Journal of Clinical Microbiology, Aug. 2010, p. 2878–2884 Vol. 48, No. 8
0095-1137/10/$12.00 doi:10.1128/JCM.00520-10
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# Clinical and Molecular Characteristics of Infections with $CO_2$-Dependent Small-Colony Variants of *Staphylococcus aureus*[▽]

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Received 11 March 2010/Returned for modification 20 May 2010/Accepted 9 June 2010

**Most *Staphylococcus aureus* small-colony variants (SCVs) are auxotrophs for menadione, hemin, or thymidine but rarely for $CO_2$. We conducted a prospective investigation of all clinical cases of $CO_2$-dependent *S. aureus* during a 3-year period. We found 14 $CO_2$-dependent isolates of *S. aureus* from 14 patients that fulfilled all requirements to be considered SCVs, 9 of which were methicillin resistant. The clinical presentations included four cases of catheter-related bacteremia, one complicated by endocarditis; two deep infections (mediastinitis and spondylodiscitis); four wound infections; two respiratory infections; and two cases of nasal colonization. Pulsed-field gel electrophoresis typing showed that the 14 isolates were distributed into 4 types corresponding to sequence types ST125-*agr* group II (*agr*II), ST30-*agr*III, ST34-*agr*III, and ST45-*agr*I. An array hybridization technique performed on the 14 $CO_2$-dependent isolates and 20 *S. aureus* isolates with normal phenotype and representing the same sequence types showed that all possessed the enterotoxin gene cluster *egc*, as well as the genes for α-hemolysin and δ-hemolysin; biofilm genes *icaA*, *icaC*, and *icaD*; several microbial surface components recognizing adhesive matrix molecules (MSCRAMM) genes (*clfA*, *clfB*, *ebh*, *eno*, *fib*, *ebpS*, *sdrC*, and *vw*); and the *isaB* gene. Our study confirms the importance of $CO_2$-dependent SCVs of *S. aureus* as significant pathogens. Clinical microbiologists should be aware of this kind of auxotrophy because recovery and identification are challenging and not routine. Further studies are necessary to determine the incidence of $CO_2$ auxotrophs of *S. aureus*, the factors that select these strains in the host, and the genetic basis of this type of auxotrophy.**

Small-colony variants (SCVs), formerly designated "G" (gonidial) variants or dwarf colonies, constitute a naturally occurring, slow-growing subpopulation of bacteria with distinctive phenotypic characteristics and pathogenic traits (25). Although SCVs have been described in a wide range of bacterial genera and species and recovered from different clinical specimens, including abscesses and soft tissues, blood and bone, joints, and the respiratory tract, those of *Staphylococcus aureus* have been most extensively studied (14). Most of these SCVs are auxotrophs for menadione, hemin, or thymidine, while others are occasionally identified as auxotrophs for $CO_2$ (14).

Although the prevalence of SCVs of *S. aureus* in clinical specimens in a general microbiology laboratory has been estimated to be around 1% (1), SCVs are recovered more frequently from certain groups of patients such as those with cystic fibrosis (14, 15). A recent study determined the prevalence of SCVs to be 17% among cystic fibrosis patients who carried *S. aureus* (2). These SCVs were thymidine auxotrophs, a characteristic related to long-term exposure to prophylactic treatment with trimethoprim-sulfamethoxazole (2, 23). Several investigators have also shown that SCVs of *S. aureus* can often be recovered from cultures of patients who have been exposed to gentamicin or other aminoglycosides (11, 14). Hemin- and menadione-auxotrophic *S. aureus* SCVs have been recovered from patients with osteomyelitis who were treated with gentamicin (23). SCVs of *S. aureus* have also been recovered from patients with device-related infections (17). In general, the characteristics of SCVs facilitate persistence and recurrence of infections (7, 13, 14, 24). Moreover, clinical cases of endocarditis, bacteremic pneumonia, and abscesses due to strains of $CO_2$-dependent staphylococcus producing dwarf colonies have rarely been described in the medical literature (16, 19–21).

After observing *S aureus* SCVs in two patients who were hospitalized in the same surgical ward, we noted that both cases were likely to have been caused by nosocomial transmission of a strain of *S. aureus* that was auxotrophic for $CO_2$. After that, we decided to conduct a prospective study of all clinical cases caused by $CO_2$-dependent *S. aureus*. Here we describe the clinical characteristics of the cases that we identified together with the phenotypic and genetic characterization of the SCVs isolated.

## MATERIALS AND METHODS

**Patients and chart review.** This study was conducted at the Hospital Universitario Doce de Octubre, a 1,300-bed tertiary care facility that comprises two separate buildings, one for children and the other for adults. This public hospital provides specialized health care to a population of approximately 550,000 residents in southern Madrid, Spain. All patients with $CO_2$-dependent SCV isolates of *S. aureus* that were recovered between 2006 and May 2009 were included in the study. We reviewed the medical charts from each of these patients and collected demographic, clinical, and microbiological data.

**Microbiology.** *Staphylococcus aureus* SCVs were identified as slow-growing, pinpoint, nonpigmented, nonhemolytic colonies after 24 to 48 h of incubation in

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▽ Published ahead of print on 16 June 2010.

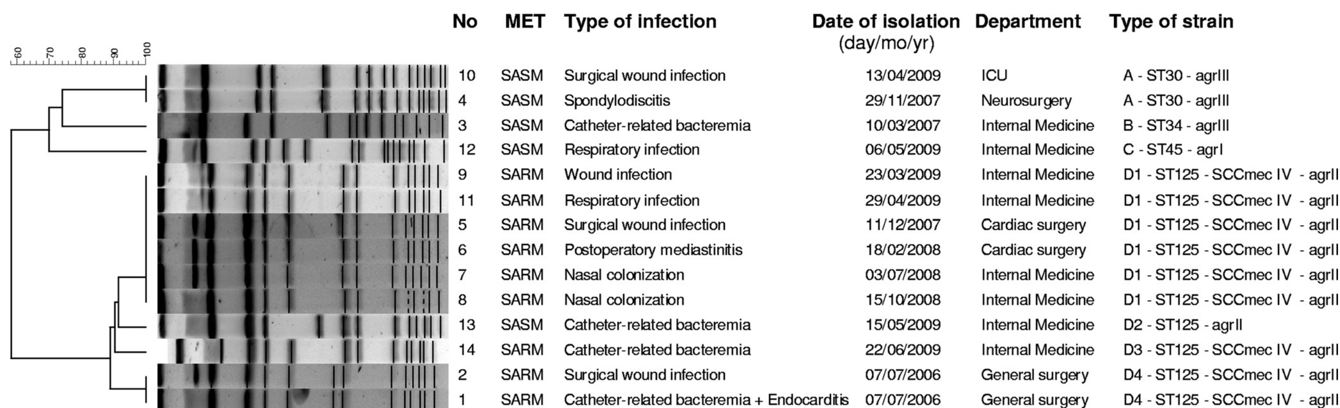


FIG. 1. Dendrogram based on PFGE patterns of $CO_2$-dependent small-colony variants of *Staphylococcus aureus*. ICU, intensive care unit; MET, methicillin; SASM, *S. aureus* susceptible to methicillin; SARM, *S. aureus* resistant to methicillin. The type of strain was defined by PFGE, ST, SCC*mec*, and *agr* typing.

air on Trypticase soy agar with 5% sheep blood. All isolates that were morphologically consistent with SCVs of *S. aureus* were subcultured on sheep blood agar at 37°C and incubated both aerobically and in an atmosphere containing 5% $CO_2$. In order to generate an anaerobic atmosphere, we used Genbox anaer (bioMérieux, Lyon, France). Identification tests comprised agglutination with Staphaurex Plus (Remel Europe, United Kingdom), coagulase analysis, and the Wider system (Soria Melguizo, Madrid, Spain). Confirmation of identification was done by sequence analysis of the 16S rRNA gene. Antimicrobial susceptibility testing was performed with Wider panels (Soria Melguizo, Madrid, Spain) (3), based on the broth microdilution method, and was interpreted using the criteria of the Clinical and Laboratory Standards Institute (5). Disk diffusion analysis was also performed at 35°C for oxacillin and cefoxitin, in air and in an atmosphere of 5% $CO_2$. All *S. aureus* SCV isolates underwent PCR analysis for the *mecA* gene (12).

Auxotrophy testing was done as previously described (7). Briefly, auxotrophy for hemin was tested using standard commercial disks, and that for thymidine and menadione was tested by impregnating disks with 15 μl of thymidine (Fluka Biochemika) at 100 μg/ml or menadione (Sigma Aldrich) at 10 μg/ml on Mueller-Hinton agar, respectively.

**Molecular typing and microarray-based genotyping.** Molecular characterization of isolates was performed by pulsed-field gel electrophoresis (PFGE) following DNA extraction and digestion with SmaI according to previously described methods (4). Computer-assisted analysis of electropherograms was carried out with Bionumerics software (Applied Maths, Kortrijk, Belgium). A 1.8% tolerance was used for comparisons of DNA patterns. Additionally, methicillin-resistant isolates of *S. aureus* (MRSA) underwent PCR characterization of the staphylococcal cassette chromosome *mec* (SCC*mec*) (12). All *S. aureus* SCV isolates were analyzed by multilocus sequence typing (MLST) as described previously (6), and sequence types (STs) were assigned by using the MLST website (http//www.mlst.net).

DNA microarrays based on the ArrayTube platform were run according to the manufacturer's instructions (Clondiag, Jena, Germany). The array used in this study covers 334 target sequences comprising allelic variants of approximately 185 distinct genes that include species markers, antimicrobial resistance genes, exotoxins, and genes encoding microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) of the host, as well as the SCC*mec* cassette, capsule, and *agr* group typing markers (9, 10).

## RESULTS

**Case reports and clinical characteristics. (i) Case 1.** A 67-year-old man, with a history of chronic obstructive pulmonary disease (COPD), was admitted to the hospital for elective surgery of gastric adenocarcinoma on 30 July 2006. On the eighth postoperative day, the patient presented with fever of unknown origin and two sets of blood cultures were drawn from a central venous catheter (CVC) and peripheral vein. Positive cultures yielded Gram-positive cocci in pairs and tetrads. The patient was treated empirically with vancomycin, and the CVC was removed. A transesophageal echocardiogram revealed vegetations on the mitral valves with abscess formation. The patient was diagnosed with mitral endocarditis and catheter-associated bacteremia, and rifampin and levofloxacin were added to the treatment. Positive blood samples were subcultured on sheep blood agar at 35°C in an aerobic atmosphere and on chocolate agar at 35°C with 5% $CO_2$. Aerobically grown culture plates examined the next day showed nonpigmented, nonhemolytic microcolonies of a diameter of approximately 0.1 mm. However, in an environment of 5% $CO_2$, the colonies grew with typical staphylococcal morphology. Both aerobic and microaerophilic isolates were identified as *S. aureus* and were resistant to methicillin, levofloxacin, erythromycin, and clindamycin. On the 13th day, a mitral valve replacement was performed, but the patient finally died 15 days after cardiac surgery.

**(ii) Case 2.** A 59-year-old man, with a history of arterial hypertension and obesity, was admitted to the surgery department with a recent diagnosis of unresectable colorectal cancer on 17 July 2006. He had surgery 2 days later for terminal colostomy but presented with signs of necrosis of the stoma. The patient needed surgical reintervention for a new colostomy 6 days later. On the 34th day, his abdominal wound exhibited seropurulent secretion, and he started antimicrobial treatment with amoxicillin-clavulanic acid and metronidazole. Culture of the abdominal wound was performed 3 days later. After 48 h of aerobic incubation at 37°C, the primary isolates on sheep blood agar showed several colony morphologies, although nonpigmented, nonhemolytic, pinpoint colonies predominated. Gram staining of different colonies showed that they were all Gram-positive cocci and all exhibited catalase activity. Subculture of pinpoint colonies onto sheep blood agar in 5% $CO_2$ led to reversion to a pigmented hemolytic colony morphology, while subculture in an aerobic atmosphere continued to yield the same atypical microcolonies. The isolates were identified as *S. aureus* and were resistant to methicillin, levofloxacin, erythromycin, and clindamycin.

Both the above patients were hospitalized in the same ward in adjacent rooms when they were infected. MRSA isolates from the two patients had identical PFGE patterns (Fig. 1),

and further epidemiological investigation showed that case 2 could be the index case for MRSA transmission to case 1 through the health care workers attending the ward.

After these two cases, $CO_2$-dependent *S. aureus* SCV isolates were obtained from another 12 patients. Among the total of 14 cases, the mean age of the patients was 66.2 years (standard deviation [SD], 17.69) and nine (64.3%) were male. Twelve cases (85.7%) were considered hospital-associated infections, and seven (50%) had previously received antibiotics. Twelve patients (85.7%) were considered to have clinical infections while two had only nasal colonization. Two patients died of causes directly related to their $CO_2$-dependent *S. aureus* infections. Selected clinical characteristics of the patients with $CO_2$-dependent *S. aureus* SCVs are shown in Fig. 1.

**Microbiological characteristics of $CO_2$-dependent *S aureus* SCVs.** During the study period 14 clinical isolates of *S. aureus* SCVs were obtained, the microbiological characteristics of which are shown in Table 1 and Fig. 2. Primary isolates of $CO_2$-dependent *S. aureus* after incubation for 24 h in an aerobic atmosphere showed some variability in appearance. In five cases (35.7%), cultures exhibited a very scant growth of only microcolonies, while in another six cases (42.9%) cultures had a heterogeneous appearance with microcolonies surrounding others of normal phenotype. In three cases (21.4%) there was no growth under aerobic conditions. All SCVs reverted to a normal phenotype after incubation in 5% $CO_2$ for 18 h. All SCVs grew anaerobically. The stability of the SCV phenotype was low, and all SCVs reverted to a normal morphology after 3 to 6 subcultures under aerobic conditions. With regard to coagulase activity, four isolates were negative at 4 h, but all were positive at 18 h of incubation. Three isolates were misidentified, two as *Staphylococcus epidermidis* and one as *Micrococcus* spp. All isolates were identified as *S. aureus* by DNA sequence analysis. The susceptibility to methicillin was also correctly reported by broth microdilution and by diffusion disk agar (with the exception of one SCV isolate) as evidenced by the presence of the *mecA* gene in all isolates identified as MRSA.

**Molecular characteristics of $CO_2$-dependent *S. aureus* SCVs.** In order to determine if a specific clone of SCV *S. aureus* was being disseminated in our hospital, the 14 isolates that we obtained were subjected to SmaI-PFGE and MLST. PFGE typing showed that the 14 isolates were distributed into 4 PFGE types that were arbitrarily designated A, B, C, and D, with 4 related subtypes within D (D1 to D4). The characterization of each isolate by MLST and SCC*mec* typing (for MRSA isolates) demonstrated that they belonged to 4 different STs: ST125-SCC*mec* IV (9 MRSA isolates and 1 methicillin-susceptible *S. aureus* [MSSA] isolate), ST30 (2 MSSA isolates), ST34 (1 MSSA isolate), and ST45 (1 MSSA isolate) (Fig. 1).

In order to investigate whether there were differences between the genetic composition of $CO_2$-dependent *S. aureus* and that of those strains that exhibit the normal phenotype, we performed microarray hybridization analysis. The 14 $CO_2$-dependent isolates and 20 *S. aureus* isolates with normal phenotype and which belonged to the same STs were analyzed to identify antibiotic resistance determinants and characterize the presence or absence of genes encoding a variety of different virulence factors (Table 2). Among the genetic markers ana-

TABLE 1. Microbiological characteristics of $CO_2$-dependent *Staphylococcus aureus*

| Isolate no. | Growth and appearance of the culture[a] | Hemolysis (TSBA)[a] | Pigment[a] | % of SCVs isolated in primary culture | Stability of SCVs on culture[b] | Reversion to normal morphology when supplemented with: | | | | Coagulase reaction[c] | | | Wider identification and methicillin resistance (% probability) | Susceptibility to methicillin by diffusion disks (mm and category)[d] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | $CO_2$ | Hemin | Menadione | Thymidine | 2 h | 4 h | 18 h | | Oxacillin | Cefoxitin |
| 1 | 6/10, heterogeneous | Yes, few colonies | Yes, few colonies | 70 | Low | Yes | No | No | No | Pos | Pos | Pos | MRSA (99.9) | 0 (R) | 0 (R) |
| 2 | 1/10, homogeneous | No | No | 100 | Low | Yes | No | No | No | Neg | Neg | Pos | MRSA (99.9) | 0 (R) | 0 (R) |
| 3 | 3/10, heterogeneous | No | No | 80 | Low | Yes | No | No | No | Pos | Pos | Pos | MSSA (99.7) | No growth | No growth |
| 4 | 2/10, heterogeneous | No | No | 80 | Low | Yes | No | No | No | Pos | Pos | Pos | MSSA (99.8) | 22 (S) | 29 (S) |
| 5 | 1/10, homogeneous | No | No | 100 | Low | Yes | No | No | No | Neg | Neg | Pos | MRSA (99.9) | 0 (R) | 0 (R) |
| 6 | 3/10, heterogeneous | Yes, few colonies | Yes, few colonies | 70 | Low | Yes | No | No | No | Pos | Pos | Pos | MRSA (99.8) | 0 (R) | 0 (R) |
| 7 | No growth | | | | Low | Yes | No | No | No | Neg | Pos | Pos | *Micrococcus* sp. (94.8) | 0 (R) | 0 (R) |
| 8 | 1/10, homogeneous | No | No | 100 | Low | Yes | No | No | No | Neg | Pos | Pos | MRSA (99.9) | 0 (R) | 0 (R) |
| 9 | 1/10, homogeneous | No | No | 100 | Low | Yes | No | No | No | Neg | Pos | Pos | MRSA (99.3) | 0 (R) | 0 (R) |
| 10 | No growth | | | | Low | Yes | No | No | No | Neg | Neg | Pos | *S. epidermidis* (99.8) | 26 (S) | 29 (S) |
| 11 | No growth | | | | Low | Yes | No | No | No | Neg | Pos | Pos | *S. epidermidis* (99.8) | 0 (R) | 0 (R) |
| 12 | 1/10, homogeneous | No | No | 100 | Low | Yes | No | No | No | Neg | Pos | Pos | MSSA (99.9) | 27 (S) | 29 (S) |
| 13 | 2/10, heterogeneous | No | Yes, few colonies | 70 | Low | Yes | No | No | No | Pos | Pos | Pos | MSSA (99.9) | 23 (S) | 25 (S) |
| 14 | 7/10, heterogeneous | Yes | Yes | 50 | Low | Yes | No | No | No | Neg | Neg | Pos | MRSA (99.9) | 0 (R) | 0 (R) |

[a] Characteristics of growth in Trypticase soy blood agar (TSBA) after 24 h of incubation at 35°C in an aerobic atmosphere. Growth is scored as 0 to 10, with 10 being growth of the culture on TSBA at 35°C in 5% $CO_2$.

[b] After 3 to 6 subcultures, the SCVs reverted to a normal phenotype.

[c] Pos, positive; Neg, negative.

[d] R, resistant; S, susceptible.

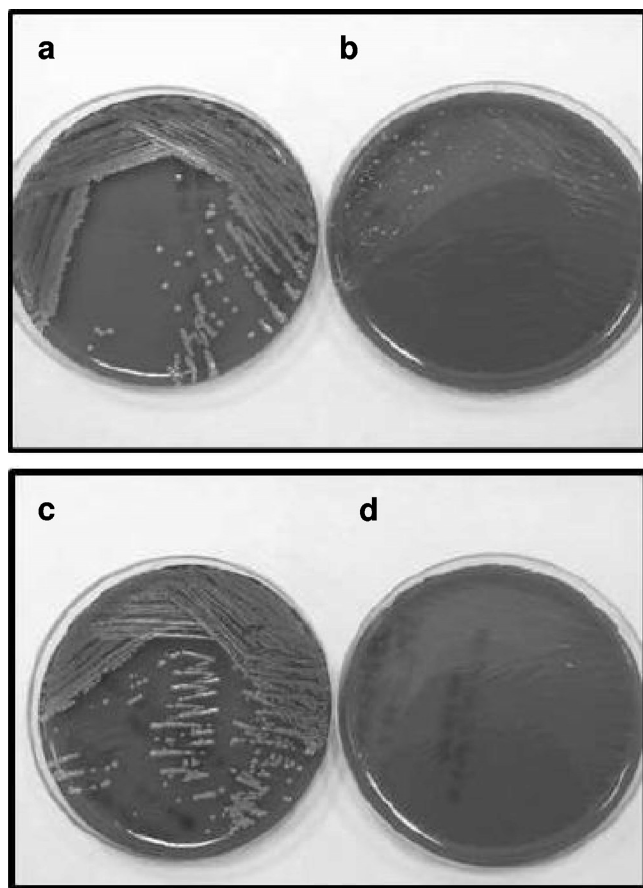


FIG. 2. Blood agar plates of two pairs of *Staphylococcus aureus* isolates (plates a and b and plates c and d) that show the normal (a and c) and the small-colony variant (b and d) phenotypes.

lyzed, none were present in all isolates with the normal phenotype that were not also present in the $CO_2$-dependent *S aureus* isolates and vice versa. All isolates, independent of phenotype, had in common the enterotoxin gene cluster *egc* (*seg*, *sei*, *sem*, *sen*, *seo*, and *seu*); α-hemolysin; δ-hemolysin; biofilm genes *icaA*, *icaC*, and *icaD*; several MSCRAMM genes (*clfA*, *clfB*, *ebh*, *eno*, *fib*, *ebpS*, *sdrC*, and *vw*); and the *isaB* gene. However, 8 of 10 clinical isolates of $CO_2$-dependent *S. aureus* SCVs belonging to ST125 more frequently had a full-length (untruncated) version of the hemolysin gene compared with 0/8 with normal phenotype. Furthermore, these $CO_2$-dependent isolates were all negative for genes encoding *entA*, *sak*, *chp*, and *scn*, while seven of the isolates with normal phenotype were positive for these genes and one was positive for *sak* and *scn*. Another difference was that only 1 of 10 $CO_2$-dependent isolates had the β-lactamase operon (*blaZ*, *blaI*, and *blaR*), while this operon was present in 6 of 8 isolates with normal morphological phenotype (Table 2).

Among $CO_2$-dependent *S. aureus* isolates, besides the variability in PFGE patterns, STs, and *agr* groups (I, II, and III), we also identified variability (presence or absence) in the antibiotic determinant genes (*mecA* and *blaZIR*), β-hemolysin, enterotoxin A, and the genes encoding staphylokinase (*sak*), chemotaxis inhibitory protein (*chp*), and staphylococcal complement inhibitor (*scn*). Overall, the $CO_2$-dependent isolates presented several differences in their array hybridization patterns, with the exception of isolates 1 and 2, which exhibited identical patterns.

## DISCUSSION

We have recently observed isolates of *S. aureus* growing as dwarf colonies or showing heterogenous appearance in culture, with satellite growth of microcolonies around others of apparently normal phenotype. These clinical isolates comply with all the requisites to be defined as SCVs of *S. aureus*. We demonstrated that 14/14 (100%) of the clinical isolates that we recovered over a 3-year period which exhibited this phenotype were $CO_2$ auxotrophs. Previous studies have demonstrated that SCVs are most frequently associated with menadione and thiamine deficiency, and only occasionally have they been reported to exhibit $CO_2$ dependency (14). The stability of the microcolony phenotype in our isolates was low, and all reverted to a normal colony morphology after several subcultures. This suggests that the exhibition of this phenotype is governed at the level of gene expression, rather than by the presence or absence of one or more specific genes.

The real incidence of infection/colonization by $CO_2$-dependent *S. aureus* is unknown. While our data suggest that the prevalence of these strains is low, with only 14 isolates in a period of almost 3 years, this may represent an underestimation of the true rate of new infections because not all cultures are incubated in a 5% $CO_2$ atmosphere. $CO_2$ SCVs are characterized by fastidious growth characteristics and an atypical, small-colony morphology (nonpigmented and nonhemolytic colonies) that may be entirely overlooked in mixed cultures. When they are recognized, SCVs may still be misidentified as coagulase-negative *Staphylococcus*, and susceptibility test results can be difficult to interpret. To reduce the potential of missing these isolates, it is important to observe the microbiological characteristics of colonies on plates incubated in a $CO_2$ atmosphere and to extend conventional cultures to at least 72 h. Nasal samples used to detect MRSA colonization are usually inoculated on mannitol-salt agar and incubated in an aerobic atmosphere for 48 h. Therefore, colonization with $CO_2$-dependent *S. aureus* can be missed if incubation is not extended and plates are not supplemented with $CO_2$. We recommend incubating at least one agar plate in a $CO_2$ atmosphere for all samples with suspicion of *S. aureus*. Some authors also suggest using Columbia blood agar and chromogenic agar media for recovery and identification of *S. aureus* SCV auxotrophs for thymidine (8). On the other hand, SCVs also present a challenge with regard to susceptibility testing. Errors can occur when these variants are resistant to oxacillin and are tested using disk diffusion methods (14). In these cases, sometimes it is necessary to increase the inoculum of bacteria and to detect the *mecA* gene by molecular methods (14). SCV isolates may also be obtained from patients with a variety of clinical manifestations, including severe infections such as catheter-related bacteremia, endocarditis, and surgical or respiratory infections in addition to nasal colonization.

In the present study, we identified two patients who were

TABLE 2. Antibiotic resistance determinants and virulence factors in *Staphylococcus aureus* with SCVs and normal phenotype

| Gene category and name | Gene product or function | Result for MLST sequence type and auxotrophy[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ST125 | | ST30 | | ST34 | | ST45 | |
| | | $CO_2$ ($n$ = 10) | No ($n$ = 8) | $CO_2$ ($n$ = 2) | No ($n$ = 6) | $CO_2$ ($n$ = 1) | No ($n$ = 2) | $CO_2$ ($n$ = 1) | No ($n$ = 4) |
| Resistance genotype | | | | | | | | | |
| *mecA* | Methicillin, oxacillin, and all beta-lactams, defining MRSA | 9/10 | + | − | − | − | − | − | − |
| *blaZIR* | Beta-lactamase operon | 1/10 | 6/8 | + | + | + | + | + | + |
| *ermC* | Macrolide, lincosamide, streptogramin | 3/10 | 2/8 | − | − | − | − | − | − |
| *msrA* | Macrolide | − | 5/8 | − | − | − | − | − | − |
| *mpbBM* | Macrolide | − | 5/8 | − | − | − | − | − | − |
| *aadD* | Aminoglycoside (tobramycin, neomycin) | 6/10 | + | − | − | − | − | − | − |
| *aphA* | Aminoglycoside (kanamycin, neomycin) | − | 5/8 | − | − | − | − | − | − |
| *sat* | Streptothricin | − | 5/8 | − | − | − | − | − | − |
| *tetK* | Tetracycline | − | − | − | − | + | − | − | − |
| *tetE* efflux | Tetracycline efflux protein (putative transport protein) | + | + | + | + | + | + | + | + |
| *cat* | Chloramphenicol | − | 1/8 | − | − | − | − | − | − |
| *fosB* | Putative marker for fosfomycin, bleomycin | + | + | + | + | + | + | − | − |
| *qacC* | Unspecific efflux pump | − | 1/8 | − | − | − | − | − | − |
| Virulence genotype | | | | | | | | | |
| *tst-1* | Toxic shock syndrome toxin | − | − | 1/2 | 4/6 | + | + | − | − |
| *entA* | Enterotoxin A | − | 7/8 | + | 2/6 | − | − | − | − |
| *entA*-320E | Enterotoxin A, allele from 320E | − | − | 1/2 | 1/6 | − | − | − | − |
| *entA*-N315 | Enterotoxin A, allele from N315 | − | 7/8 | − | − | − | − | − | − |
| *entC* | Enterotoxin C | − | − | − | − | − | − | + | + |
| *entH* | Enterotoxin H | − | − | − | − | + | + | − | − |
| *entL* | Enterotoxin L | − | − | − | − | − | − | + | + |
| *egc* cluster | Enterotoxins from *seg*, *sei*, *sem*, *sen*, *seo*, and *seu* | + | + | + | + | + | + | + | + |
| PVL | Panton-Valentine leukocidin | − | − | − | 1/6 | − | − | − | − |
| *lukF* | γ-Hemolysin, component B | + | + | + | + | + | + | + | + |
| *lukS* | γ-Hemolysin, component C | + | + | + | + | + | + | − | 1/4 |
| *hlgA* | γ-Hemolysin, component A | 7/10 | + | − | 2/6 | − | + | − | + |
| *lukD* | Leukocidin D component | + | + | − | − | − | − | − | − |
| *lukE* | Leukocidin E component | 8/10 | 7/8 | − | − | − | − | − | − |
| *lukX* | Leukocidin/hemolysin toxin family protein | 6/10 | 7/8 | 1/2 | 4/6 | + | + | − | + |
| *hla* | α-Hemolysin (alpha toxin) | + | + | + | + | + | + | + | + |
| *hld* | δ-Hemolysin | 9/10 | + | + | + | + | + | + | + |
| *hlb* | β-Hemolysin (phospholipase C) | 1/10 | 4/8 | − | 3/6 | − | + | − | − |
| Untruncated *hlb* | β-Hemolysin (phospholipase C/untruncated) | 8/10 | − | − | 2/6 | − | 1/2 | − | − |
| *sak* | Staphylokinase | − | + | + | 4/6 | + | 1/2 | + | + |
| *chp* | Chemotaxis inhibitory protein (CHIPS) | − | 7/8 | + | 3/6 | + | 1/2 | + | + |
| *scn* | Staphylococcal complement inhibitor | − | + | + | 4/6 | + | 1/2 | + | + |
| *aur* | Aureolysin | 9/10 | + | + | + | + | + | − | − |
| *aur* (other than 252) | Aureolysin, allele from other than MRSA252 | + | + | − | − | − | − | − | − |
| *aur*-MRSA252 | Aureolysin, allele from MRSA252 | − | − | + | + | + | + | + | + |
| *splA* | Serine protease A | + | + | − | − | − | − | − | − |
| *splB* | Serine protease B | + | + | − | − | − | − | − | − |
| *splE* | Serine protease E | − | − | + | 4/6 | + | + | − | − |
| *sspA* | Glutamyl endopeptidase/V8 protease | 9/10 | + | + | + | + | + | + | + |
| *sspB* | Staphopain B | + | + | + | + | + | + | + | + |
| *sspP* | Staphopain A (staphylopain A) | + | + | + | + | + | + | + | + |
| Capsule/biofilm | | | | | | | | | |
| Capsule 5 | Capsule type 5 | + | + | − | − | − | − | − | − |
| Capsule 8 | Capsule type 8 | − | − | + | + | + | + | + | + |
| *icaA*, *icaC*, *icaD* | Intercellular adhesion proteins A and C, biofilm PIA[b] synthesis protein D | + | + | + | + | + | + | + | + |
| MSCRAMMs/adhesion factors | | | | | | | | | |
| *bbp*-all | Bone sialoprotein-binding protein | + | 7/8 | + | 5/6 | + | 1/2 | − | + |
| *clfA*-all | Clumping factor A | + | + | + | + | + | + | + | + |
| *cna* | Collagen-binding adhesin | − | − | + | + | − | − | + | + |
| *ebh*-all | Cell wall-associated fibronectin-binding protein | + | + | + | + | + | + | + | + |

*Continued on following page*

TABLE 2—*Continued*

| Gene category and name | Protein | Result for MLST sequence type and auxotrophy[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ST125 | | ST30 | | ST34 | | ST45 | |
| | | $CO_2$ (*n* = 10) | No (*n* = 8) | $CO_2$ (*n* = 2) | No (*n* = 6) | $CO_2$ (*n* = 1) | No (*n* = 2) | $CO_2$ (*n* = 1) | No (*n* = 4) |
| *eno* | Enolase, phosphopyruvate hydratase | + | + | + | + | + | + | + | + |
| *fib* | Fibrinogen-binding protein | + | + | − | − | − | − | − | − |
| *fib*-MRSA252 | Fibrinogen-binding protein, allele from MRSA252 | − | − | + | + | + | + | + | 3/4 |
| *ebpS* | Cell wall-associated fibronectin-binding protein | + | + | + | + | + | + | + | + |
| *fnbA*-all | Fibronectin-binding protein A | + | + | + | + | + | + | + | + |
| *fnbB*-COL+Mu50+MW2 | Fibronectin-binding protein B, allele from COL/Mu50/MW2 | + | + | − | 1/6 | − | − | + | + |
| *fnbB*-Mu50 | Fibronectin-binding protein B, allele from Mu50 | + | + | − | 1/6 | − | − | − | + |
| *map* | Major histocompatibility complex class II analog protein | + | + | + | + | + | + | + | + |
| *sdrC*-all | Ser-Asp-rich fibrinogen-binding, bone sialoprotein-binding protein C | + | + | + | + | + | + | + | + |
| *sdrD*-COL+MW2 | Ser-Asp-rich fibrinogen-binding, bone sialoprotein-binding protein D | − | − | − | − | − | − | − | + |
| *sdrD*-Mu50 | Ser-Asp-rich fibrinogen-binding, bone sialoprotein-binding protein D, allele from Mu50 | 8/10 | + | − | − | − | − | − | − |
| *sdrD*-other | Ser-Asp-rich fibrinogen-binding, bone sialoprotein-binding protein D, allele from other | − | − | + | 4/6 | + | + | − | − |
| *sdrD*-other than 252 + 122 | Ser-Asp-rich fibrinogen-binding, bone sialoprotein-binding protein D, allele from other than MRSA252/RF122 | + | + | + | 5/6 | + | + | − | + |
| *vwb*-all | von Willebrand factor-binding protein | + | + | + | + | + | + | − | − |
| *vwb*-MRSA252 | von Willebrand factor-binding protein, allele from MRSA252 | − | − | + | + | + | + | + | + |
| *vwb*-Mu50 | von Willebrand factor-binding protein, allele from MU50 | + | + | − | − | − | − | − | − |
| *sasG* | *S. aureus* surface protein G | + | + | − | − | − | − | − | − |
| Immunoevasion and miscellaneous | | | | | | | | | |
| *isaB* | Immunodominant antigen B | + | + | + | + | + | + | + | + |
| *mprF* | Probable lysylphosphatidylglycerol synthetase (defensin resistance) | 7/10 | + | 1/2 | 5/6 | + | + | − | − |
| *isdA* | Heme/transferrin-binding protein | + | + | + | + | + | + | + | + |
| *lmrP* | Putative transporter protein | + | + | + | + | + | + | + | + |

[a] In cases where the gene was not present in all isolates, data are shown as number of isolates with the gene present/total number of isolates.
[b] PIA, polysaccharide intercellular adhesin.

admitted to the same clinical ward and who were infected with the same strain of SCV *S. aureus*, as demonstrated by MLST, PFGE, and DNA microarray analysis. The secondary case developed catheter-related bacteremia complicated with endocarditis, and the patient finally died in spite of treatment with vancomycin and rifampin and valve replacement. This was an example of nosocomial transmission with important clinical consequences. Although several studies using animal models have indicated that *S. aureus* SCVs might be less virulent than strains with the normal phenotype, as determined by measurement of lethal doses and fatality rates, under the right conditions, SCVs can persist for long periods, thereby facilitating recurrent infections and clinical complications (14). Some authors propose that the formation of SCVs is a strategy used by the bacterium to resist antibiotic therapy by becoming a facultative intracellular pathogen (25). Compared to typical *S. aureus* strains, SCVs show increased uptake by host cells, as well as resistance to and reduced stimulation of intracellular host defenses (14, 18).

In order to gain insights into the genetic makeup of $CO_2$-dependent *S. aureus* SCVs, we first investigated the clonality of these isolates. We observed that SCV isolates were primarily nonclonal in origin, belonging to different sequence types (ST125, ST30, ST34, and ST45). Notably, clones belonging to these STs and exhibiting a normal phenotype were also circulating in our community and hospital. We also studied the antibiotic resistance and virulence determinants of these isolates by using an array hybridization assay. No common pattern of genetic markers was evident, and we found remarkable variability with regard to antibiotic resistance markers, *agr* groups, SCC*mec* types, exotoxin genes, capsule types, and MSCRAMM genes. With few exceptions,

isolates that belonged to the same ST shared almost the same array hybridization pattern, independent of the phenotype (SCV or not). We found in all SCV isolates belonging to ST125 that the genes encoding *entA*, *sak*, *chp*, and *scn* were absent, while these genes were present in most isolates with normal phenotype. What accounts for the differences is the fact that these genes are carried by β-hemolysin-converting bacteriophages, which leads to the disruption of the β-hemolysin (22). Probably, any *S. aureus* strain belonging to any ST can develop SCV $CO_2$ auxotrophy, although we do not know what alterations in bacterial metabolism may cause this type of variant or the genetic mechanism for reversion to a rapidly growing form. In 1970 investigators who studied two strains of $CO_2$-dependent microcolony variants of *S. aureus* by electron microscopy showed that the cell walls of the isolates when grown in air were thick and irregular (19). In contrast, in a $CO_2$ atmosphere, their ultrastructure appeared to be that of normal staphylococci.

Our study confirms the importance of $CO_2$-dependent SCVs of *S. aureus* as significant pathogens in a variety of clinical presentations, ranging from severe infections such as catheter-related bacteremia, endocarditis, and surgical or respiratory infection to nasal colonization. Clinical microbiologists must be made aware of this kind of auxotrophy because of the difficulties in isolating these variants by using routine laboratory methods. Nevertheless, further studies will be necessary to understand the true prevalence of $CO_2$ auxotrophs of *S aureus*, the factors which select this phenotype in the host, and the genetic basis of this type of auxotrophy.

## ACKNOWLEDGMENTS

We thank Tobin Hellyer for reviewing the manuscript.

This work was supported by the Spanish Network for Research in Infectious Diseases (REIPI RD06/0008/0011) and by PI07/90239 and PI081520 from the Instituto de Salud Carlos III, Spain.

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