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Variation of the Polymorphic Region X of the Protein A Gene during Persistent Airway Infection of Cystic Fibrosis Patients Reflects Two Independent Mechanisms of Genetic Change in *Staphylococcus aureus*

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Variation of the polymorphic region of the protein A gene (*spa*) was observed during long-term persistence of *Staphylococcus aureus* in the airways of 10 cystic fibrosis patients and occurred at a rate of one genetic change every 70 months. Independent mutational events were observed eight times in 142 isolates: four deletions, two duplications of repeats, and two point mutations.

DNA sequence-based approaches, such as multilocus sequence typing of seven housekeeping genes and single-locus DNA sequencing of the variable repeat region X of the protein A gene (*spa*), are being used more frequently as molecular typing methods for *Staphylococcus aureus* population studies (2, 3, 5, 9). An important advantage of sequence-based typing methods is the ease of access of sequencing data for interlaboratory comparison via the Internet (e.g., http://www.mlst.net or http://www.ridom.de/spaserver/). In a recent study, *spa* has been shown to function as a genetic marker, and the discriminatory power of *spa* typing is comparable to those of pulsed-field gel electrophoresis (PFGE) and whole-genome DNA microarray (7). Furthermore, to detect genetic variation that accumulates rapidly and slowly by two independent mechanisms, Koreen et al. (7) used *spa* typing at the level of *spa* types and *spa* lineages for which *spa* types with similar repeat profiles were grouped together. Thus, *spa* typing might serve as a useful method not only for outbreak investigations but also for long-term epidemiological and population-based studies. However, the occurrence of mutational events of the polymorphic *spa* region in consecutive strains of individual patients in vivo has not been studied extensively.

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In this study, 142 isolates collected during a longitudinal study of 10 cystic fibrosis (CF) patients with persistent infections were chosen for sequence analysis of the *spa* region (6 to 25 isolates per patient) (6). These 10 patients were persistently infected (median, 56 months; range, 41 to 75 months) by single *S. aureus* clones as determined by PFGE. The PFGE fragment patterns of consecutive isolates from the individual patients were identical (isolates from five patients) or displayed differences in fragment patterns consistent with the occurrence of one or two independent genetic events (differences of two to six bands), thereby indicating that the strains are related (10). Such changes most likely emerged due to genomic changes during long-term persistence in the host (4, 10). Thirty-nine of the strains from six patients that were analyzed were small colony variant strains, which were isogenic to the phenotypically normal *S. aureus* strain as shown by PFGE (6).

The *spa* region was amplified with the following primers: spa-1113f (5′-TAAAGACGATCCTTCGGTGAGC-3′) and spa-1514r (5′-CAGCAGTAGTGCCGTTTGCTT-3′). DNA sequences were obtained with an ABI 3100 Avant sequencer (Applied Biosystems, Foster City, Calif.). *spa* types were determined with the Ridom StaphType software (5). Numerical *spa* repeat and type codes were used.

Sixteen different *spa* types with 4 to 13 repeats (median, 10) for the 142 isolates were resolved by sequencing (Table 1). All repeats consisted of 24 bp, except repeat 44, which was only 21 bp (AAAGAAGACAACAAGCCTGGT). Sequential isolates of half of the patients (five patients) had identical *spa* types, while consecutive isolates of the other half were grouped into two or three *spa* types for each patient (Table 1). There was no association of variation of the *spa* region and variation of the whole genome as determined by PFGE fragment pattern differences: the isolates of two of five patients with identical PFGE patterns displayed different *spa* types (Fig. 1A), while the isolates of two of five patients with PFGE fragment pattern differences showed the same *spa* types (Fig. 1B). Variation of the *spa* type of consecutive strains from individual patients was not associated with specific differences in the susceptibility patterns of the strains, clinical scores of the patients, or with normal or small colony variant phenotypes of the strains (data not shown).

Different *spa* types of individual patients showed an overall similar composition of repeats (Table 1). The changes in the composition of the repeats were consistent with deletion or duplication of repeats and point mutations. Specifically, deletion of repeats occurred four times independently and was demonstrated in nine isolates, duplication of repeats took

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TABLE 1. *spa* types of consecutive *S. aureus* strains from the airways of individual CF patients

Patient[a]	*spa* type[b]	Repeats[c]	No. of isolates[d]	Comments[e]
1	8	r11r19r12r21r17r34r24r34r22r25	6	
2	15	r08r16r02r16r34r13r17r34r16r34	12	Ancestor
	50	r08r16r02r16r34r34r17r34r16r34	3	pm, AAC to AAA, (Asp-Lys) at pos 6
3	71	r26r23r23r17r34r17r20r17r12r17r16	10	Ancestor
	2	r26r23r17r34r17r20r17r12r17r16	1	del of r23 at pos 3
	72	r26r23r23r17r12r17r16	2	del of r34r17r20r17 at pos 5
4	154	r04r44r33r31r12r16r34r22r17r25r22r22r34	10	
5	84	r07r23r12r34r34r12r12r23r02r12r23	11	Ancestor
	85	r07r23r12r34r34r12r23r02r12r23	1	del of r12 at pos 7
6	80	r09r02r16r34r42r17r16r34	11	
7	91	r07r23r21r17r34r12r23r02r12r23	24	Ancestor
	250	r07r16r23r21r17r34r12r23r02r12r23	1	dupl with pm at pos 2, GGC to GGG (Gly-Gly)
8	78	r04r21r12r41r20r17r12r12r17	18	Ancestor
	87	r04r21r12r41r20r17r12r12r17r17	1	dupl of r17 at pos 10
	79	r04r21r12r41	5	del of r20r17r12r12r17 at pos 5
9	9	r11r12r21r17r34r24r34r22r24r34r22r33r25	15	
10	91	r07r23r21r17r34r12r23r02r12r23	11	

[a] Individual CF patients.
[b] Numerical *spa* types.
[c] Repeats of the respective *spa* types.
[d] Number of isolates that were sequenced and were found to display the different *spa* types.
[e] pm, point mutation; del, deletion; pos, position of the repeat; dupl, duplication.

place two times and was shown in two isolates, and point mutations happened two times and were identified in four isolates. The duplication of a single repeat in one isolate was coupled with a point mutation in the duplicated repeat. Thus, eight independent mutational events occurred in the strains analyzed, and these events were demonstrated for 14 of 142 isolates (10%). Four isolates with changed repeats occurred only once, while isolates with three mutations were observed in consecutive isolates (Table 2), indicating differences in the stability of the mutation. The two point mutations observed in our study resulted in one synonymous change (Gly-Gly) and one nonsynonymous change (Asn-Lys) of amino acids. The rate of genetic change (clock speed) of the variable *spa* region was 70 months (number of months of persistence of *S. aureus* in all patients/number of independent genetic events = 556/8). In particular, every 93 months, deletions or duplications occurred in the strains analyzed, and every 280 months, point mutations occurred in the strains analyzed. The variability of the number of repeats is thought to be caused by slipped-strand mispairing (SSM), which seems to occur in combination with inadequate DNA mismatch repair systems (12). In this way, repeats can be deleted or inserted during DNA polymerase-mediated DNA duplication, depending on the orientation of the strand. Variations by SSM occurred more often in the isolates analyzed (six times in 11 isolates) than point mutations (twice in 4 isolates). While point mutations are an indicator of the background rate of nucleotide mutation in the repeats, SSM has been documented as an important prerequisite for bacterial phase variation and adaptation (12).

By using consecutive isolates from individual patients who were persistently infected by a single *S. aureus* clone as determined by PFGE, the chance that sequential isolated strains have evolved from a common ancestor is high. Therefore, it is very likely that differences in the resolved *spa* types from consecutive isolates resulted from mutational changes during persistence in the host. The evolutionary events that appeared in our isolates had been hypothesized previously by Brigido et al. (1), who proposed a model for the evolution of region X from an ancestral 24-bp region through multiple processes, such as duplication, deletion, and point mutations. Changes of repeat regions as demonstrated in the sequential *S. aureus* strains have also been shown to occur in *Haemophilus influenzae* repeat regions during persistence in the airways of CF patients (8).

In this study, we determined a rather high rate of mutational events that occurred in 10% of the strains analyzed. This is in contrast to other studies, which demonstrated stability of the *spa* region in vitro and in vivo (3, 9). While the in vitro stability was established during multiple passages on blood agar plates in the laboratory (3, 9), the data on in vivo stability of the *spa* region were retrieved from three methicillin-resistant *S. aureus* strains collected over a 5-year period from only one CF methicillin-resistant *S. aureus* carrier (3). The conclusions that can

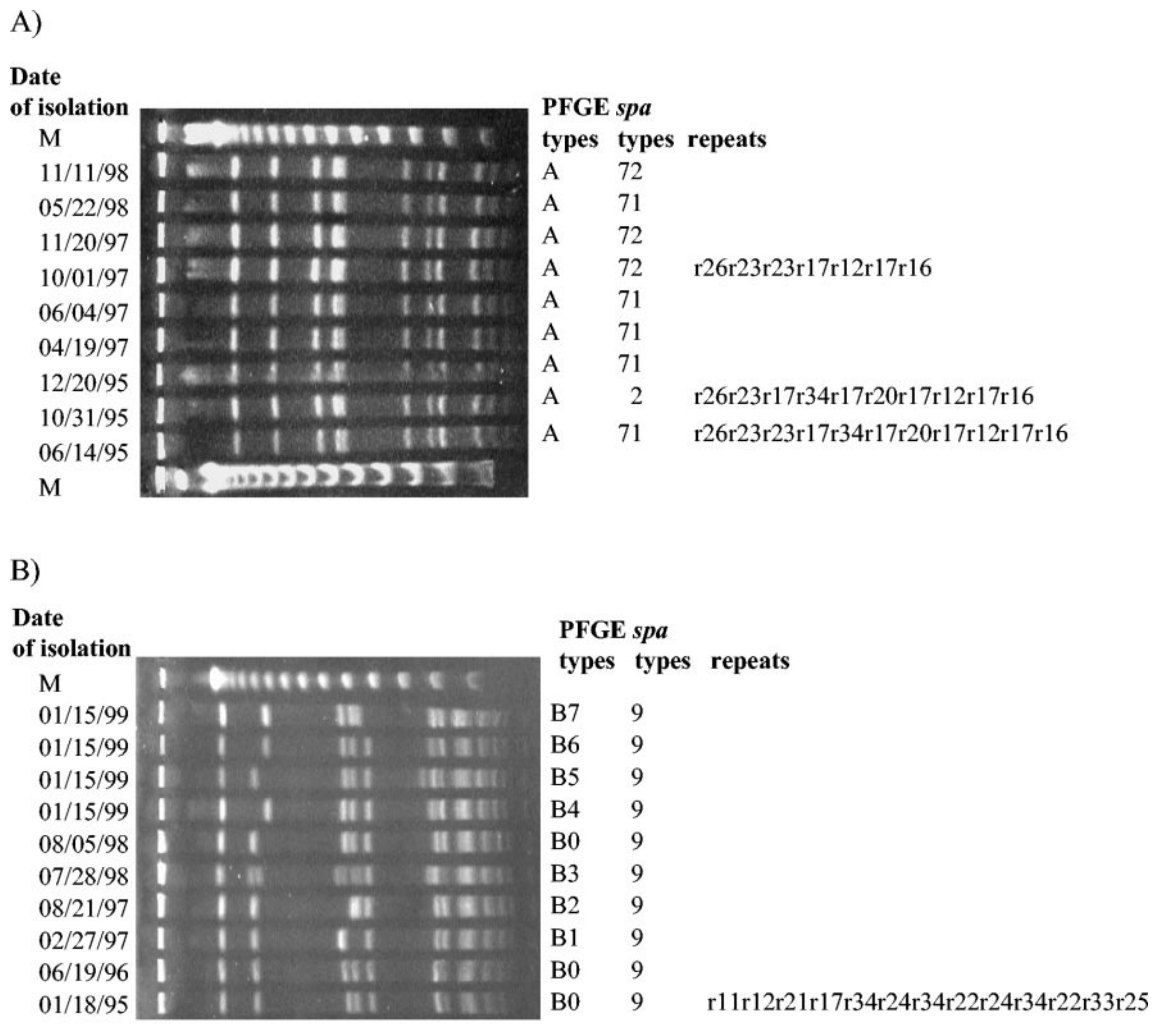

FIG. 1. Comparison of PFGE fragment patterns and *spa* types of consecutive isolates from patients 3 and 9. The dates of isolation (month/day/year) of the strains are shown to the left of the gel. The PFGE types and *spa* types of the strains, including the composition of repeats, are shown to the right of the gel. The marker (M) is a molecular weight marker of concatemers of lambda phage DNA. (A) Identical PFGE fragment patterns for isolates from patient 3. The *spa* type 71 changed to *spa* type 2 by the loss of repeat r23 at position 2 or 3 and to *spa* type 72 by the loss of 4 repeats, i.e., r34r17r20r17 at position 5. (B) Consecutive isolates from patient 9 with minor differences of PFGE fragment patterns indicating that the isolates are closely related. The *spa* types of all isolates were identical.

be drawn from these data are limited compared to the large number of long-term persistent isolates were sequenced in this study.

van Belkum et al. raised concerns about using the *spa* region as an epidemiological marker, because in their analysis of isolates collected from 20 nasal *S. aureus* carriers over time, clonally related strains as determined by phage typing and random amplification of polymorphic DNA analysis displayed heterogeneous numbers of *spa* repeats (11). These researchers concluded that the *spa* region may behave in a hypervariable, unstable manner which is unrelated to the overall evolution of the *S. aureus* genome. Unfortunately, these researchers did not sequence the *spa* region to provide information of the kind of variability that they found in their isolates. It is likely that the results in their study would parallel the results that we found in our analysis.

Using *spa* typing on isolates collected during a longitudinal study, it was possible for the first time to monitor evolution of the *spa* region during long-term persistence of *S. aureus* in the host. The changes that occurred in the *spa* region resulted in different *spa* types of consecutive isogenic or closely related strains as determined by PFGE. The different *spa* types of consecutive isolates consisted of highly similar repeat profiles, which could be explained by loss or gain of repeats and point mutations, thus confirming the hypothesis of Koreen et al. that strains with similar repeat profiles are closely related (7). Furthermore, our study enabled us to determine the rate of genetic change of this region. Although the rather high rate of variability in the *spa* region as demonstrated for the CF isolates may not be generalized to the frequency and persistence of *S. aureus* because of the special ecological niche and selective pressure present in the airways of CF patients, these data may be useful in modeling the evolution of the polymorphic *spa* region of *S. aureus*.

TABLE 2. Analysis of *spa* types of consecutive isolates of *S. aureus* from individual patients over time

Patient[a]	*spa* type[b] of consecutive isolates from individual patients at the indicated month:																								
	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60	63	66	69	72	75
1	8				8			8		8															
								8		8															
2	15	15				15					15		15				15				15				
	15					15					50		15								50				
						15					50		15												
3	71	2		71	71	71			72		71		71												
	71	71		71		71							72												
4	154			154	154		154						154		154					154	154				
																				154	154				
5	84		84	84		84		84	84		84	84	85												
			84	84							84														
6	80					80				80				80			80	80							
	80													80			80	80							
																		80							
7	91	91		91	250	91	91	91	91	91		91	91		91										
	91			91		91	91	91		91		91	91		91										
						91		91				91													
								91																	
8	78	78			78			78		78	78		78		78		78						78	78	78
					78			78		87					79		78						79		78
								78		78					79		78								
															79										
9	9					9			9		9				9		9								
						9			9		9				9		9								
											9						9								
											9						9								
10	91	91	91	91			91		91		91	91				91									
		91	91																						

[a] Individual CF patients.
[b] Numerical *spa* types.

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REFERENCES

1. **Brigido Mde, M., C. R. M. Baradi, C. A. Bonjardin, C. L. S. Santos, M. L. Junqueira, and R. R. Brentani.** 1991. Nucleotide sequence of a variant protein A of *Staphylococcus aureus* suggests molecular heterogeneity among strains. J. Basic Microbiol. **31:**337–345.
2. **Enright, M. C., N. P. Day, C. E. Davies, S. J. Peacock, and B. G. Spratt.** 2000. Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of *Staphylococcus aureus*. J. Clin. Microbiol. **38:**1008–1015.
3. **Frenay, H. M., A. E. Bunschoten, L. M. Schouls, W. J. Van Leeuwen, C. M. Vandenbroucke-Grauls, J. Verhoef, and F. R. Mooi.** 1996. Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. Eur. J. Clin. Microbiol. Infect. Dis. **15:**768–770.
4. **Goerke, C., S. Mathias y Papenberg, S. Dasbach, K. Dietz, R. Ziebach, B. C. Kahl, and C. Wolz.** 2004. Increased frequency of genomic alterations in *Staphylococcus aureus* during chronic infection is in part due to phage mobilization. J. Infect. Dis. **189:**724–734.
5. **Harmsen, D., H. Claus, W. Witte, J. Rothgänger, H. Claus, D. Turnwald, and U. Vogel.** 2003. Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting using novel software for *spa* repeat determination and database management. J. Clin. Microbiol. **41:**5442–5448.
6. **Kahl, B. C., A. Duebbers, G. Lubritz, J. Haeberle, H. G. Koch, B. Ritzerfeld, M. Reilly, E. Harms, R. A. Proctor, M. Herrmann, and G. Peters.** 2003. Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. J. Clin. Microbiol. **41:**4424–4427.
7. **Koreen, L., S. V. Ramaswamy, E. A. Graviss, S. Naidich, J. M. Musser, and B. N. Kreiswirth.** 2004. *spa* typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. J. Clin. Microbiol. **42:**792–799.
8. **Renders, N., L. Licciardello, C. IJsseldijk, M. Sijmons, L. van Alphen, H. Verbrugh, and A. van Belkum.** 1999. Variable numbers of tandem repeat loci in genetically homogenous *Haemophilus influenzae* strains alter during persistent colonisation of cystic fibrosis patients. FEMS Microbiol. Lett. **173:**95–102.
9. **Shopsin, B., M. Gomez, S. O. Montgomery, D. H. Smith, M. Waddington, D. E. Dodge, D. A. Bost, M. Riehman, S. Naidich, and B. N. Kreiswirth.** 1999. Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. J. Clin. Microbiol. **37:**3556–3563.
10. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
11. **van Belkum, A., N. Riewerts Eriksen, M. Sijmons, W. van Leeuwen, M. VandenBergh, J. Kluytmans, F. Espersen, and H. Verbrugh.** 1996. Are variable repeats in the *spa* gene suitable targets for epidemiological studies of methicillin-resistant *Staphylococcus aureus* strains? Eur. J. Clin. Microbiol. Infect. Dis. **15:**768–769.
12. **van Belkum, A., S. Scherer, L. van Alphen, and H. Verbrugh.** 1998. Short-sequence DNA repeats in prokaryotic genomes. Microbiol. Mol. Biol. Rev. **62:**275–293.