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Thymidine-Dependent *Staphylococcus aureus* Small-Colony Variants Are Associated with Extensive Alterations in Regulator and Virulence Gene Expression Profiles

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Chronic airway infection is a hallmark of cystic fibrosis (CF) and many CF patients are infected persistently by *Staphylococcus aureus*. Thymidine-dependent trimethoprim-sulfamethoxazole (SXT)-resistant *S. aureus* small-colony variants (SCVs), often in combination with isogenic normal *S. aureus* phenotypes, are highly prevalent and persistent in airway secretions of CF patients due to long-term SXT therapy (B. Kahl, M. Herrmann, A. S. Everding, H. G. Koch, K. Becker, E. Harms, R. A. Proctor, and G. Peters, J. Infect. Dis. 177: 1023–1029, 1998). In this report, SCVs were compared to normal *S. aureus* by transcription analysis of important regulator (*sigB*, *sarA*, and *agr*) and virulence (α-hemolysin, *hla*, and protein A, *spa*) genes. Growth curve analyses revealed longer doubling times and lower final densities for SCVs than for normal strains. *sigB* activity was measured by transcription analysis of the *sigB* target gene *asp23*. For nearly all SCVs, expression of all regulators was decreased as assessed by *asp23* reverse transcription-PCR for *sigB* and Northern analysis for *sarA* and *agr*. These results are in agreement with diminished *hla* signals in all SCVs and increased *spa* signals in 5 of 10 SCVs compared to the isogenic normal *S. aureus*. Both supplementation of SCVs with thymidine and activation of the *agr* quorum-sensing system by the supernatant of the isogenic normal strain reversed transcription to almost normal levels. In conclusion, multiple changes in growth characteristics and in regulator and virulence gene expression render SCVs less virulent and allow them to survive in the hostile environment present in the airways of CF patients, thereby illustrating adaptation of the bacteria during long-term persistence.

Staphylococcus aureus is a major human pathogen responsible for a variety of community-acquired and nosocomial infections ranging from mild skin diseases, abscesses, and soft-tissue diseases to life-threatening infections such as osteomyelitis, endocarditis, pneumonia, and sepsis (19). Cystic fibrosis (CF) is characterized by chronic suppurative airway disease with progressive pulmonary insufficiency (16). Depending on the study, 50 to 80% of children and adolescents with CF in particular are chronically colonized or infected by *S. aureus* (4, 5, 9, 13, 15, 23). However, the pathogenesis of persistent *S. aureus* infection in CF patients is complex and not fully elucidated.

The persistence of *S. aureus* in CF and other persistent infections such as osteomyelitis and device-related infections has been associated with the isolation of *S. aureus* small-colony variants (SCVs) (11, 15, 27). In contrast to the normal *S. aureus* phenotype, SCVs grow as tiny, nonpigmented, and nonhemolytic colonies. SCVs (i) produce greatly reduced amounts of α-hemolysin; (ii) persist within host cells in in vitro assays (26); (iii) are auxotrophic for substrates such as menadione, hemin, thiamine, or thymidine; and (iv) can revert to their normal phenotype (1, 28). In a 6-year prospective study, persistence of clonal SCVs and/or normal *S. aureus* has been shown in the airways of CF patients over extended periods, as disclosed by pulsed-field gel electrophoresis (15). From this study, 212 SCVs were available for auxotrophism testing. A total of 122 SCVs were thymidine dependent, and 26 SCVs were both thymidine and hemin dependent. As a result of long-term trimethoprim-sulfamethoxazole (SXT) treatment, SXT-resistant and thymidine-dependent SCVs emerged, while the corresponding normal strains remained SXT susceptible (13). While SXT interferes with the tetrahydrofolic acid pathway, tetrahydrofolic acid acts as a coenzyme for thymidylate synthetase, which catalyzes the synthesis of dTMP from dUMP (25). Since dTMP is essential for DNA synthesis, susceptible *S. aureus* strains are affected by SXT therapy. However, thymidine-dependent SCVs are resistant to SXT and survive if extracellular thymidine is provided. Phenotypically, thymidine-dependent SCVs display two different colony types, (i) fried-egg SCVs with translucent edges surrounding a smaller, elevated pigmented center and (ii) pinpoint colonies, which are nearly 10 times smaller than the normal *S. aureus* colony (14).

S. aureus has the capability to synthesize a variety of extracellular and cell wall-bound proteins, many of which are involved in pathogenesis (22). The expression of most of these virulence factors is under the control of global regulators such as *agr* (accessory gene regulator) (17) and *sarA* (staphylococcal accessory regulator) (7). The *agr* system of *S. aureus* represents a quorum-sensing system. A secreted autoinducing peptide (AIP) induces activation of the *agr* operon at a given threshold,

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thereby up-regulating extracellular protein production while down-regulating synthesis of cell wall-associated proteins, which serve as adhesins for the pathogen to host tissues (17). The *sarA* locus encodes the DNA binding protein SarA, which activates the synthesis of both extracellular and cell wall-associated proteins via *agr*-dependent and *agr*-independent pathways (8). The *sarA* locus is composed of three overlapping transcripts with *sarA* P1 (0.58 kb) and *sarA* P2 (1.15 kb) maximally transcribed during the exponential phase (tapering toward the stationary phase), while *sarA* P3 (0.84 kb) is expressed primarily during the late-exponential and stationary phases (2). Based on transcriptional analysis, the *sarA* P3 promoter has been shown to be *sigB* dependent (10, 20). In contrast to the primary sigma factor SigA, which is responsible for the transcription of housekeeping genes, the alternative sigma factor SigB (*sigB*) plays an important role in regulating gene expression upon environmental stresses (e.g., stationary phase, heat, and osmotic stresses).

While the phenotypic characteristics of clinical and laboratory SCVs have been described by several groups (21, 28, 29), a detailed analysis of alteration in gene regulation putatively contributing to or resulting in the SCV phenotype has not been attempted. Therefore, in this report, we characterized the transcription patterns of important regulator (*sigB*, *sarA*, and *agr*) and virulence genes (*hla* and *spa*) of long-term persisting thymidine-dependent *S. aureus* SCVs isolated from the airways of CF patients in comparison to the normal isogenic strain, which was isolated at the same time.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Ten normal and *S. aureus* SCV strain pairs previously described were used for further analysis (15). These strain pairs were cultured from individual CF patients persistently infected by *S. aureus*. All SCVs were thymidine dependent and clonal to their normal counterpart, as determined by pulsed-field gel electrophoresis as described previously (13). To ensure stability of the SCV phenotype, the first four strain pairs were subcultured at least 10 times in the laboratory. To confirm the transcription patterns of these pairs, which have been repetitively subcultured in the laboratory, six other isogenic thymidine-dependent SCV-normal strain pairs from other patients were chosen for further analysis. These strain pairs were subcultured only once after primary storage at −70°C. Five SCVs displayed the fried-egg phenotype with translucent edges surrounding a smaller, elevated pigmented center; the other five SCVs exhibited pinpoint colonies, which are nearly 10 times smaller than the normal *S. aureus* colony size.

Normal *S. aureus* isolates and SCVs were grown in brain heart infusion (BHI; Merck, Darmstadt, Germany) broth, which supports the growth of SCVs, while *Escherichia coli* was grown in Luria-Bertani broth. *E. coli* InvαF′ (Invitrogen, Karlsruhe, Germany) was used as a host strain for the TA cloning vectors pCRII and pCR 2.1. For the growth curve analysis, cultures were grown at 35°C on a rotary shaker. For the determination of the optical density at 578 nm (OD_{578}) beyond a value of 2, the value of the ODs was calculated from readings of diluted cultures. Ampicillin was used at a concentration of 50 μg/ml. For supplementation studies, thymidine (Fluka Chemie, Buchs, Switzerland) was added to BHI broth at a concentration of 100 μg/ml.

DNA probes. The internal fragments of the following genes were amplified by PCR (Perkin Elmer 2400, Überlingen, Germany): *sarA*, nucleotides (nt) 864 to 1218 (GenBank accession no. U46541) RNAIII, nt 1260 to 1571 (GenBank accession no. X52543); *hla*, nt 583 to 1211 (GenBank accession no. X01645); and *spa*, nt 776 to 1160 (GenBank accession no. X61307). PCR products were cloned into pCRII or pCR 2.1 (Invitrogen), sequenced for control with the ABI PRISM 310 Genetic Analyser (Perkin Elmer, Weiterstadt, Germany), and used as probes, which were generated by the Dig Probe Synthesis kit (Roche, Mannheim, Germany), for Northern blot analysis. Plasmid DNA was extracted with the QIAprep Spin Mini Kit (QIAGEN, Hilden, Germany).

Isolation of RNA and Northern analysis. Prior to RNA extraction, growth curves were performed for normal isolates, SCVs, and SCVs supplemented with thymidine. RNA was extracted according to early log, late log, and stationary phases for the particular strains. Overnight cultures of normal colonies and *S. aureus* SCVs were set to 0.1 at an optical density at 578 nm (OD_{578}) in BHI and were then allowed to grow to the respective growth phases. For supplementation of SCVs, 100 μg/ml thymidine was added to the BHI broth.

The cells were pelleted, and total cellular RNA was isolated with the FastPrep system (Fast Prep FP120 instrument; Qbiogene, Heidelberg, Germany) (2). Since SCVs have a tendency to revert to normal growth, the growth of SCVs after the shaking liquid culture was controlled by streaking the respective cultures onto Columbia blood agar plates, which were incubated for 24 h at 35°C. The extracted RNA was processed further only if the SCVs did not revert to the normal phenotype or if <30% of the cultures reverted. Ten micrograms of RNA was electrophoresed through a 1.5% 0.66 M formaldehyde gel in morpholinepropanesulfonic acid (MOPS) running buffer (20 mM MOPS, 10 mM sodium acetate, 2 mM EDTA [pH 7.0]). Every gel was controlled for equal loading of RNA. RNA was transferred onto a Hybond N+ membrane (Amersham Pharmacia Biotech Europe GmbH, Braunschweig, Germany) under mild alkaline conditions with a turboblotter system (Schleicher and Schuell, Dassel, Germany). RNA was fixed to the membrane by baking (80°C for 0.5 h), hybridized under aqueous conditions at 65°C overnight with the digoxigenin-labeled DNA probes, washed, and detected by chemiluminescence (Roche, Mannheim, Germany). One fried-egg SCV and one pinpoint SCV with their respectie isogenic normal strains were chosen for optimal representation and reproducibility of the transcription patterns of the different tested strain pairs.

***asp23* transcription by reverse transcription-PCR.** For RNA isolation from culture, *S. aureus* was grown in BHI medium to the desired growth phase and controlled for potential reversion of the phenotype as described above. Bacteria were mechanically disrupted (Fast Prep FP120 instrument; Qbiogene, Heidelberg, Germany), and RNA was isolated (RNeasy mini kit; QIAGEN, Hilden, Germany). After treatment with RNase-free DNase I (QIAGEN), total RNA samples were amplified in an ABI PRISM 7000 SDS with SYBR Green PCR Master Mix (Applied Biosystems, Weiterstadt, Germany) and *gyrB* primers (gyrB f1, 5′-GACTGATGCCGATGTGGA-3′; gyrB r1, 5′-AACGGTGGCTGTGCA ATA-3′) to check for the absence of genomic DNA. Previously transcribed cDNA served as a positive control. RNA was then reverse transcribed (High Capacity cDNA Archive kit; Applied Biosystems). cDNA was used for real-time amplification with *asp23*-specific primers (asp23 fl, 5′-CAAGAACAAAATCAA GAGCCTCAAT-3′; asp23 r1, 5′-CTTCACGTGCAGCGATACCA-3′) and 100 ng of cDNA per reaction mixture. The level of mRNA expression of *asp23* was normalized against the internal control *gyrB* expression, which is constitutively expressed (12). The amount of *asp23* transcript was expressed as the *n*-fold expression difference of *asp23* relative to the expression of the control gene *gyrB* ($2^{-\Delta CT}$, where ΔCT represents the difference in threshold cycle between the target and control genes). All determinations were performed in duplicate.

***agr* activation of *S. aureus* SCVs by the isogenic normal *S. aureus*.** For the activation of SCVs by the AIP of the isogenic normal strain, the culture supernatant (SN) from the normal strain was prepared after overnight culture and sterile filtration. Bacteria were inoculated from an overnight culture to an initial OD_{578} of 0.1 in BHI and grown for 1 h at 35°C. One-tenth of the sterile SN or 1/10 of BHI (as a control) was added to 9/10 of the culture, after which the bacteria were allowed to grow for another 2 h or 4 h. RNA was prepared as described above. The activity of *agr* was assessed by Northern blot analysis with an RNAIII probe as described above.

RESULTS

SCVs have an extended lag phase and do not reach the densities of normal strains. For each normal colony/SCV strain pair, growth curves were analyzed. Five of the tested SCVs belonged to the fried-egg phenotype, while the other five SCVs exhibited pinpoint colonies (Table 1). All SCVs differed in their growth phase characteristics from normal strains (Fig. 1A and 2A) by extended lag phases (mean difference from the normal *S. aureus* colony, 2.85 h; range, 1 to 6 h) and lower final densities (mean OD at 578 nm [OD_{578}], 4.5; range, 2 to 8 compared to a mean OD_{578} of 12.3; range, 9 to 14 for the normal *S. aureus* colony) (Table 1). The maximum densities for pinpoint SCVs were even lower than for fried-egg SCVs (Table 1; Fig. 1A and 2A). If SCVs were supplemented with thymi-

TABLE 1. Results of growth and transcriptional analysis of 10 thymidine-dependent SCV-normal *S. aureus* strain pairs isolated from airway secretions of CF patients

No.	Phenotype	SCV type	OD_{578} maximum[a]	Hemolysis[b]	Pigment[c]	Northern analysis[d]												RT-PCR[e]		
						RNAIII			*sarA* P3			*hla*			*spa*			*asp23*		
						el	ll	sp	el	ll	sp	el	ll	sp	el	ll	sp	el	ll	sp
1	Normal		9	−	Y[f]	−	−	−	−	−	+/−	+/−	+	++	++	+	+	1.3	2.7	8.0
	SCV	FE[g]	5	−	W[h]	−	−	−	−	−	−	+/−	−	−	++	+	+	1.0	0.8	0.6
2	Normal		12	++	Y	+/−	+++	+++	−	++	++	+	+++	+++	++	+	++	2.7	9.9	18.9
	SCV	FE	5	+/−	W	−	−	−	−	−	−	+	+	++	++	++	++	1.1	ND[j]	23.9
3	Normal		13	++	Y	++	+++	+++	−	++	++	+	+++	+++	−	−	−		ND	
	SCV	PP[i]	3	−	W	+/−	+/−	+/−	+	+/−	+	−	−	−	−	−	−		ND	
4	Normal		14	+/−	W	+/−	+++	+++	+	++	++	+	++	+++	+++	++	+	1.0	7.0	6.7
	SCV	PP	2	+/−	W	−	−	−	++	++	++	+	+	++	+++	+++	++	0.6	2.4	2.6
5	Normal		12	++	Y	+	+++	+++	+	++	++	+	++	++	++	+	−	0.2	5.0	1.5
	SCV	FE	8	+	W	+/−	+	+/−	+/−	+/−	+/−	+	+/−	+/−	++	++	++	0.1	0.4	0.7
6	Normal		14	++	Y	−	+/−	+/−	−	+	++	++	++	++	++	+++	+	3.6	67.2	43.3
	SCV	FE	6	+/−	W	++	+/−	+/−	−	−	+/−	+	−	−	+	+	+/−	1	0.8	1.7
7	Normal		11	++	Y	++	+++	+++	+	+	++	+	+++	+++	++	+/−	−	1.0	4.3	3.5
	SCV	PP	6	+/−	W	++	ND	+/−	+	ND	+	+	ND	+	++	ND	++	2.0	1.8	0.5
8	Normal		14	−	W	−	+/−	+	+	++	++++		++	+++	+++	+++	++	1.5	12.1	16.6
	SCV	FE	6	−	W	−	−	−	+	−	+/−	+/−	+/−	+/−	++	++	++	0.9	2.7	1.4
9	Normal		12	++	W	+	+	++	++	++	++	++	+++	+++	++	+	+	8	129.8	178
	SCV	PP	2	−	W	−	−	−	+	+	+	+	++	++	+++	+++	+++	18.9	99.9	35
10	Normal		12	++	Y	+	+++	+++	+/−	+	++	+	+++	+++	+++	++	−	3.2	11.9	3.7
	SCV	PP	2	−	W	−	−	−	+/−	+/−	+	+/−	+/−	−	++	+++	+++	0.5	1.9	1.4

[a] Maximum OD_{578} during growth curve analysis.
[b] Hemolysis on Columbia blood agar (sheep blood). Hemolysis was determined as follows: −, none; +/−, weak; +, median; ++, strong.
[c] Pigmentation of *S. aureus* colonies on Columbia blood agar.
[d] Northern analysis of RNA extracted at early log (el), late log (ll) and stationary phase (sp). Blots were probed with digoxigenin-labeled gene probes for RNAIII, *sarA, hla*, and *spa*. Signals were detected by chemiluminescence and characterized as follows: −, none; +/−, weak; +, median; ++, strong; +++, very strong.
[e] Fold increase of *asp23* over the constitutively expressed gyrase.
[f] Y, yellow pigment of *S. aureus* on Columbia blood agar.
[g] FE, SCV with fried-egg appearance.
[h] W, white pigment of *S. aureus* on Columbia blood agar.
[i] PP, SCV with pinpoint appearance.
[j] ND, not done.

dine, the bacteria yielded higher densities than nonsupplemented SCVs. There was a difference in growth after supplementation between the fried-egg and pinpoint phenotypes. While the maximum density of most fried-egg SCVs was reached within <12 h (four of five SCVs) upon supplementation with thymidine, the maximum optical density of all pinpoint SCVs (five of five) was not reached within this period and was determined for two pinpoint SCVs at 19 h, as shown in Fig. 2A for one particular pinpoint SCV.

Expression of *sigB*, *sarA*, and *agr* is decreased in SCVs. As SigB represents an alternate sigma factor, which reacts to environmental stress especially in response to growth phase, heat shock, or starvation, we evaluated transcription of this important regulator. Since the activity of SigB is determined by the amount of free SigB dissociated from its anti-sigma factor RsbW, SigB activity is best determined by expression of *asp23*, a SigB target gene (3). *asp23* transcription was expressed as fold increase compared to the expression of the constitutively transcribed *gyrB* gene. The expression of *asp23* was decreased in eight of nine SCVs tested, compared to isogenic normal strains (Table 1). Only SCV 2 expressed nearly the same levels of *asp23* as the normal strain. Interestingly, there was a high rate of variation of *asp23* activity among the different normal strains, ranging from 1.5- to 178-fold increase over *gyrB* gene expression during the stationary phase of normal strains. Since *sigB* activity is important for the pigmentation of *S. aureus*, we determined the color of the strains grown on Columbia blood agar plates (Table 1). Seven of 10 SCVs displayed fewer pigmented colonies than the normal strains. These differences in pigmentation between normal and SCVs were consistent with low levels of *asp23* in SCVs. However, normal strain 9, which had the highest activity of *asp23*, displayed white colonies on the agar plate, indicating that additional factors also have an impact on pigmentation (Table 1). The *asp23* activity of nine SCVs was additionally tested by supplementing the medium with 100 μg/ml of thymidine. Five of these SCVs were fully restored and three were partially restored in their *asp23* activity, while only one SCV was not supplemented (data not shown).

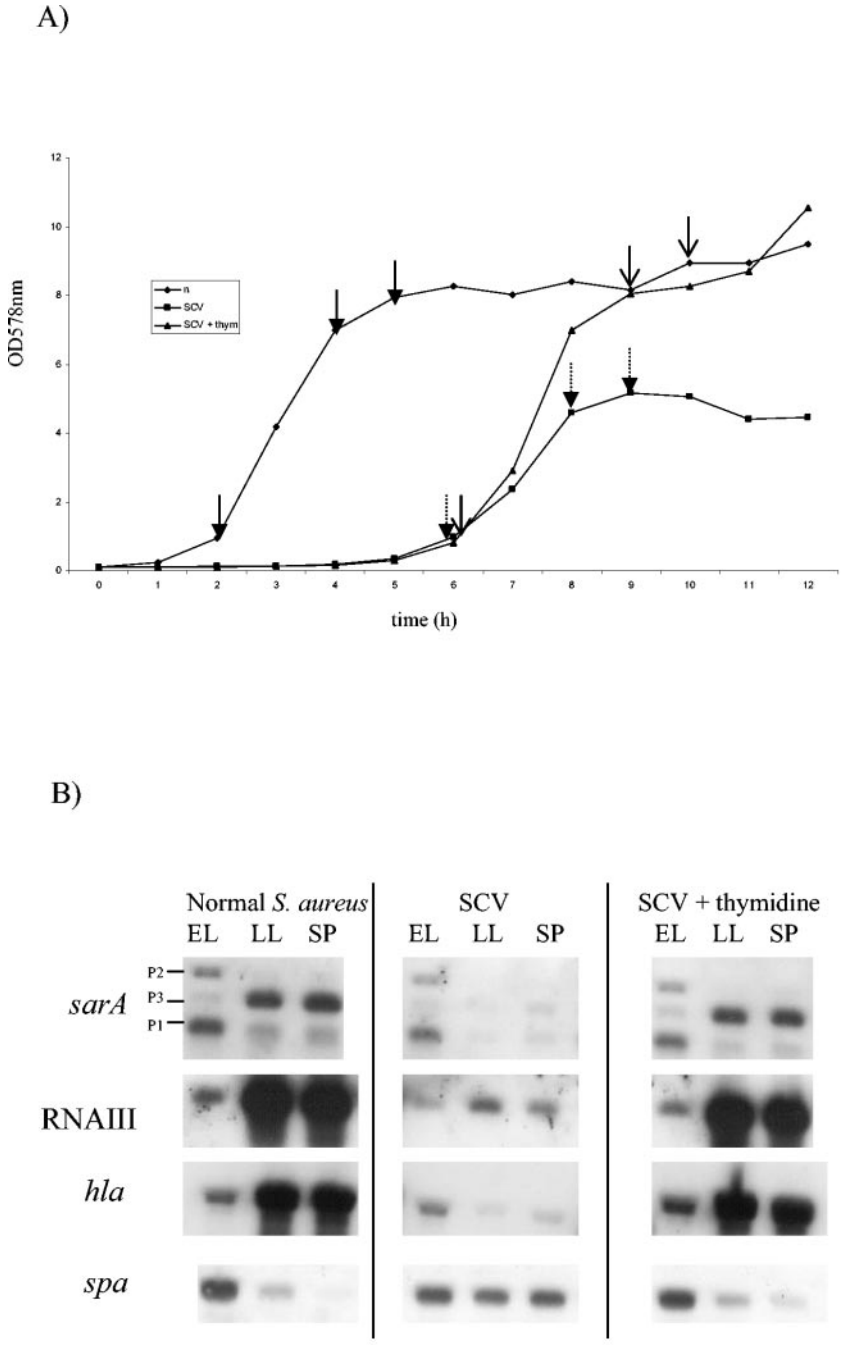

FIG. 1. Growth phase analysis and transcription profiles of a normal *S. aureus* strain, the isogenic thymidine-dependent SCV with fried-egg appearance, and the SCV supplemented with thymidine. (A) For growth phase analysis (strain pair 1), bacteria were cultured in BHI or BHI supplemented with 100 μg/ml thymidine on a rotary shaker at 37°C. Every hour, bacteria were harvested and the optical density at 578 nm was determined. (B) Transcription analysis of *sarA*, RNAIII, *hla*, and *spa* of the normal *S. aureus* strain 5, its isogenic thymidine-dependent SCV with fried-egg appearance, and the SCV supplemented with 100 μg/ml thymidine, representative for typical transcription patterns as found in other SCV-normal strain pairs with fried-egg appearances. RNA was harvested at early log phase (EL), late log phase (LL), and stationary phase (SP). Ten micrograms of total RNA was applied to each lane.

To assess the activity of the *sarA* regulon in SCVs versus normal strains, we analyzed *sarA* transcription by Northern blot analysis. The normal clinical strains displayed quite typical *sarA* transcription patterns: *sarA* P1 and *sarA* P2 were maximally expressed during early log phase, declining towards stationary phase, while *sarA* P3 was most active during stationary phase (Fig. 1B and 2B). *sarA* P3 expression of the SCVs was not detectable or maximal during stationary phase and always decreased compared to the level of the normal *S. aureus* (Fig. 1B and 2B) in 9 of 10 SCVs tested (Table 1). Only SCV 4

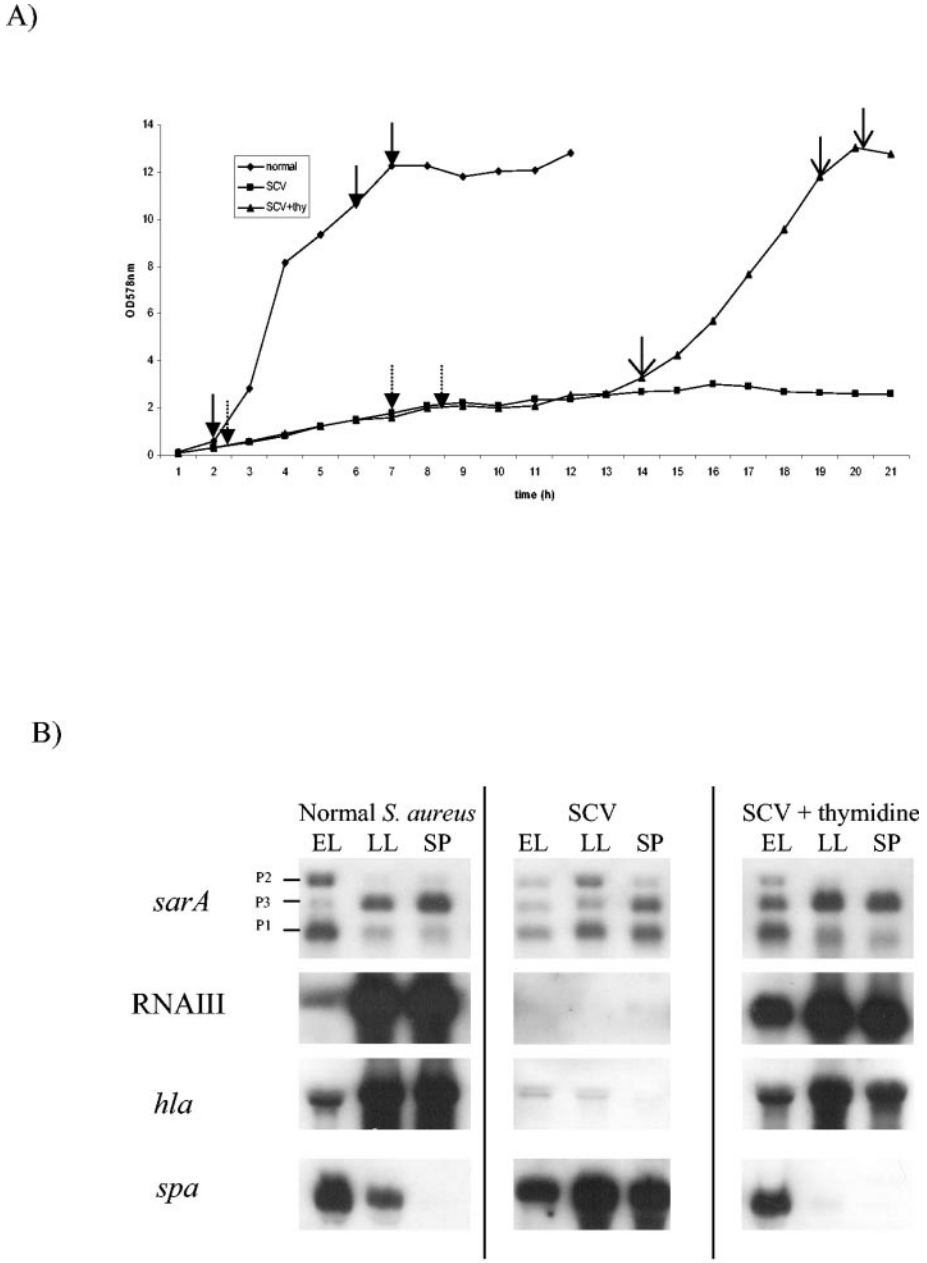

FIG. 2. Growth phase analysis and transcription profiles of a normal *S. aureus* strain, the isogenic thymidine-dependent SCV with pinpoint phenotype, and the SCV supplemented with thymidine (strain pair 10). (A) For growth phase analysis, bacteria were cultured in BHI or BHI supplemented with 100 μg/ml thymidine on a rotary shaker at 37°C. Every hour, bacteria were harvested and the optical density was determined at 578 nm. (B) Transcription analysis of *sarA*, RNAIII, *hla*, and *spa* of a normal *S. aureus* strain, the isogenic thymidine-dependent SCV with pinpoint phenotype, and the SCV supplemented with 100 μg/ml thymidine, representative for typical transcription patterns as found in other pinpoint SCV-normal strain pairs. RNA was harvested at early log, late-log, and stationary phases. Ten micrograms of total RNA was applied to each lane.

displayed the same *sarA* P3 transcription level as the normal *S. aureus* (Table 1). The differential effect of SCVs and normal *S. aureus* phenotypes was less apparent on *sarA* P1 and *sarA* P2 transcription. Upon supplementation with thymidine, transcription for *sarA* P3 was partially restored in two of seven SCVs and fully restored in five of seven SCVs (Fig. 1B and 2B).

To determine the activity of the *agr* regulon, which is partly dependent on *sarA* activity, we consecutively used an RNAIII probe for Northern analysis. While *agr* transcription was highest in late exponential and stationary phases in normal *S. aureus* strains, *agr* transcription was absent or decreased in eight of nine SCVs (Fig. 1B and 2B). SCV 6 displayed a stronger RNAIII transcript than the normal strain 6, whereas no RNAIII transcript was detectable for either phenotype of strain pair 1 (Table 1). In four of seven SCVs supplemented with thymidine, RNAIII transcription was fully restored, as

shown in the representative strain pairs (Fig. 1B and 2B) and partially restored in SCV 2 (data not shown). Upon thymidine supplementation of SCV 6, which displayed a higher level of RNAIII transcript than the normal *S. aureus*, the RNAIII transcript was highly expressed during all growth phases (data not shown).

α-Hemolysin and protein A transcription are altered in SCVs compared to normal *S. aureus*. The presence and transcription of *sarA*- and *agr*-dependent virulence genes such as *hla* and *spa* were analyzed. Despite the presence of the α-hemolysin gene (*hla*) in all studied SCVs and normal strains as determined by Southern blot analysis (data not shown), transcription of *hla* was found to be significantly lower in all SCVs than in the normal cohort strains (Table 1; Fig. 1B and 2B). As anticipated, transcription of *hla* was maximal in late logarithmic and stationary phases in normal strains, while transcription of *hla* was decreased or almost absent in all SCVs (Fig. 1B and 2B; Table 1). Upon supplementation with thymidine, *hla* expression was fully restored in all SCVs (Fig. 1B and 2B).

Southern blot analysis disclosed the presence of *spa* in all strains (data not shown). In exponential growth phase, transcription of *spa* in SCVs was comparable to the normal strains (Fig. 1B and 2B). *spa* transcription of the normal strains declined towards late log phase, and no *spa* signal was detectable during stationary phase. In contrast, *spa* signals of many SCVs increased toward late log phase and were still present or even increased during stationary phase in five of nine SCVs (Fig. 1B and 2B). In SCVs 6 and 8, *spa* signals were weaker than in the normal strains, while in SCVs 1 and 2 no difference in *spa* transcription between the SCV and normal phenotype was apparent (Table 1). Strain pair 3 did not express *spa* (Table 1). The transcription results concerning *spa* in SCV and normal strain pairs were consistent with the expression of Spa, as determined by Western blot analysis after extraction of cell wall-associated proteins, which were probed with chicken anti-protein A antibodies (data not shown). Upon supplementation with thymidine, transcription of *spa* was fully restored for SCVs 5 and 10 (Fig. 1B and 2B) and partially restored for SCV 7 (data not shown). No difference in transcription was seen in SCVs 1, 2, and 8. In strain pair 6 with the enhanced *spa* signal in the normal strain, the thymidine-supplemented SCV expressed *spa* only during the early log phase, while the SCV expressed *spa* at all time points.

***agr* activation of an SCV by the AIP of the normal *S. aureus* strain.** At a certain threshold, *S. aureus* secretes an AIP growth phase dependently into the medium to communicate and synchronize the expression of virulence genes. During growth phases, SCVs reached only low cell densities. Therefore, we hypothesized that the threshold for the activation of the *agr* regulon may not be reached in SCVs. Accordingly, we analyzed whether the AIP of the normal strain is able to activate the *agr* response of SCVs. To test this, the SN of an isogenic normal *S. aureus* was prepared after overnight culture and added to an early growth phase culture of normal and *S. aureus* SCVs, which were further grown in the presence or absence of the AIP. The activity of *agr* was determined by Northern blot analysis. While no RNAIII signal was detected after 2 h or 4 h in the SCV grown in BHI only, the addition of the AIP of the normal strain activated the *agr* response of the SCV strain at 2 h and 4 h (Fig. 3), indicating a potentially functional *agr* locus.

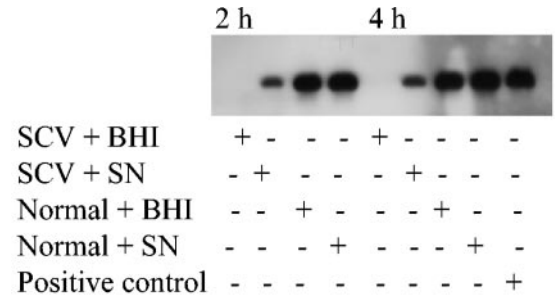

FIG. 3. *agr* activation of SCV strains by the AIP of the isogenic normal *S. aureus* strain. To determine if the *agr* regulon of SCVs can be activated by the AIP of the isogenic normal *S. aureus*, the supernatant of the normal strain was prepared after overnight culture and added to SCV and to normal growth cultures at the indicated time points. As a control, BHI broth alone was added to SCV and normal cultures.

DISCUSSION

Thymidine-dependent SCVs are highly prevalent and persistent in airway secretions of CF patients if the patients are treated or have been treated by long-term SXT (11, 13, 15). In this report, we demonstrated profound alterations of growth characteristics and regulator and effector gene expression in clinical thymidine-dependent SCVs compared to their isogenic normal *S. aureus* strains isolated at the same time. Both fried-egg and pinpoint SCV phenotypes displayed slower growth curves than the isogenic normal *S. aureus* strains. Interestingly, the two phenotypes of SCVs could be distinguished not only by their different morphotypes but also by their growth characteristics, because pinpoint SCVs had even longer doubling times and reached much lower densities than the fried-egg SCVs. Additionally, differences between these phenotypes were also obvious upon supplementation with thymidine, since the growth curves of most fried-egg SCVs reverted earlier to the growth characteristics of a normal phenotype upon supplementation than the pinpoint SCVs, probably due to faster growth and earlier uptake of external thymidine. However, transcription profiles of both phenotypes were comparable, depending on the respective background of the normal strain.

The alternative sigma factor SigB represents a powerful regulatory effector in response to environmental stress (18). Using *asp23* transcription as a marker for *sigB* activity, we assessed *sigB* activity of SCVs versus isogenic normal *S. aureus* strains. Since environmental changes such as long-term antibiotic therapy have resulted in the SCV phenotype, we expected an increased stress response in SCVs. In contrast to our expectations, the expression of *sigB* was decreased compared to the normal *S. aureus* strains, probably due to the action of other upstream-located regulators or as an indication that limited sources of thymidine, which are crucial for the survival of thymidine-dependent SCVs, are not sensed by *sigB*. Low levels of *sigB* expression were consistent with the nonpigmentation of SCVs in comparison to the normal *S. aureus*. The *sarA* P3 promoter has been shown to be under the control of *sigB* (2, 10, 20). The regulatory network of the *sigB-sarA-agr* regulators is very complex. While Chien et al. have shown that functional SarA controls *agr* expression (8), Bischoff et al. demonstrated that *sigB* increases *sarA* expression while RNAIII levels were

reduced simultaneously (3). The data of these two groups indicate that *sigB* has *sarA*-dependent as well as *sarA*-independent effects upon *agr* transcription. Our data are consistent in view of the *sigB-sarA* hierarchy resulting in a decreased level of *sarA* P3 transcription, presumably due to low *sigB* activity in SCVs compared to the normal *S. aureus* strains, as shown for 9 of 10 SCVs. Furthermore, low levels of *agr* transcription in SCVs are consistent with low levels of *sarA* P3 expression. Bischoff et al. demonstrated that low *sigB* transcription resulted in up-regulation of *agr*. However, *agr* transcription in SCVs was almost absent, indicating *sigB*-independent regulation of *agr* in SCVs.

The fact that thymidine supplementation of SCV 6 resulted in enhanced RNAIII transcription compared to the very weak RNAIII transcript present in the respective isogenic normal strain, which was isolated at the same time, suggests that this SCV emerged earlier from an isogenic normal strain, which presumably displayed strong *agr* activity. Interestingly, the isogenic normal strain, which was isolated at the same time as the SCV, apparently underwent changes of the *agr* regulon during long-term persistence, resulting in an almost negative *agr* status. Changes in the *agr* regulon have been shown to occur during serial passages in vitro, due to mutations in the nucleotide sequence of *agr* (24). It is conceivable that such changes also occur due to long-term persistence of *S. aureus* in the host. The assumptions that the SCV emerged from an earlier normal strain with an active *agr* regulon are also consistent concerning the *spa* transcription, because after supplementation of the SCV, the *spa* transcript was strongest during the early log phase and not present in the later growth phases (in contrast to the *spa* transcript of the isogenic normal strain isolated at the same time, which also expressed a strong signal during stationary phase).

As with SCVs associated with hemin and/or menadione auxotrophy (1, 28), thymidine-dependent SCVs do not readily produce α-hemolysin on Columbia agar (14). Although *hla* was present in SCVs as confirmed by Southern blotting, the expression of *hla* was strongly down-regulated in SCVs at the transcriptional level. These results are not surprising, considering the decrease in *sarA* activity, as well as in *agr* activity, which concertedly controls *hla* expression. Although it has been shown that *sigB* affects *hla* transcription, resulting in increased hemolysis in a *sigB* mutant strain, low levels of *sigB* activity in SCVs were not associated with a hemolytic phenotype (6), indicating *sigB*-independent regulation of *hla* in these SCVs.

Taken together, thymidine-dependent SCVs display an *agr* mutant phenotype in light of an inactive *agr* locus (RNAIII) associated with increased *spa* transcription and decreased *hla* transcription. Interestingly, upon thymidine supplementation of SCVs, transcription of the *agr* locus and the *agr*-dependent virulence genes *spa* and *hla* were fully or partially restored in most SCVs, confirming that the *agr* locus was potentially functional in SCVs. Moreover, it was possible to activate the *agr* response of SCVs by the AIP of the isogenic normal *S. aureus* strain, suggesting that the *agr* system was inactive, probably due to the decreased growth rate and low density of AIP present in SCVs. The fact that *sigB* and *sarA* expression, which is independent of *agr*, was also restored after thymidine supplementation points to other regulatory mechanisms responsible for the various alterations that occur in thymidine-dependent SCVs.

In conclusion, multiple changes in growth characteristics and in regulator and virulence gene expression render SCVs less virulent and allow them to survive in the hostile environment present in the airways of CF patients, thereby illustrating adaptation of the bacteria during long-term persistence.

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