JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 2006, p. 3274–3278 Vol. 44, No. 9
0095-1137/06/$08.00+0 doi:10.1128/JCM.00847-06
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Fourier-Transform Infrared Spectroscopic Analysis Is a Powerful Tool for Studying the Dynamic Changes in *Staphylococcus aureus* Small-Colony Variants

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Received 21 April 2006/Returned for modification 5 June 2006/Accepted 7 July 2006

Infections due to small-colony variants (SCVs) of *Staphylococcus aureus* in patients with chronic and recurrent infections are an emerging problem; however, studies with this subpopulation are hampered by the fact that SCVs may exhibit unstable phenotypes, making them difficult to study, particularly in broth media. In this study, two *S. aureus* sets comprising the (i) normal and the (ii) SCV phenotype (clonal with normal phenotype) recovered from clinical specimens, as well as (iii) corresponding site-directed mutants displaying the SCV phenotype (knockout of *hemB*) and (iv) their complemented mutants were examined by Fourier-transform infrared (FTIR) spectroscopy. Phenotypes were defined on solid and in broth media. Using first-derivative infrared spectra to calculate spectral distances, hierarchical clustering based on spectral information resulted in a dendrogram with clear discrimination between SCV and normal phenotypes. The SCVs gave an FTIR fingerprint that was easily recognizable and that was much closer to other SCVs than to their parent strains. This technique offers for the first time a noninvasive approach to investigate dynamic processes of reversion of SCVs to the normal phenotype and vice versa. Thus, FTIR spectroscopy allowed a rapid and reproducible tool for the examination of different subpopulations of *S. aureus* on solid and in broth media for diagnostic and research purposes.

Staphylococcus aureus small-colony variants (SCVs), recognized as emerging pathogens, represent a naturally occurring, slow-growing subpopulation, and they are defined by nonpigmented and nonhemolytic colonies about 10 times smaller than the parent strain, hence their name (14, 16). On solid agar media, *S. aureus* has long been recognized to produce colonies that may lack pigment, change their pigmentation as the colony ages (from yellow to orange), and have smooth versus scalloped edges. These are relatively minor compared to the changes seen in SCVs, where the colonies are more dramatically altered but the phenotype much less stable (16). *S. aureus* exhibiting typical colony size, pigmentation, and hemolysis on Columbia blood agar after overnight growth is herein referred to as "normal" *S. aureus*.

Many of the atypical characteristics of SCVs can be explained by a common mechanism, i.e., defects in electron transport (26). The ability to interrupt electron transport, to persist intracellularly, and to form a variant subpopulation affords *S. aureus* a number of survival advantages that extend beyond simply increased resistance to antibiotics (15, 24). The significance of SCVs as causative organisms in chronic, recurrent, and antibiotic-resistant infections, particularly in patients with chronic osteomyelitis or cystic fibrosis, has been demonstrated in several prospective studies (8, 9, 25). Furthermore, staphylococcal SCVs were cultivated from patients with persistent skin infections, deep-seated abscesses, and foreign body-associated infections (17, 18, 24).

In general, studies on SCVs recovered from patients with persistent infections are hampered by the fact that SCVs are difficult to recover and to identify. In addition, they frequently exhibit an unstable phenotype, i.e., reversion into the normal phenotype may occur. On the other hand, while stable laboratory-generated mutants reproduce the SCV phenotype, we do not know the genetic basis for the clinical SCVs, even though multiple lines of evidence point toward defects in hemin or menadione biosynthesis (16).

On solid agar, however, a reversion of the SCV morphotype into the normal phenotype is more easily detected by the naked eye; a reversion in broth media rapidly leads to overgrowth of the SCV population and is not traceable. Because many diagnostic and research investigations rely upon studies in broth media, e.g., transcriptional and metabolic analyses, a tool to follow the dynamic changes found in SCVs is needed.

Fourier-transform infrared (FTIR) spectroscopy is a noninvasive technique that allows one to study populations of bacteria under different culture conditions, including solid and fluid media. Thus, the purposes of this study were to establish a reliable method that is able (i) to evaluate the discriminatory power of FTIR for accurate differentiation between normal and SCV phenotypes of *S. aureus*, (ii) to evaluate SCV status in broth media, and (iii) to observe and investigate the phase variation from SCV phenotype into the normal phenotype and vice versa.

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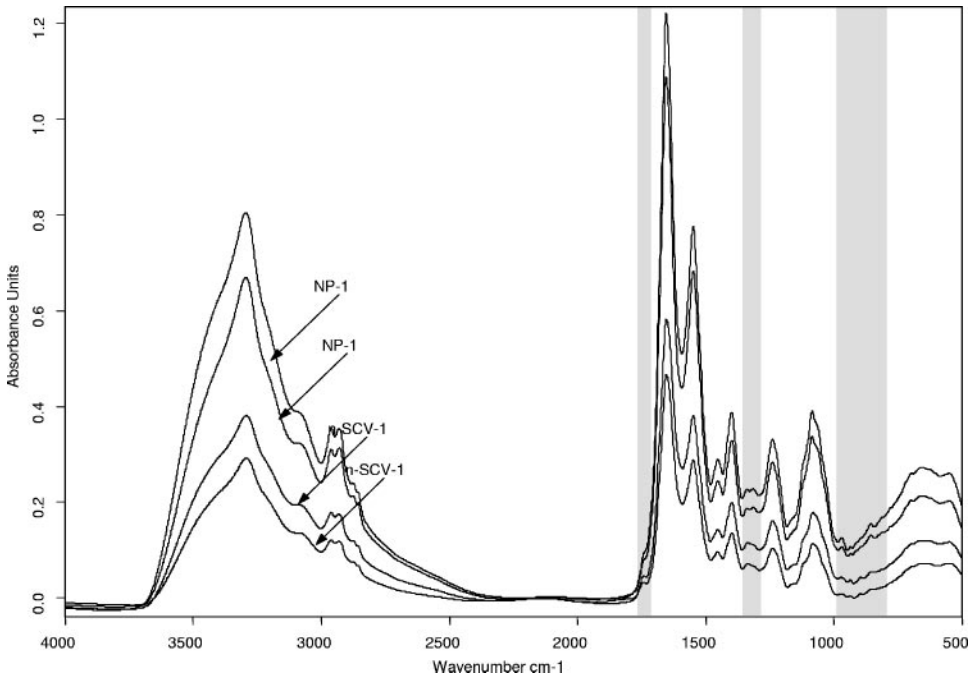

FIG. 1. FTIR spectra as measured from an *S. aureus* strain exhibiting the normal phenotype (NP-1) and from the isogenic *S. aureus* strain displaying the small-colony-variant phenotype (n-SCV-1). Prominent differences between the spectra of both phenotypes are marked.

MATERIALS AND METHODS

Bacterial strains. Two epidemiologically unrelated *S. aureus* strains with normal phenotypes (NP-1 and NP-2) recovered from clinical specimens (25), which were isolated simultaneously or sequentially with clonally identical natural SCVs (n-SCV-1 and n-SCV-2), were used in this study. NP-1 and NP-2 were also selected as parent strains for construction of site-directed mutants displaying the SCV phenotype (HemB-1 and HemB-2). These stable, genetically defined mutants with SCV phenotypes were constructed by interrupting one of the hemin biosynthetic genes, *hemB*, in normal-phenotype *S. aureus* NP-1 and NP-2 by inserting an *ermB* cassette as previously described (26). Following construction, these mutants were complemented with the *hemB* gene (c-HemB-1 and c-HemB-2) as described previously (26). Whereas one of the clinically derived natural SCVs (n-SCV-1) exhibited stable pinpoint colonies, n-SCV-2 was characterized by a high rate of spontaneous reversion into the normal phenotype. To determine the clonal relationship, SmaI digests of total bacterial DNA were resolved and analyzed by pulsed-field gel electrophoresis as previously described (6, 20).

Sample preparation. When solid media were used, bacterial cells were subcultured at 37°C on Mueller Hinton agar (MHA) for 48 h. Using a vibrating bacterial loop (Labor Technik Barkey, Bielefeld, Germany), two loopfulls of each subculture on solid media were suspended in 80 μl distilled water. For experiments using fluid media, bacterial cells were cultivated overnight in Trypticase soy broth (TSB) at 37°C. Subsequently, 35 μl of the suspension was transferred to the sample wheel (Bruker Analytik, Karlsruhe, Germany) and dried under vacuum (−0.90 bar) in a desiccator. All examinations were performed at least in triplicate. For broth culture experiments, the respective isolates cultured in parallel on solid media served as controls.

To study mixed cultures, suspensions of *S. aureus* SCVs (n-SCV-1) prepared as described above were spiked with suspensions of normal-phenotype *S. aureus* (NP-1) in the ratios of 1:10, 1:20, 1:50, 1:100, and 1:1,000.

In order to record spontaneous reversions of clinically derived SCVs into the normal phenotype by FTIR, TSB was inoculated with n-SCV-2 and cultivated as described above. To monitor reversion, subcultures from TSB were plated onto MHA during and after the incubation period.

FTIR spectroscopic analysis and data processing. FTIR spectra were recorded on an IFS 28/B FT-IR spectrometer (Bruker Analytik). The preprocessing and processing of FTIR spectral data were performed using OPUS NT 3.1 software (Bruker Analytik). Preprocessing was done on the total spectral region (500 and 4,000 cm^{-1}), and 64 scans were averaged at a resolution of 6.0 cm^{-1}. After baseline elastic correction of the total spectral region, vectorial normalization was performed followed by calculation of the first derivative using the Savitsky-Golay algorithm. The first derivation of the digitized original spectra was used to diminish the difficulties arising from unavoidable baseline shifts and to improve the resolution of complex bands (13). The setting of the spectral windows that were used for analysis were based on the arrangement for the spectral analysis as follows (frequency ranges [weights]): 3,197 to 3,600 cm^{-1} (1), 698 to 902 cm^{-1} (1), 900 to 1,200 cm^{-1} (1), 1,199 to 1,500 cm^{-1} (1), 1,498 to 1,801 cm^{-1} (1), 2,797 to 3,003 cm^{-1} (1), and 3,001 to 3,300 cm^{-1} (1). For hierarchical cluster analysis, average linkage was used to construct dendrograms. The spectral distance was defined as a measure of the similarity of the spectra of two strains corresponding to the size of nonoverlapping areas of both spectra.

RESULTS

Generation of the *hemB* mutants and their complemented mutants. Stable mutants in electron transport were generated by interrupting *hemB* in two clinical isolates of *S. aureus* (HemB-1 and HemB-2). For this purpose, an *ermB* cassette was inserted into the *hemB* gene by allelic exchange (26). After homologous recombination, the *hemB* mutants HemB-1 and HemB-2 were isolated by growth at a nonpermissive temperature and by selection for erythromycin resistance. The *hemB* mutants showed slow growth with pinpoint colonies after 24 to 48 h of incubation on tryptic soy agar plates as well as decreased pigment formation and reduced hemolytic activity, thus reproducing the major phenotypic features of naturally occurring clinical SCVs.

In addition, each *hemB* mutant was complemented with the *hemB* gene. The PCR-amplified *hemB* gene was cloned in *S. carnosus* using vector pCX19 containing a xylose-inducible promoter (26). Yielded plasmid (pCE12) was used to transform *hemB* mutants, resulting in complemented mutants (c-HemB-1 and c-HemB-2) exhibiting normal growth character-

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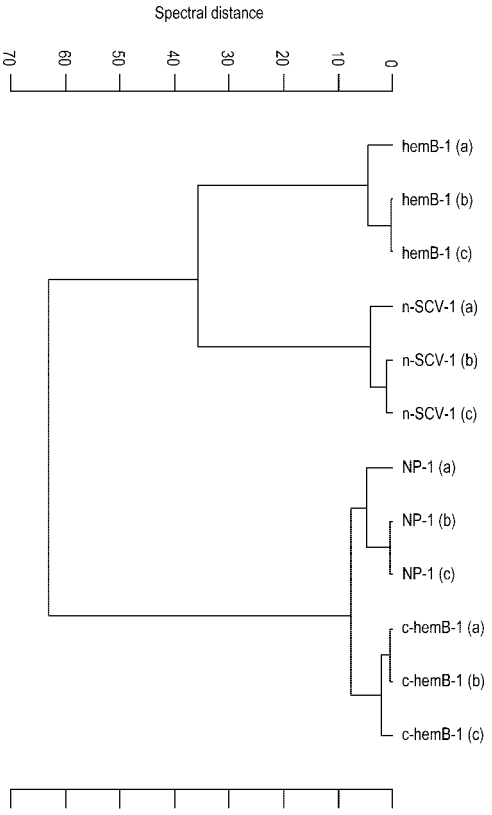

FIG. 2. FTIR dendrograms derived from the spectra of an isogenic clone harvested from broth media consisting of a clinically derived, natural *S. aureus* SCV (n-SCV-1), its parent strain with normal phenotype (NP-1), the *hemB* mutant displaying the SCV phenotype (hemB-1), and the *hemB*-complemented mutant (c-hemB-1). Spectra were analyzed in triplicate (a to c). The isolates with normal phenotypes (NP-1 and c-hemB-1) were grouped in one cluster, which was clearly separated from that of the isolates exhibiting the SCV phenotype (n-SCV-1 and hemB-1).

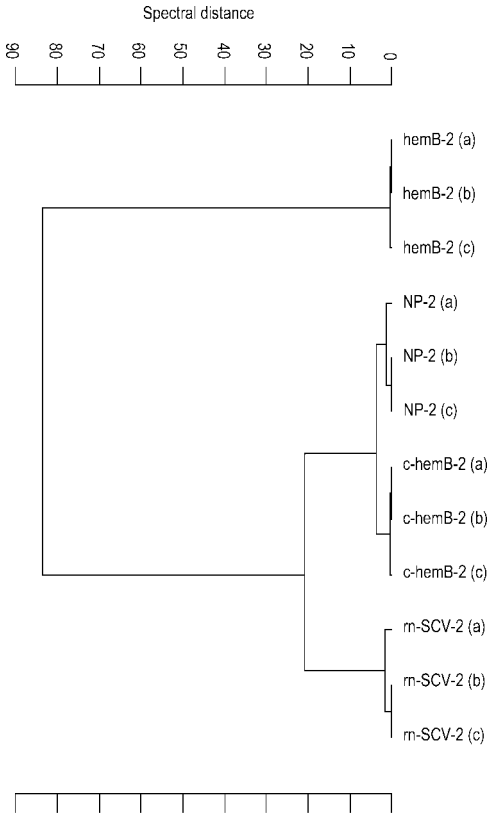

FIG. 3. Tracing the switch of the SCV phenotype into the normal phenotype by FTIR in broth. The spectra of the clinically derived n-SCV-2 reverted during cultivation in brain heart infusion broth (rn-SCV-2) were grouped into the cluster comprising those isolates exhibiting the normal phenotype, i.e., the parent strain (NP-2) and the *hemB*-complemented mutant (c-hemB-2). For comparison, the spectra of the isogenic *hemB* mutant displaying the SCV phenotype (hemB-2) were included. Spectra were analyzed in triplicate (a to c).

istics. Curing of complemented mutants of pCE12 restored the SCV phenotype.

Thus, originating from two parent clinical *S. aureus* strains, two clonal sets were established, each comprised of (i) naturally occurring SCVs (isolated from clinical specimens), (ii) stable *hemB* mutants with the SCV phenotype, (iii) *hemB* gene complemented mutants with the normal phenotype, and (iv) parent strains with the normal phenotype.

FTIR spectroscopy. The two clonal sets of *S. aureus* isolates described above were used to investigate the feasibility of FTIR spectroscopy for tracing the SCV phenotype in broth media. In preliminary tests, colonies harvested from MHA plates that demonstrated either the normal or SCV phenotype produced clearly different FTIR spectra. First-derivative infrared spectra to calculate spectral distances were found to be useful for hierarchical clustering (Fig. 1). Based on spectral information in three different spectral ranges, clustering resulted in a dendrogram that showed a clear discrimination between both phenotypes, i.e., the normal and the SCV phenotypes of *S. aureus*. Two distinct clusters comprising the clinical and mutant SCV phenotypes on one hand and the normal phenotype (isolate with normal phenotype and complemented mutant) on the other hand were produced (data not shown).

Based on the results of the preliminary tests, spectroscopic patterns of cells of the isogenic sets harvested from broth media were gained and analyzed. As shown for the results based on material from solid media, the isolates exhibiting the SCV phenotype (clinical SCVs and *hemB* mutants) assorted into one cluster, which was generated clearly separated from another cluster comprising those isolates exhibiting the normal phenotype, i.e., the parent strains and the complemented *hemB* mutants (Fig. 2). The FTIR spectroscopy results were in accordance with the solid media controls.

In artificially mixed cultures consisting of SCV suspensions and diluted suspensions of its isogenic normal parent strain, normal-phenotype cells were detectable up to a dilution of 1:1,000 by FTIR (data not shown).

Using TSB cultures of the clinically derived natural strain n-SCV-2, FTIR spectroscopy showed that spontaneously reverted SCV cells exhibited the same spectra as the normal parent strain (NP-2) as well as the respective complemented mutant c-HemB-2. The switch of the phenotype was confirmed by coexistence of normal and SCV colonies on subcultures performed in parallel during and after incubation of the broth medium. In contrast, the respective SCV mutant (HemB-2) was clearly separated by hierarchical clustering (Fig. 3).

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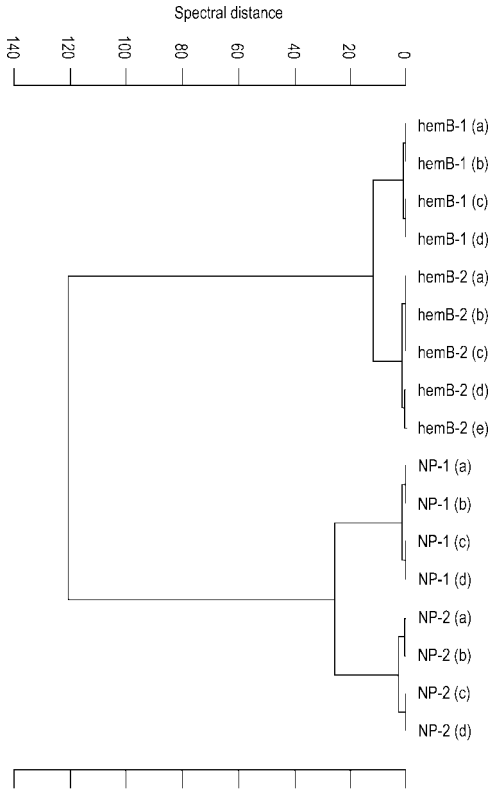

FIG. 4. FTIR dendrogram clustering the spectra of two epidemiologically unrelated pairs consisting both of a normal parent strain (NP-1 and NP-2) and its isogenic *hemB* mutant exhibiting the SCV phenotype (hemB-1 and hemB-2). Spectra were analyzed at least in quadruplicate (a to e). Despite their different clonality, isolates with the normal phenotype were grouped in one cluster, whereas isolates with the SCV phenotype were grouped in a second cluster.

In analyses of two strain pairs of different clonality, consisting of the normal parent strains and their site-directed *hemB* mutants exhibiting the SCV phenotype, isolates were found to cluster predominantly according to their phenotype rather than to their genotype (Fig. 4).

DISCUSSION

Biophysical methods based upon vibrational spectroscopic techniques, such as FTIR spectroscopy, are rapid, cost- and time-effective, require little biomass, and necessitate little or no sample pretreatment (3, 12). This approach permits the user to collect complex biochemical fingerprint-like spectra derived from the total cell composition without destruction of the biological structures studied (11).

SCVs are slow-growing subpopulations of bacteria with distinctive phenotypic and pathogenic traits (16). On solid media, the unique SCV phenotype may be recognized visually if cells grow unambiguously as pinpoint colonies. However, naturally occurring SCVs are unstable, and most studies on the SCV phenotype are based upon stable site-directed mutants in electron transport (2, 19, 23, 26, 27). Because the mutations in clinical strains have yet to be defined, these mutants do not necessary reflect the complete phenotype of natural SCVs. The use of FTIR offers another method for the study of clinically derived SCVs. To meet the requirement of real-time readout of the SCV nature under liquid culture conditions, an FTIR approach was established. Spectroscopic patterns of SCV cells harvested from broth media were shown to sort into the same cluster as SCV cells derived from solid media, thus offering for the first time a direct approach to ensure and monitor the SCV phenotype under sessile as well as planktonic conditions. FTIR clearly discriminated, within a short time period (150-min overall working time), between the staphylococcal SCV phenotype and the normal phenotype. The spectra of the SCVs from different genetic backgrounds were much more similar to one another than the spectra from any of their parent strains. Hence, an SCV fingerprint was established allowing a clear discrimination between the SCV and the normal phenotype by hierarchical cluster analysis.

Various studies have shown the applicability of FTIR spectroscopy to classify and identify bacterial and fungal microorganisms as well as prions (1, 4, 5, 10, 21, 28). Furthermore, FTIR spectroscopy was used successfully as a fingerprinting technique for the assessment of intraspecies variability (7, 13, 22). However, as shown here for staphylococci, intraspecific fingerprinting by FTIR spectroscopy is only reliable if the isolates exhibit the same phenotype or morphotype.

This technique may offer for the first time a nondestructive approach to trace and investigate directly the so far not fully understood process of reversion from the normal phenotype into the SCV phenotype and vice versa. For *Bradyrhizobium japonicum*, structural changes during growth have been successfully monitored by FTIR (29). Lin et al. were able to detect biochemical differences between intact and injured cells of *Listeria monocytogenes* (11). In future studies, this technique may also provide an approach for tracing a bacterial phenotype in infected tissues, in particular with regard to a therapeutic approach affecting the phenotype. In conclusion, FTIR spectroscopy allows a rapid, reliable, and well-defined discrimination of different phenotypes of *S. aureus* in liquid media applicable for diagnostic and research purposes.

ACKNOWLEDGMENTS

We are grateful to Michaela Brück, Elke Kruse, Martina Schulte, Angela Terliesner, and Susanne Weber for excellent technical assistance. We thank A. Schmalreck (MBS, Munich, Germany) for technical support.

This work was supported in part by grants from BMBF (Pathogenomic Network) to K.B., G.P., and C.v.E., from the Deutsche Forschungsgemeinschaft (EI 247/7-1) to C.v.E., and from the National Institutes of Health (AI42072) to R.A.P.

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