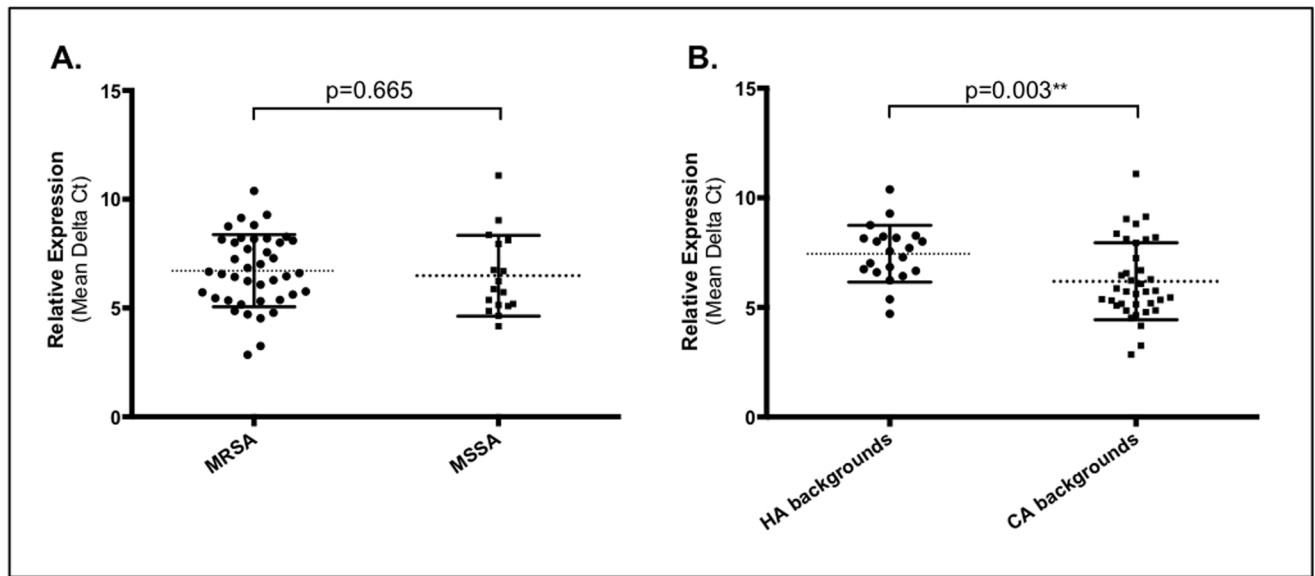

Figure 4. Distribution of the relative *hla* expression. Mean of relative expression of three independent readings. Expression comparison between a) MRSA and MSSA and b) HA and CA backgrounds using the Two-tailed Student's t-test. Statistically significance (p≤0.05) (**).
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MLST genes or *hla* sequences of strains belonging to ST59 and ST93, suggesting that their displacement in the two trees could derive from different phenomena, like random mutation.

It was previously suggested that CA-MRSA expressed more *hla* than HA-MRSA [9]. Results from our study allowed us to extend this conclusion to virtually all epidemic CA, but also in two particular cases of HA genetic backgrounds. The CA strains belonging to ST398, ST25, ST121 and ST93 showed uniformly high relative expression rates and strains belonging to ST36, ST45, ST239, ST247 and ST250 showed uniformly low expression rates. To understand if in fact these patterns of expression are characteristic of these clones, more strains within each clone should be studied for *hla* expression. Nevertheless, we could not correlate the *hla* expression rate with any particular polymorphism within the promoter or any aa substitution in the *hla* gene. The results suggest that *hla* regulation is probably a result of combination of factors which are redundant, rather than associated to a single genetic event. In fact, it has been demonstrated by several authors that alpha-hemolysin is part of a complex regulatory network, that includes the main two-component systems (TCS) – Agr – that in turn is controlled by a diverse pool of regulatory networks that coordinately interact in response to external stimulus and cell signals, namely others TCS (SaeRS, ArlRS and SrrAB), alternative sigma factors (σ^B), and transcription factors (e.g. SarS, SarT, Rot, SarA, SarZ) [33,34].

We showed that *hla* evolved together with the genetic background. Moreover, the most epidemic CA-MRSA genetic backgrounds express more *hla* than the most epidemic HA-MRSA genetic backgrounds. However, the finding of frequent strain-to-strain variation in the expression level of *hla* within strains of the same clonal types suggests that *hla* polymorphisms cannot be used as genetic markers of virulence and investigators should remain cautious when inferring conclusions for the entire MRSA population from studies performed with a limited number of strains.

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Supporting Information

Figure S1 Phylogenetic trees of the *hla* gene, promoter gene and concatenated sequences of both. The tree was constructed using MEGA 5 with Neighbour-joining method and bootstrap values provided as percents over 1000 replications. Branch length values are indicated and the percentage of replicate trees (bootstrap test) are shown next to the branches. The dashed line indicates the separation of the two evolutionary branches (L and H).
(TIF)

Figure S2 **I.** Growth curves for triplicates of each *S. aureus* strain – Set C. **II.** Growth curves for triplicates of each *S. aureus* strain – Set D. **III.** Growth curves for triplicates of each *S. aureus* strain – Set E.
(TIFF)

Figure S3 Internal sequences of *hla* promoter (highlighted blue) and *hla* gene (highlighted orange) used for analysis in this study. Primers used are highlighted. The sequence shown corresponds to the promoter and *hla* regions of USA300 strain from our collection blasted against USA300_TCH1516.
(TIF)

Table S1 Molecular characterization of the 73 MRSA and MSSA strains included in this study [35–50].
(DOC)

Author Contributions

Conceived and designed the experiments: HdL MM. Performed the experiments: AT. Analyzed the data: AT MM ACP JBN KS. Contributed reagents/materials/analysis tools: HdL MM HW. Wrote the paper: AT MM. Manuscript revision: HdL HW KS JBN KB SR ACP.

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