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In Vivo Mutations of Thymidylate Synthase (Encoded by *thyA*) Are Responsible for Thymidine Dependency in Clinical Small-Colony Variants of *Staphylococcus aureus*[▽]

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Trimethoprim-sulfamethoxazole (SXT)-resistant *Staphylococcus aureus* thymidine-dependent small-colony variants (TD-SCVs) are frequently isolated from the airways of cystic fibrosis (CF) patients, often in combination with isogenic normal strains if patients were treated with SXT for extended periods. As SXT inhibits the synthesis of tetrahydrofolic acid, which acts as a cofactor for thymidylate synthase (*thyA*), the survival of TD-SCVs depends exclusively on the availability of external thymidine. Since the underlying mechanism for thymidine dependency is unknown, we investigated if alterations in the *thyA* nucleotide sequences were responsible for this phenomenon. Sequence analysis of several clinical TD-SCVs and their isogenic normal strains with reference to previously published *S. aureus thyA* nucleotide sequences was performed. Three clinical TD-SCVs were complemented by transforming TD-SCVs with the vector pCX19 expressing ThyA from *S. aureus* 8325-4. Transcriptional analysis of metabolic and virulence genes and regulators (*agr*, *hla*, *spa*, *citB*, *thyA*, and *nupC*) was performed by quantitative reverse transcription-PCR. The previously published sequences of *thyA* and two normal clinical strains were highly conserved, while *thyA* of four normal strains and four SCVs had nonsynonymous point mutations. In 8/10 SCVs, deletions occurred, resulting in stop codons which were located in 4/10 SCVs close to or within the active site of the protein (dUMP binding). Complementation of TD-SCVs with *thyA* almost fully reversed the phenotype, growth characteristics, and transcription patterns. In conclusion, we demonstrated that mutations of the *thyA* gene were responsible for the phenotype of TD-SCVs. Complementation of TD-SCVs with *thyA* revealed that a functional ThyA protein is necessary and sufficient to change the SCV phenotype and behavior back to normal.

Cystic fibrosis (CF) is one of the most common hereditary diseases in the Caucasian population. Patients with CF suffer from chronic suppurative airway infections, which eventually lead to progressive lung insufficiency and premature death. Only a few bacterial species are associated with CF (17). Interestingly, there is an age-related sequence of colonizing and infecting pathogens (6). In most patients, during early infancy and adolescence the leading pathogens are *Staphylococcus aureus* and *Haemophilus influenzae*, which are later replaced by *Pseudomonas aeruginosa*, which, especially in its mucoid form, indicates deterioration of the disease (17).

Colonization and infection with *S. aureus* are usually monoclonal in CF patients (14). The same clone can be isolated for months or years in spite of appropriate antibiotic treatment. Due to the hostile environment and repeated exposure to antibiotics, especially during long-term treatment with trimethoprim-sulfamethoxazole (SXT), in CF patients, particularly SXT-resistant, thymidine-dependent small-colony variants (TD-SCVs) emerge (2, 8, 11). Menadione-dependent SCVs, hemin-dependent SCVs, and TD-SCVs have been recovered from patients with other chronic infections, such as osteomyelitis (21). Compared to normal *S. aureus*, SCVs produce much smaller pinpoint or fried-egg colonies, are less pigmented, and are less hemolytic on conventional Columbia blood agar plates (13). The transcription patterns of important virulence regulators and metabolic and stress-related genes of TD-SCVs are dramatically changed, and these strains have a less virulent but more persistent phenotype (12) with decreased expression of α-hemolysin and increased expression of cell wall-associated proteins, such as protein A. Moreover, SCVs are more resistant to antibiotics and defensins (21, 22, 24) and survive intracellularly in various cell types, such as keratinocytes and epithelial and endothelial cells (11, 32, 33). The intracellular locus provides protection against antibiotic treatment and host defense.

Recently, we showed that TD-SCVs exhibit superior stationary-phase survival during long-term culture compared to normal *S. aureus* due to a lack of a functional tricarboxylic acid (TCA) cycle (5). Reversible inactivation of the TCA cycle in wild-type organisms has been suggested to be a survival strategy used to circumvent oxidative stress induced during host-pathogen interactions (29).

Many bacteria depend on the synthesis of tetrahydrofolic acid (THF). THF is a required cofactor for the conversion of dUMP to dTMP by thymidylate synthase (encoded by *thyA*), which is essential for DNA synthesis. TMP interferes with THF

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TABLE 1. Bacterial strains used in this study

Strain(s) or vector	Genotype, relevant characteristics, or nucleotide sequence	Reference
S. aureus strains		
8325-4	Wild-type standard laboratory strain	20
Normal-1 to Normal-4	Phenotypically normal *S. aureus* strains isolated from airway secretions of four individual CF patients	12
SCV-1 to SCV-4	SCV *S. aureus* strains isolated from airway secretions of four individual CF patients	12
Normal-5 to Normal-6	Phenotypically normal *S. aureus* strains isolated from airway secretions of two individual CF patients	This study
SCV-5 to SCV-8	SCV *S. aureus* strains isolated from airway secretions of four individual CF patients	This study
SCV/pCX19*thyA*	Clinical TD-SCV *S. aureus* strain harboring pCX19*thyA* expressing *thyA*	This study
SCV/pCX19empty	Clinical TD-SCV *S. aureus* strain harboring the empty vector pCX19empty	This study
E. coli InvαF′	Host strain for the TA cloning vector pCR2.1	
Vectors		
pCR2.1	Cloning vector for sequencing	
pCX19	Xylose-inducible expression vector	10
pCX19*thyA*	pCX19 with *thyA*	This study
pCX19empty	pCX19 without *thyA*	This study

synthesis at two different steps, thereby inhibiting THF synthesis; sulfamethoxazole as a sulfonamide competitively inhibits the bacterial modification of *p*-aminobenzoic acid to dihydrofolate, whereas trimethoprim inhibits bacterial dihydrofolate reductase (34). If THF is not synthesized, dUMP is not converted to dTMP. Thus, the bacteria die due to a lack of thymidine (23). However, if external thymidine is available, SXT-resistant TD-SCVs emerge from wild-type *S. aureus* strains during long-term SXT treatment by an unknown mechanism. In the airways of CF patients, TD-SCVs survive for extended periods due to the occurrence of abundant pus and destroyed cells, which provide sufficient amounts of thymidine.

The underlying mechanism of TD-SCVs is not known. Since TD-SCVs are resistant to SXT, we hypothesized that *thyA* is mutated. Therefore, in this study *thyA* in several clinical TD-SCVs was sequenced and compared to *thyA* in isogenic normal *S. aureus* strains and to *thyA* sequences in previously published *S. aureus* genomes. To further strengthen our hypothesis, clinical TD-SCVs were complemented by a vector expressing wild-type ThyA, and then the phenotypes, growth characteristics, and transcription patterns of important virulence regulators and metabolic genes were analyzed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. For sequence analysis, four pairs of normal and SCV strains were used, which were described previously and have been frozen and thawed several times (12). To ensure that similar changes occurred in vivo and were not a result of reculturing of strains, fresh isolated SCVs or pairs of normal and SCV strains from airway secretions of CF patients were analyzed. The newly isolated strains were analyzed by *spa* typing to determine the clonality of pairs of strains (9), and the members of each pair belonged to the same *spa* type; Normal-5 and SCV-5 were type t118, Normal-6 and SCV-6 were type t040, SCV-7-1 and SCV-7-2 were type t084, and SCV-8-1 and SCV-8-2 were type t100. All new TD-SCVs were SXT resistant (MIC, >32 μg/ml, as determined by an Etest [AB Biodisk, Solna, Sweden]) and thymidine dependent on chemically defined medium agar (11). Normal *S. aureus* and TD-SCV strains 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 vector pCR2.1. For bacterial growth, cultures were grown at 35°C on a rotary shaker. Bacterial growth was assessed by measuring the optical density at 568 nm (OD_{568}). Ampicillin was used at a concentration of 50 μg/ml.

Sequencing strategy. For sequencing, *thyA* of TD-SCVs and isogenic normal strains was amplified with primers which also included regions up- and downstream of *thyA* (Table 2). The resulting amplicons were cloned into the cloning vector pCR2.1 and sent to MWG Biotech AG, Martinsried, Germany, for sequencing using the M13 vector primers.

Complementation of clinical TD-SCVs. For complementation, the pCX19 vector was used, which is a derivative of staphylococcal low-copy-number plasmid pC194 with lipase cloned into BamHI and SmaI restriction sites under the

TABLE 2. Primers used in this study

Primer	Sequence (5′ → 3′)
Complementation primers	
*thy*Ex2-rev	ATATCCCGGGGCAATGACTACACTGCTATTGG[a]
*thy*Ex1-up	ATATGGTACCTGAGGTGTTATCGCATATCTTG[b]
Cloning primers	
thy-1475592-up[c]	CCTACCACATCGTAACGTGA
thy-1474240-rev[c]	TGTGCAACTAGAATGGATAAAGTCA
Real time RT-PCR primers	
yqiL-1	TGCGATGATGTTAGTCATGT
yqiL-2	TTCAAAGCCTTTTCTACAGC
nupC-1	CTATTTAGCTCAGACAGGAAAAA
nupC-2	AGCAAGATAAATGCAAAGATAAG
RNAIII-5	TGAATTTGTTCACTGTGTCG
RNAIII-6	AGGAAGGAGTGATTTCAATG
hla-3	TTTCACCAGACTTCGCTACAG
hla-4	CCAATTTGTTGAAGTCCAATG
citB-1	TCAAAATCCATCATTCTTCC
citB-2	TCACCGAATTTACCCATAAC
spa-1	TGGTTTTATCCAAAGCCTTA
spa-2	TTTGGAGCTTGAGAGTCATT
thyA-3	TGTGGACTTGAAGTAGGAGAA
thyA-4	ACGTGCTAATTGTGTTTGAAT

[a] The SmaI restriction site is underlined.
[b] The BamHI restriction site is underlined.
[c] The number in the designation is the number of base pairs in the genome of strain COL.

TABLE 3. Sequencing results and mutations in *thyA* in clinical normal strains and TD-SCVs

Strain	Alteration(s) in nucleotide sequence	Predicted results
Normal-1[a]		
SCV-1[a]	Δ247AAT249	3-bp deletion → deletion of Asn
Normal-2[a]		
SCV-2[a]	Δ247AAT249	3-bp deletion → deletion of Asn
Normal-3[b]	T76C	Point mutation Cys → Arg (amino acid 26)
SCV-3[b]	C139T, G337A, A493G, ΔA565	Point mutations Leu → Por (amino acid 47), Gly → Ser (amino acid 113), and Asn → Asp (amino acid 165) and 1-bp deletion → stop codon (amino acid 188)
Normal-4[a]	T730G, G835A	Point mutations Ser → Ala (amino acid 244) and Gly → Ser (amino acid 279)
SCV-4[a]	T82A, Δ592CTTCCGCCTTG601	11-bp deletion (amino acid 198 → frameshift mutation → truncated protein
Normal-5[b]	G308A, C838T	Point mutations Gly → Asp (amino acid 103) and Leu → Phe (amino acid 280)
SCV-5	ΔA51	1-bp deletion → frameshift mutation → stop codon (amino acid 19)
Normal-6[b]	C383T, A482G, G886A	Point-mutations Ser → Phe (amino acid 128), Asp → Gly (amino acid 161), and Val → Ile (amino acid 296)
SCV-6[b]	G359A, ΔG743	Point-mutation Arg → Gln (amino acid 120) and 1-bp deletion → frameshift mutation → stop codon (amino acid 251)
SCV-7-1[b]	T7A, C521T, G806A, ΔA893	Point-mutations Tyr → Asn (amino acid 3), Tyr → Ile (amino acid 174), and Gly → Asp (amino acid 269) and 1-bp deletion → frameshift mutation → stop codon (amino acid 311)
SCV-7-2[b]	T7A, T616C	Point-mutation Tyr → Asn (amino acid 3) and point mutation → stop codon (amino acid 206)
SCV-8-1	ΔT121	1-bp deletion → frameshift mutation → stop codon (amino acid 50)
SCV-8-2[b]	Δ547ACATCGT553	7-bp deletion → frameshift mutation → stop codon (amino acid 212)

[a] Allele 2 (C at nucleotide position 300, Asn at amino acid position 100, and same *thyA* sequence as *S. aureus* strain MW2).
[b] Allele 1 (A at nucleotide position 300, Lys at amino acid position, and same *thyA* sequence as *S. aureus* strain COL).

control of an *xylA* promoter and a chloramphenicol resistance cassette (15). To complement TD-SCVs, *thyA* was amplified from *S. aureus* 8325-4 using the primers listed in Table 2 and cloned into the expression vector pCX19. Prior to ligation of *thyA* into pCX19, the lipase gene was removed from the vector by restriction with SmaI and BamHI. The resulting plasmid, pCX19*thyA*, was transformed into three clinical TD-SCVs by protoplast transformation. For complementation with *thyA*, SCV-1, SCV-2, and SCV-4 were used (Table 3). The *thyA* mutations of these TD-SCVs are indicated in Table 3. To eliminate sequencing mistakes introduced by PCR or transformation procedures, *thyA* was sequenced. To ensure that the observed phenotypic changes were due to *thyA* and not to the plasmid itself, a vector without *thyA* (pCX19empty) was constructed and also transformed into the three TD-SCVs.

Quantitative RT-PCR. Prior to RNA extraction, *S. aureus* was grown in BHI medium until the desired growth phase, either early log phase, late log phase, or stationary phase. Bacteria were mechanically disrupted (Fast Prep FP120 instrument; Qbiogene, Heidelberg, Germany), and RNA was isolated with an RNeasy mini-kit (Qiagen, Hilden, Germany). cDNA was synthesized from RNA using a Quantitect reverse transcription (RT) kit (Qiagen, Hilden, Germany) by following the manufacturer's recommendations.

Real-time amplification was performed using specific primers (Table 2) with 25 ng of cDNA as the template in each reaction mixture, and it was carried out with an iCycler iQ real-time PCR system (Bio-Rad, Hercules, CA) using iQ SYBR green Supermix (Bio-Rad). The levels of mRNA expression of the different genes were normalized using the expression of the internal control gene *yqiL* (encoding acetyl-coenzyme A acetyltransferase), which is a housekeeping gene successfully used for quantitative RT-PCR (16, 26). The levels of transcripts were expressed as increases (n-fold) relative to the values for the internal control (4).

RESULTS

In silico analysis of the *thyA* sequence in *S. aureus* genomes. *thyA* in *S. aureus* consists of 957 bp encoding a 318-amino-acid protein. The previously published sequences of *thyA* of strains COL, MRSA252, MSSA476, MU50, MW2, and N315 were aligned with the program clustalW (http://www.ebi.ac.uk/clustalW/) and analyzed. *thyA* was highly conserved in all strains at the base pair level, but some base pair changes resulted in nonsynonymous mutations. Interestingly, two alleles were detected at position 300, which encoded lysine in COL and MRSA252 but asparagine in the other strains. There are two predicted binding sites in *thyA*, a binding site for folate and dUMP at positions 142 to 174 coding for PLLTTKKVSFL (amino acids 48 to 58) and a binding site at position 586 to 618 coding for MALPPCHTMFQ (amino acids 196 to 206) (3). Both of these binding sites show 82% homology to similar regions in *Bacillus subtilis* at the protein level (19).

Sequence analysis of *thyA* in clinical TD-SCVs reveals different mutations compared to the normal clinical strains. Since there is a strong association between the emergence of TD-SCVs and SXT treatment, we hypothesized that *thyA*, which is responsible for thymidine synthesis from dUMP, would be affected in TD-SCVs. To test this hypothesis, *thyA*, including up- and downstream sequences, was amplified from six normal clinical strains and 10 TD-SCVs, cloned into the cloning vector pCR2.1, and sequenced, and the sequences were compared to the *thyA* sequences of previously sequenced strains. The same alleles found at position 300 in the previously sequenced strains were detected in the clinical strains, and 50% of the clinical strain pairs had one of these alleles and 50% had the other. In two normal strains, the *thyA* sequence was identical to the *thyA* sequence of N315. In the other normal strains (n = 4), one to three nonsynonymous point mutations were detected in the gene (Table 3). The *thyA* sequences of TD-SCVs had several different mutations. In four SCVs, one to three nonsynonymous point mutations occurred. Furthermore, two 3-bp in-frame deletions in two SCVs from two different patients occurred at the same position; six 1-bp (n =

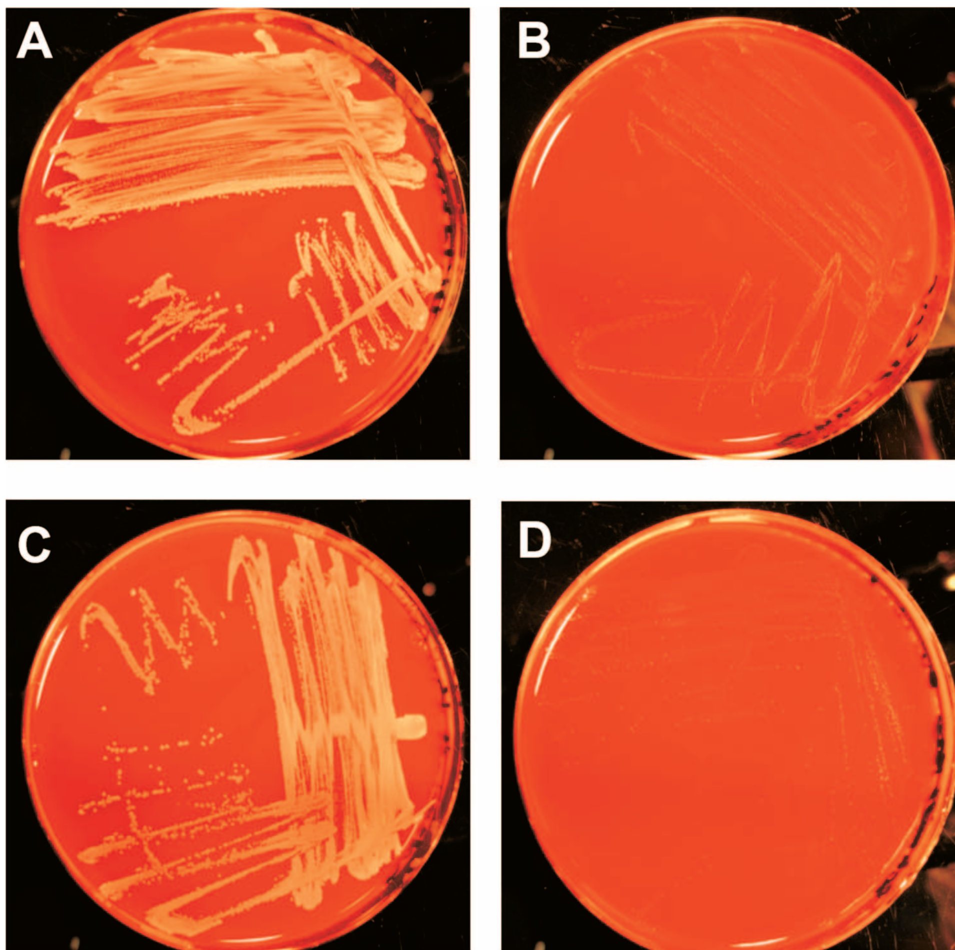

FIG. 1. Clinical TD-SCV is phenotypically complemented by a functional *thyA* gene. (A) Normal *S. aureus* grown on Columbia blood agar. (B) TD-SCV grown on Columbia blood agar exhibiting the SCV phenotype with small, less pigmented colonies. (C) TD-SCV transformed with pCX19*thyA* expressing functional ThyA, leading to the normal phenotype with large, pigmented colonies. (D) TD-SCV transformed with pCX19empty with the SCV phenotype, indicating the importance of functional ThyA for reversion to the normal phenotype. The images are representative of three sets of TD-SCV, normal *S. aureus*, and SCV/pCX19*thyA* strains.

5) or 7-bp ($n = 1$) deletions and one point mutation resulting in a stop mutation (Table 3) were detected. In one TD-SCV, an 11-bp deletion was observed, which resulted in a frameshift mutation. In 4 of 10 TD-SCVs (SCV-3, SCV-4, SCV-7-2, and SCV-8-2 [Table 3]), mutations were located within or close to the dUMP-binding site (positions 586 to 618), which has been suggested to be the active site of *thyA*. The in-frame deletion, which was detected in two independent SCVs, occurred at the same position where three Asn residues were encoded in the wild-type gene.

Complementation of clinical TD-SCVs with a functional *thyA* gene. The analysis of *thyA* of 10 clinical TD-SCVs revealed that various mutations occurred in *thyA*, most of which caused stop mutations in the gene (Table 3). In order to prove that the observed phenotypes of TD-SCVs were due to *thyA* mutations, the entire *thyA* gene from *S. aureus* 8325-4 was cloned into a xylose-inducible expression vector, which was subsequently transformed into three clinical TD-SCVs. The three *S. aureus* TD-SCVs complemented with the functional *thyA* gene were indistinguishable from the isogenic normal strains (exemplified by SCV-1) (Fig. 1). Induction by xylose did not affect the phenotype on Columbia blood agar, showing the “leaky” behavior of the pCX19 vector (data not shown). However, if the complemented TD-SCVs lost the plasmid after several subcultures, the strains displayed the SCV phenotype again. To ensure that the altered phenotype depended on the expression of ThyA, an empty vector lacking *thyA* (Table 1) was transformed into the TD-SCVs. This transformation did not change the SCV phenotype (Fig. 1). Thus, we confirmed that the observed TD-SCV phenotypes were due to the presence of various mutations in the *thyA* nucleotide sequence, as the SCV phenotypes could be reverted to normal by complementation of the TD-SCVs with functional *thyA*.

Complementation with *thyA* reversed the phenotype, growth, and transcription patterns of TD-SCVs. Since in TD-SCVs there are significant changes in important virulence regulators and their target genes (12), transcription of the important virulence regulator *agr* (encoding accessory gene regulator) and its target virulence genes, *hla* (encoding alpha-toxin) and *spa* (encoding protein A), was determined in normal, TD-SCV, the TD-SCV/pCX*thyA*, and TD-SCV/pCX*thyA* strains induced by xylose. Prior to RNA extraction, a bacterial growth analysis

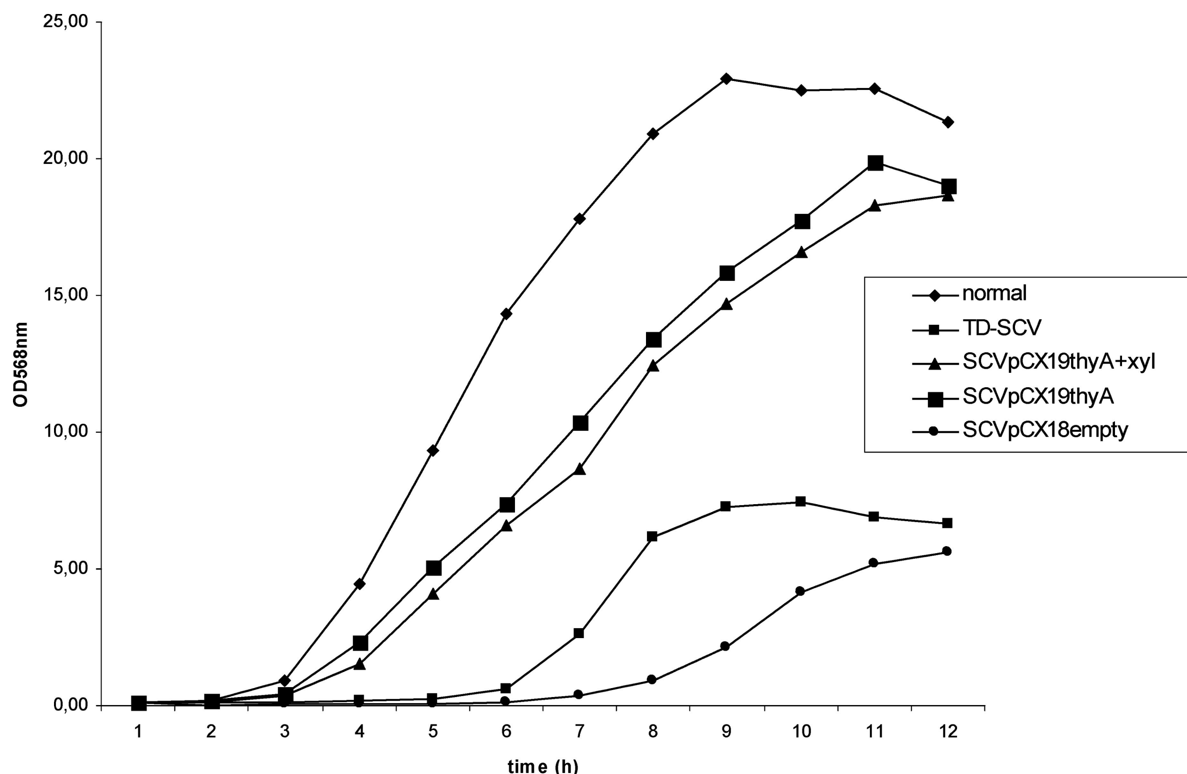

FIG. 2. Analysis of growth of normal *S. aureus*, TD-SCV, SCV/pCX19*thyA*, SCV/pCX*thyA* induced with xylose, and SCV/pCX19empty. Bacteria were grown in BHI broth with or without 0.5% xylose on a rotary shaker at 35°C. Every hour, the OD_{568} of the cultures were determined. The data are representative of two sets of TD-SCV, normal *S. aureus*, and SCV/pCX19*thyA* strains.

of the complemented strain with and without xylose was performed, and this strain displayed the same characteristics as the normal strain (Fig. 2). Interestingly, the log phase of the SCV/pCX*thyA* strain without xylose and the xylose-induced SCV/pCX*thyA* strain started after 2 h at the same time as the log phase of the normal strain, and the bacteria reached almost the same cell density (OD_{568}, 20) as the normal strain. In contrast, the log phase of the SCV/pCXempty strain was even more delayed than the log phase of the TD-SCV strain, and the SCV/pCXempty strain reached only the cell density of the TD-SCV strain (OD_{568}, 5), indicating the importance of functional ThyA for growth and replication.

As the transcription of *agr*, the transcription of *hla*, and the transcription of *spa* are differentially regulated (the transcript levels of *agr* and *hla* increase during stationary phase, while *spa* transcription is inhibited), the results for *agr* and *hla* transcription were analyzed during stationary phase and the results for *spa* were analyzed during late log phase using real-time RT-PCR. The transcription levels of RNAIII, the effector gene of *agr* (Fig. 3A), and *hla* (Fig. 3C) were increased in the SCV/pCX19*thyA* strain compared to the TD-SCV strain, while the level of *spa* was decreased compared to the level in the TD-SCV strain (Fig. 3B). These transcription patterns were very similar to that of the normal strain, indicating that there was complementation of the TD-SCV strain by *thyA* for these virulence genes. Recently, we showed that enhanced stationary-phase survival of TD-SCVs was associated with an impaired TCA cycle, which is important for entry into the bacterial death phase (4, 5, 28). To analyze if *thyA* is also able to complement this important metabolic pathway, we determined the transcriptional levels of aconitase (*citB*), the first enzyme of the TCA cycle in *S. aureus*. As shown previously (5), *citB* transcription was significantly decreased in the TD-SCVs compared to the normal strain (Fig. 3D). As expected, the transcriptional level of *citB* in the SCV/pCX19*thyA* strain partially reverted back to the level in the normal strain, indicating that there was complementation of this important metabolic pathway by *thyA*. Xylose induction of the TD-SCV/pCX19*thyA* strain had an effect only on RNAIII transcription. All other genes tested showed the same transcriptional levels in the TD-SCV/pCX19*thyA* strain with xylose as in the TD-SCV/pCX19*thyA* strain without xylose.

Furthermore, we wanted to know if *thyA* is expressed in the TD-SCVs. The levels of *thyA* transcript expression were highest during stationary phase (Fig. 4A). Compared to the normal strain, *thyA* expression of the TD-SCV strain was higher during the early log and late log growth phases, with the highest level of expression during early log phase (Fig. 4A). As in the normal strain, transcription of *thyA* in the SCV/pCX19*thyA* strain was highest during stationary phase but reached the level of *thyA* transcription in the late log and stationary phases in the normal strain only if it was induced with xylose (Fig. 4A).

Since *thyA* is essential for survival, TD-SCVs need to take up thymidine from an extracellular site. To accomplish this, thymidine has to be transported into the bacterial cell. One important transporter for nucleotides is NupC (25). As assessed

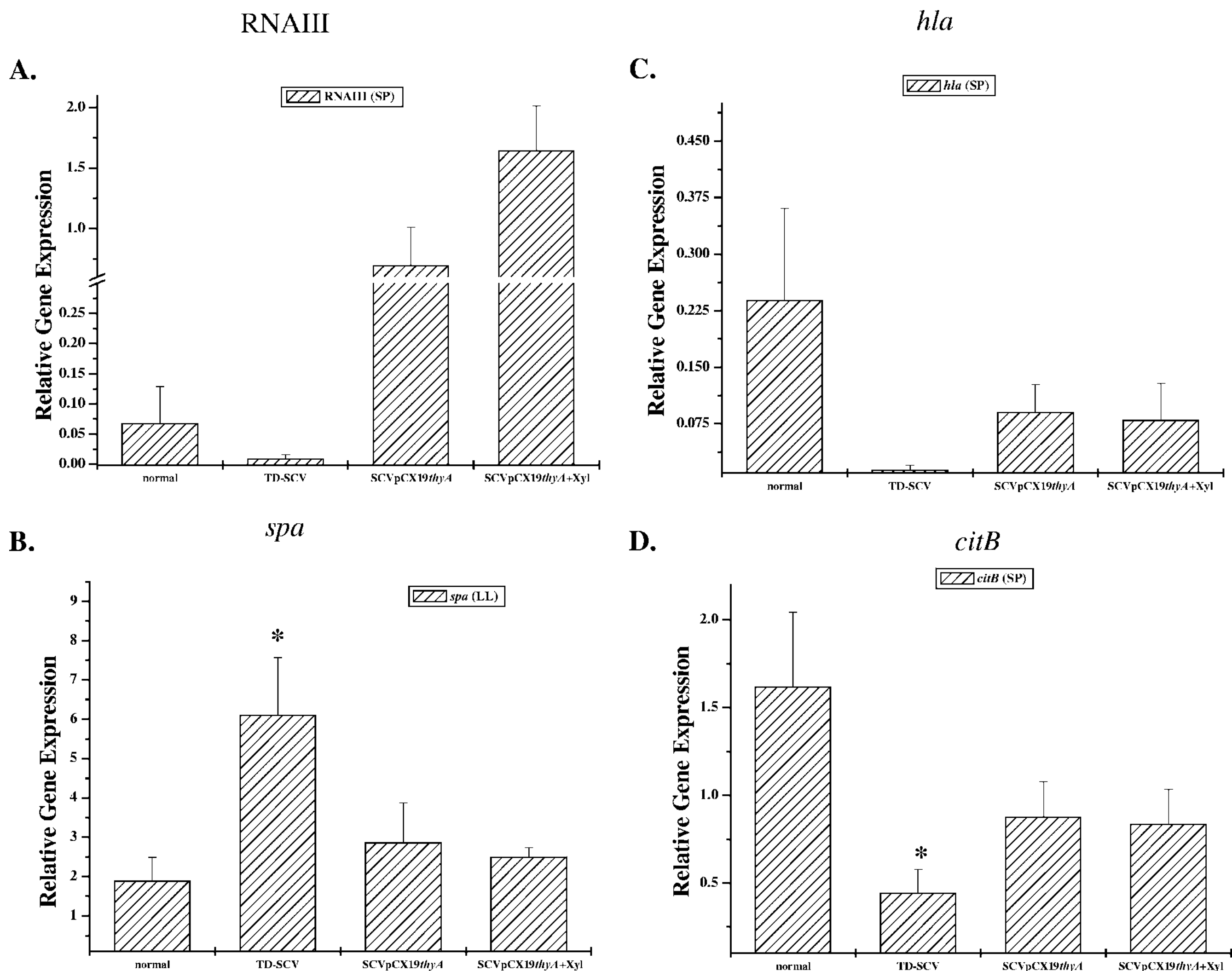

FIG. 3. Real-time RT-PCR quantification of expression in normal, TD-SCV, and SCV/pCX19*thyA* with or without 0.5% xylose of RNAIII as the effector gene for *agr* (A), *hla* (C), and *citB* (D) in stationary phase (SP) and of *spa* in late log phase (LL) (B). The levels of mRNA expression of the different genes were normalized using the expression of an internal control, *yqiL*. The transcript quantities are expressed as increases (*n*-fold) relative to the values for the internal control. The data are means and standard errors of the means obtained in three independent experiments. Asterisk, $P < 0.05$ compared to the normal strain (*t* test).

by real-time RT-PCR, expression of *nupC* peaked during early log phase in the normal strain (Fig. 4B). Compared to the normal strain, *nupC* in the TD-SCV strain showed increased expression during all growth phases, with the highest level during late log phase (Fig. 4B). In contrast, *nupC* expression in the SCV/pCX19*thyA* strain exhibited the same pattern as *nupC* expression in the normal strain, with the highest level of expression during early log phase and low levels of expression during the late log and stationary phases (Fig. 4B). Xylose induction of the SCV/pCX19*thyA* strain did not affect the levels of *nupC* expression.

DISCUSSION

TD-SCVs emerge during long-term treatment with SXT in the airways of CF patients, are resistant to SXT, and are often isolated together with the normal *S. aureus* strains. Often, TD-SCVs survive with the isogenic normal *S. aureus* strains for years even in the absence of antibiotic pressure, most likely due to optimized adaptation to the hostile environment (14). In this study, we tried to determine the underlying mechanism of TD-SCVs. To do this, we sequenced *thyA*, the gene which we hypothesized is affected in TD-SCVs due to a lack of THF, as THF is not synthesized in bacteria if patients are treated with SXT.

Sequence analysis of *thyA* in TD-SCVs and the isogenic normal strains revealed that various mutations occurred at different positions in the gene. These data are in line with data of Besier et al., who recently described a great variety of mutations in *thyA* in TD-SCVs (1). Unexpectedly, in four normal strains one to three point mutations occurred at different loci compared to the TD-SCVs, and all of them resulted in nonsynonymous changes in amino acids. Since the normal strains were also under antibiotic pressure during SXT therapy of the patients, such changes prob-

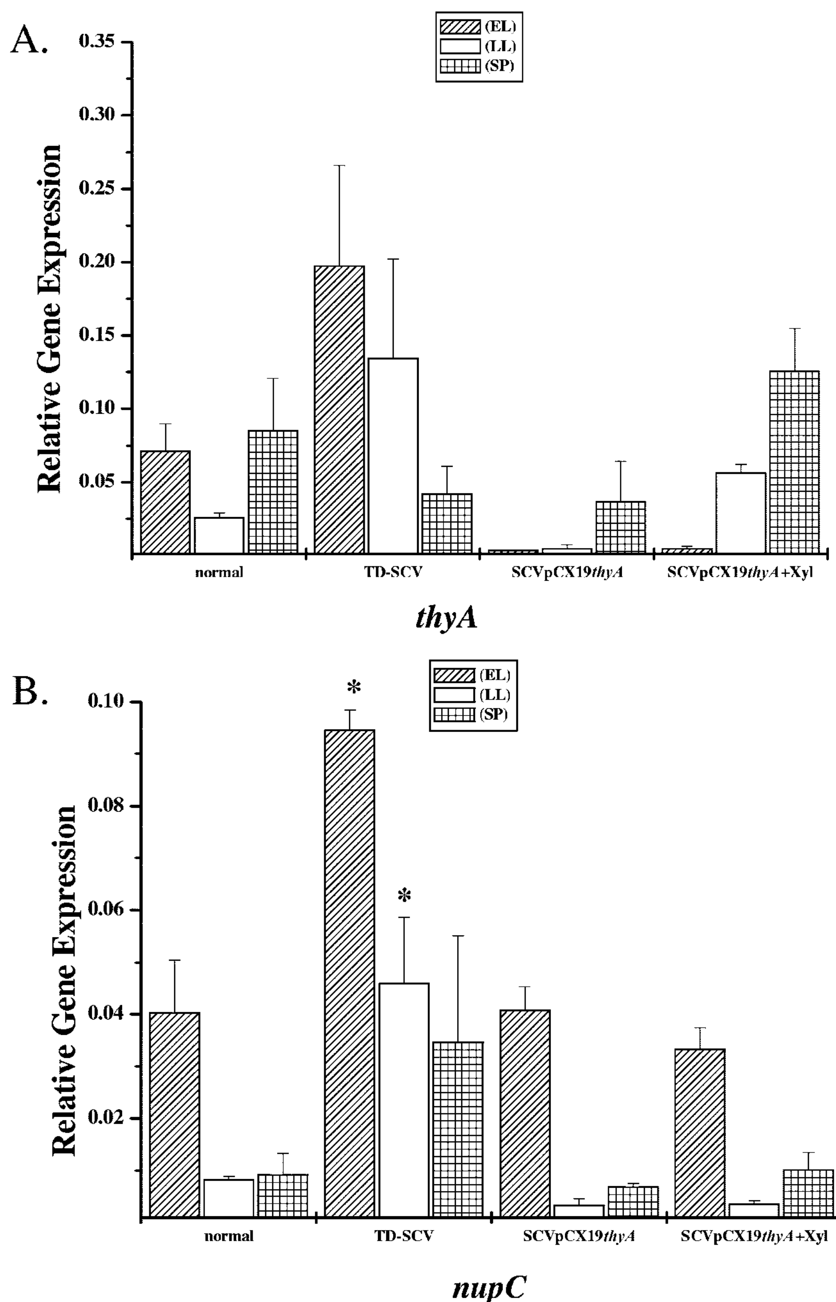

FIG. 4. Real-time RT-PCR quantification of expression in normal, TD-SCV, and SCV/pCX19*thyA* with or without 0.5% xylose of *thyA* (A) and *nupC* (B) in early log phase (EL), late log phase (LL), and stationary phase (SP). The levels of mRNA expression of the different genes were normalized using the expression of an internal control, *yqiL*, and the transcript quantities are expressed as increases (*n*-fold) relative to the values for the internal control. The data are means and standard errors of the means obtained in three independent experiments Asterisk, $P < 0.05$ compared to the normal strain (*t* test).

ably occur as a response to this selective pressure. It is not known if the activity of the protein is affected by these changes. Surprisingly, in our study, most mutations in TD-SCVs were scattered around the dUMP-binding site. This may be due to the fact that the dUMP-binding site has been described as the active site of the protein in other bacteria and species (7). Therefore, we assume that mutations occurring at this site have the most dramatic effects on the activity of the protein. Although *thyA* is severely mutated in TD-SCVs, this gene is transcribed at high levels. These results could be explained as follows. First, thymidine synthesis is tightly regulated, and increasing *thyA* expression occurs in TD-SCVs due to the missing negative feedback by thymidine. Second, mutations in the dUMP-binding site in TD-SCVs prevent binding of dUMP, which then is not captured by the more abundant nonfunctional protein and thus is available for other important pathways. Finally, increased transcription of *thyA* leading to increased translation of the protein could also be important to overcome the inactivity of the protein.

Interestingly, we detected one in-frame deletion, which occurred at the same site in two independent SCVs and occurred in a region where three asparagines were encoded in the gene. Such deletions are most likely due to slipped-strand mispairing, which seems to occur in combination with inadequate DNA mismatch repair systems (31). In this way, repeats can be deleted or inserted during DNA polymerase-mediated DNA duplication depending on the orientation of the strand. However, it is not clear if this in-frame deletion leads to an inactive protein.

As we detected various mutations in *thyA* in the clinical TD-SCVs, we tried to determine the impact of *thyA* by transforming clinical SCVs with a functional *thyA* gene. Supporting our hypothesis, transformation of TD-SCVs with a vector expressing ThyA changed the phenotype to that of the normal strain. These results were corroborated by the fact that the SCV phenotype of TD-SCVs did not revert to normal if SCVs were transformed with an empty expressing vector and by the fact that the SCV phenotype emerged again if TD-SCVs were cured of the ThyA-expressing vector. Moreover, replication of TD-SCVs transformed by pCX19*thy* was fully complemented with the typical growth phenotype of normal strains, and a cell density that was the same as that of the normal strain was reached. Interestingly, these results differ from the growth phenotype of TD-SCVs which were supplemented with thymidine (12). Supplementation of TD-SCVs with thymidine did not affect the lag phase of the SCVs, which was extended in the supplemented SCVs, as it was in the TD-SCVs. Only if the supplemented TD-SCVs reached the log phase did SCVs revert to the normal phenotype and reach the same cell density as the normal strain. This observation indicates that *thyA* affects more important functions required for proper bacterial growth. Interestingly, most effects analyzed in the SCV/pCX19*thyA* mutant were present without induction by xylose, indicating that the pCX19 vector is really leaky.

Mutations in essential genes, such as *thyA*, are usually lethal for bacteria (23). The emergence of TD-SCVs that has been described to occur in vivo for *S. aureus*, *E. coli*, and *Salmonella* after treatment with SXT (8, 11, 18, 27, 30) is possible only if bacteria have access to external thymidine. In the presence of thymidine, *thyA* mutations can occur due to uptake of extracellular thymidine. Since nucleotides do not pass through bacterial cell walls and membranes by diffusion, uptake is an active process, which is accomplished by nucleotide transporters, such as *nupC* (25). Accordingly, we studied the expression of *nupC* using real-time RT-PCR, and this expression was shown to be increased in the TD-SCVs compared to the normal strain and decreased in the SCV/pCX19*thyA* strain.

In conclusion, mutations in *thyA* have been shown to be responsible for the SCV phenotype in TD-SCVs. By transforming clinical TD-SCVs with a vector expressing ThyA in *trans*, we convincingly showed that a functional *thyA* gene is necessary and sufficient to complement the phenotype, growth characteristics, expression of genes for virulence regulators and virulence genes, and also important metabolic pathways.

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