Characterization of Small-Colony-Variant *Stenotrophomonas maltophilia* Isolated from the Sputum Specimens of Five Patients with Cystic Fibrosis

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Cystic fibrosis (CF) patients are predisposed to chronic respiratory infection by nonfermentative gram-negative bacilli, including *Stenotrophomonas maltophilia*. *S. maltophilia* is highly resistant to most antibiotics, with the exception of sulfamethoxazole-trimethoprim (SXT). SXT-resistant *S. maltophilia* has been reported, but the mechanism of resistance is not well defined. Repeated findings of suspected small-colony-variant (SCV) *S. maltophilia* isolates from the sputa of five CF patients were confirmed by partial 16S rRNA gene sequencing. The SCV *S. maltophilia* isolates were the only *S. maltophilia* isolates in these cultures, and none were clonally related. DNA fingerprint analysis confirmed that once established, the SCV *S. maltophilia* strains persisted. Nutritional studies of SCV *S. maltophilia* have suggested auxotrophy in hemin, methionine, and thymidine associated with resistance to multiple antibiotics, including SXT. The phenotypic switch from wild-type to SCV *S. maltophilia* was reproducible in vitro by exposure to SXT, suggesting that prolonged exposure to antibiotics may select for both the SCV *S. maltophilia* phenotype and SXT resistance by interference with the dihydrofolate reductase pathway. Recovery of SCV *S. maltophilia* from the sputum of CF patients has implications for both laboratory testing and patient management.

Cystic fibrosis (CF) is an inherited genetic disorder that results in production of viscous mucus in the airway, with eventual infection of the respiratory tract by nonfermentative gram-negative bacilli, including *Pseudomonas aeruginosa*, the *Burkholderia cepacia* complex, and *Stenotrophomonas maltophilia*. Once established in the lungs, these infections are highly resistant to antibiotic treatment and contribute to a gradual decline in lung function. The pathogenic effects of *P. aeruginosa* and *B. cepacia* complex in individuals with CF have been well described (8). In contrast, the significance of *S. maltophilia* in CF airway disease has not been determined. It has been demonstrated that *S. maltophilia* is capable of long-term airway colonization (21), and anecdotal reports from clinicians suggest that its contribution to morbidity may be significant. However, identification of a consistent link between *S. maltophilia* culture positivity and deterioration in lung function is lacking (7, 9, 10, 21). Like *B. cepacia* complex, *S. maltophilia* is highly resistant to most antibiotics, with the notable exception of sulfamethoxazole-trimethoprim (SXT). This may contribute to its persistence and potential for causing morbidity in CF.

Small-colony variants (SCV) of several CF pathogens, including *Staphylococcus aureus* and *P. aeruginosa*, have been reported (11, 12). The SCV phenotype is characterized by small colony size, slow growth on agar media compared to wild-type isolates, and the inability to generate in vitro susceptibility results (broth MIC, Kirby-Bauer, or Etest) under standard conditions as defined by CLSI (Clinical and Laboratory Standards Institute) (4). Recent work with *S. aureus* and *Escherichia coli* has suggested that the switch to the SCV phenotype is a response to antibiotic pressure, involving down-regulation of the bacterial electron transport and/or dihydrofolate reductase (DHFR) pathway (12, 15, 20). Individuals with CF receive frequent courses of antibiotics; thus, adaptation to conditions in the CF airway is likely to result in the SCV phenotype via a similar mechanism of nutritional dependency and growth retardation.

In the laboratory of the Seattle CF Center at Children’s Hospital and Regional Medical Center, we recently recognized a novel, SCV form of *S. maltophilia* from CF sputum specimens. The clustering of SCV *S. maltophilia* isolates in sputum samples from five separate patients over a 2-month period prompted further evaluation to ensure that there was neither person-to-person spread nor laboratory contamination. To characterize these isolates, we performed genotyping and evaluated growth kinetics, nutritional requirements, and antibiotic susceptibility.

**MATERIALS AND METHODS**

**Isolate characterization.** Suspected SCV *S. maltophilia* isolates recovered from CF clinical sputum cultures were identified through a short set of biochemical reactions: positive for maltose oxidation and lysine decarboxylation but nonreactive in triple-sugar-iron agar slants, as well as a negative or weak oxidase reaction. Identification was confirmed by 16S rRNA gene amplification and partial sequencing (14, 17, 18). For initial characterization, all SCV *S. maltophilia* isolates were compared to a wild-type *S. maltophilia* strain (ATCC 51331) based on growth characteristics after 48 h of incubation at 35°C in ambient air on sheep

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blood agar (SBA), chocolate agar, brucella agar, and MacConkey agar plates (Remel, Lenexa, KS).

**Growth and auxotrophy.** Fresh growth of SCV *S. maltophilia* isolates on SBA plates was suspended in sterile saline to a density equivalent to a 0.5 McFarland standard and streaked confluentely with a cotton swab on M9 minimal agar plates (Teknova, Hollister, CA). Filter paper disks containing 10 μl of aqueous thymidine, methionine, or menadione solutions at the limit of aqueous solubility (48 mg/ml, 4 mg/ml, and 272 mg/ml, respectively) were then applied to the inoculated plates. Additionally, a paper strip containing hemin (Taux X Factor strips; Becton Dickinson, Sparks, MD) was applied to each plate. The plates were incubated (35°C, ambient air) and checked for growth daily for a maximum of 6 days. Nutritionally dependent growth of the SCV *S. maltophilia* isolates was analyzed with single-agent discs/strips placed distally (>35 mm) or proximally (10 to 15 mm) to one another.

**Growth rate assay.** Three *S. maltophilia* isolates (strain ATCC 51331, a phenotypically wild-type CF clinical isolate, and an SCV CF clinical isolate) were incubated overnight, and turbidity was adjusted to a 0.5 McFarland standard in 200-ml aliquots of warmed brain heart infusion broth (Remel). The broth cultures were aerated with 100 rpm motion during incubation (Barnstead Lab-Line Shaker, 35°C, ambient air), and turbidity (absorbance at 600 nm) was measured with a spectrophotometer (Spectronic 20D+; ThermoSpectronic Corp.) at multiple time points through a total of 80 h.

**Genotyping.** Genetic DNA fingerprinting of SCV *S. maltophilia* isolates was performed primarily by enterobacterial repetitive intergenic consensus (ERIC) PCR using primer ERIC2, 5'-AAGTAAGTGACTGGGGTGAGCG-3' (6), and isolates from the five patients were confirmed by pulsed-field gel electrophoresis (PFGE) using XbaI restriction fragmentation (21). For ERIC PCR, bacterial DNA was amplified by standard PCR techniques, and the resultant amplification products were analyzed based on mobility profiles by gel electrophoresis. Isolates that differed by two or more bands were interpreted as unrelated (1, 21).

**MIC determinations.** The MICs of SXT, levofloxacin, and minocycline for SCV *S. maltophilia* isolates were determined with Etest strips (AB Biodisk, Piscataway, NJ). Cation-adjusted Mueller-Hinton (CAMH) agar or broth, *Haemophilus* test medium (HTM), and chocolate agar plates (PML Microbiologicals, Wilsonville, OR) were employed to assess the effects of the nutritional complexity of growth media on the MICs. The test plates were incubated for up to 48 h, accommodating the slow growth of the SCV *S. maltophilia* isolates. *E. coli* ATCC 25922 was used to control for Etest MIC reference values on HTM and chocolate agar, in addition to the standard CAMH agar.

FIG. 1. Comparison of bacterial growth between a wild-type strain (ATCC 51331) and a typical SCV *S. maltophilia* isolate from patient A on various routine agar media after 48 h of incubation at 35°C. ATCC 51331 was inoculated to the left of the SCV *S. maltophilia* isolate. BSA, sheep blood agar; Choc, chocolate agar; Bru, brucella agar; Mac, MacConkey agar.
Antibiotic induction of the SCV phenotype in vitro. A 1:1,000 dilution of a 0.5 McFarland standard-adjusted saline suspension of S. maltophilia ATCC 51331 was used to inoculate CAMH broth containing either tobramycin or SXT at 0.5 times the MIC (2 μg/ml of sulfamethoxazole, 0.4 μg/ml of trimethoprim, and 256 μg/ml of tobramycin). The resultant broth cultures were incubated (35°C, ambient air), and aliquots were plated on SBA at day 1, day 2, and finally at day 20. The subcultured plates were incubated for 2 days, and suspected SCV S. maltophilia colonies were streaked for isolation on fresh SBA plates. On this subculture plate, the parent strain was streaked alongside the suspected SCV S. maltophilia colony for the purpose of side-by-side comparison of colony sizes. The SCV S. maltophilia colonies generated in the induction assay described above were confirmed as S. maltophilia by partial 16S rRNA gene sequencing. Genetic relatedness between the parent strain and the descendant SCV S. maltophilia isolates was confirmed by DNA fingerprinting with the products of ERIC PCR.

RESULTS

Bacterial isolates. Suspected SCV S. maltophilia isolates were isolated in sputum cultures from a cluster of five CF patients seen at the same clinic over a 2-month period in 2004. Four of the subjects had two colony sizes of SCV S. maltophilia in a single sputum sample, and one had a single SCV S. maltophilia morphotype; none had wild-type S. maltophilia isolated from the sample as the index SCV S. maltophilia. Patients C, D, and E also had documented wild-type S. maltophilia isolates prior to their SCV S. maltophilia findings, while patient B was a recent transfer from another CF center.

Comparison of S. maltophilia wild-type and SCV phenotypes on various media and growth restoration by nutritional supplementation. The SCV S. maltophilia isolates exhibited significantly less growth on SBA, brucella agar, and chocolate agar, and no visible growth on MacConkey agar, compared with ATCC 51331 (Fig. 1). All SCV S. maltophilia isolates also showed no visible growth on M9 minimal medium. Visible growth of SCV S. maltophilia on M9 medium was supported by the supplementation of hemin, thymidine, or methionine alone but was not supported by menadione. Growth was enhanced in the shared area between the three discs containing hemin, thymidine, and methionine (Fig. 2).

Growth curves. Results of a representative growth curve experiment are shown in Fig. 3. The wild-type ATCC strain and the wild-type S. maltophilia CF clinical isolate exhibited similar growth kinetics, as both reached stationary phase by 24 h. In contrast, the SCV S. maltophilia clinical isolate showed only a slight increase in optical density between 56 and 80 h, suggesting growth impairment in broth culture that correlated with the SCV phenotype on solid media.
Genetic fingerprinting of SCV *S. maltophilia* isolates. DNA fingerprinting by ERIC PCR was first performed on the cluster of isolates obtained from five patients during a 2-month period to examine the relatedness of the phenotypically similar SCV *S. maltophilia* isolates (data not shown). Based on the interpretive criteria described above (see Materials and Methods), the SCV *S. maltophilia* isolates from each of the five patients were sufficiently different, as later confirmed by PFGE (Fig. 4). Interestingly, the banding patterns of the SCV *S. maltophilia* isolates from any single patient are identical. The two strains from patient B (B1 and B2) were very slow growing, and only B2 produced enough discriminative DNA material on PFGE. However, ERIC PCR results for these two isolates from patient B suggested that they were clonally related. The independent clonal types among patients suggested that person-to-person transmission of a single strain of SCV *S. maltophilia* was unlikely, as was the possibility of laboratory contamination introduced at the time of culture setup.

Archived isolates from patient A were further examined. The persistence of SCV *S. maltophilia* in that patient over a 4-year period was demonstrated by a specific fingerprinting pattern on multiple isolates (Fig. 5). Of the 18 isolates from a...
total of 11 longitudinally collected sputum samples, 15 were recognized, by growth and colony size, as SCV S. maltophilia. Three transiently recovered wild-type S. maltophilia strains, two from January 2001 and one from March 2002, demonstrated two unique ERIC PCR patterns that were time specific and genotypically distinct, separated by 14 months.

**MIC determinations under nonstandard conditions.** Susceptibility testing of a single SCV S. maltophilia isolate from each patient failed to produce results by any method (disk diffusion, Etest, or broth microdilution MIC) when inoculated onto CAMH medium and incubated for 18 to 24 h, as specified by CLSI (4), or for up to 48 h (data not shown). HTM agar supported the growth of SCV S. maltophilia isolates from only two of the five subjects after 48 h of incubation, while chocolate agar supported the growth of all isolates after 48 h of incubation. MICs of levofloxacin, SXT, and minocycline were determined by using Etest strips on chocolate agar at 48 h. The results are summarized in Table 1. Irrespective of the medium and incubation time, reference Etest MICs of SXT and levofloxacin against E. coli ATCC 25922 fell within the acceptable ranges permitted under standard conditions (5), but those of minocycline did not. Assuming the stability of the antibiotics and the diffusion equilibrium established upon extended incubation (48 h), these results suggest that none of the SCV S. maltophilia isolates were resistant to minocycline and that only one of the five was resistant to levofloxacin, as defined by CLSI breakpoints (5).

**Induction of the SCV phenotype in antibiotic-containing medium.** At time points on day 1, day 2, and day 20, the antibiotic-containing S. maltophilia cultures were subcultured onto SBA plates. The day 20 subculture of the SXT-treated culture yielded a small number of SCV colonies. No SCV S. maltophilia colonies were recovered from any of the tobramycin or preceding SXT subcultures.

**DISCUSSION**

The microbiology laboratory of Children’s Hospital and Regional Medical Center processes a large number of CF respiratory specimens annually. The recognition of SCV forms of diverse CF pathogens is now a frequent occurrence. Molecular techniques developed in this laboratory and others have enabled reliable species identification of these small-colony isolates, which has led to their characterization (17, 22). However, even with correct identification, the lack of available methods for susceptibility testing of SCV isolates adds to the difficulty of patient treatment in CF.

It is worth noting that the term “small-colony-variant S. maltophilia,” as we apply it to these isolates, represents a spectrum of growth characteristics and morphotypes distinct from those of the wild type, rather than a single set of phenotypic parameters. At present, based on the characterization of only a small number of isolates, we define SCV S. maltophilia as follows: (i) viable growth by small colony sizes consistently observed on rich media only, such as SBA or chocolate agar, after prolonged incubation; (ii) little or no growth on MacConkey agar medium after 48 h; (iii) biochemically inert for phenotypic identification; and (iv) intractability to standard susceptibility tests due to slow growth and/or the inability to grow in Mueller-Hinton media. As more SCV S. maltophilia morphotypes are isolated and characterized, presumably a consensus will evolve for the definition of SCV S. maltophilia isolates.

The present study examined the molecular epidemiology and phenotypic characteristics of SCV S. maltophilia isolates from five individuals with CF at our center. In addition, a subset of the isolates was used to evaluate nonstandard methods for susceptibility testing of SCV S. maltophilia strains. Distinctive genetic fingerprinting patterns ruled out the possibility of either patient-to-patient spread among the five patients or a single-source outbreak. This finding also ruled out specimen carryover contamination due to material handling in the laboratory.

Similar growth kinetics were demonstrated for a CF clinical isolate of S. maltophilia and the ATCC 51331 type strain (both with wild-type colonial morphologies), each reaching stationary phase by 24 h. In contrast, the SCV S. maltophilia culture showed no significant increase in optical density until after 48 h and was unable to reach stationary phase by the final time point at 80 h. This assay confirmed, in a quantitative fashion, that the SCV S. maltophilia isolates characterized on agar media were the result of their impaired growth rate or prolonged cell doubling time.

Although SCV forms of S. aureus and P. aeruginosa have been reported from CF specimens (11, 12, 19), this is the first report of SCV S. maltophilia in the CF population. This is

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<th>Isolate</th>
<th>Levofloxacin</th>
<th>SXT</th>
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<td>S. maltophilia ATCC 51331</td>
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<td>A (SCV)</td>
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<td>B (SCV)</td>
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<sup>a</sup> RI, reincubated due to inadequate growth at the indicated time point for MIC determination; NG, no growth.

<sup>b</sup> Acceptable range of levofloxacin MICs for E. coli ATCC 25922 incubated according to CLSI standards: 0.008 to 0.06 μg/ml.

<sup>c</sup> Acceptable range of SXT MICs for E. coli ATCC 25922 incubated according to CLSI standards: ≤0.5 to 9.5 μg/ml.

<sup>d</sup> Acceptable range of minocycline MICs for E. coli ATCC 25922 incubated according to CLSI standards: 0.25 to 1 μg/ml.
particularly important because of the implications of our inability to perform standard susceptibility testing against a highly antibiotic-resistant pathogen such as *S. maltophilia*. The three non-β-lactam agents recommended by CLSI for in vitro testing with this organism are SXT, levofloxacin, and minocycline (5). However, in vitro SXT susceptibility is markedly dependent on the medium used for testing. Since the antibacterial activity of SXT is exerted by inhibition of the DHFR pathway, the use of medium that supplies the end product of this pathway results in inaccurate MICs. In addition, the slow growth rate of SCV *S. maltophilia* under CLSI-recommended conditions precludes MIC determinations. For these reasons, SCV *S. maltophilia* isolates recovered from clinical specimens at our institution are reported with the following comment: “In vitro susceptibility is not valid for this organism. Thymidine auxotrophy suggests sulfamethoxazole-trimethoprim resistance”.

There are important clinical management implications for CF caregivers confronted with an organism known to be highly antibiotic resistant but for which susceptibility testing cannot be performed. This situation becomes more complicated when the first-line antibiotic (SXT) may not be clinically efficacious. For these reasons, we sought to use modified growth conditions for in vitro susceptibility testing and to test antibiotic alternatives to SXT. The favorable in vitro activities of levofloxacin and minocycline against *S. maltophilia* CF isolates have been described previously in the literature (3). In the present study, the susceptibilities of SCV *S. maltophilia* isolates to SXT, levofloxacin, and minocycline were examined by Etest under nonstandard conditions (HTM and chocolate agar after 48 h of incubation). Chocolate agar was the only medium that supported the growth of the SCV *S. maltophilia* isolates from all five patients. Of those five isolates, four had an SXT MIC of >32 μg/ml on chocolate agar. In contrast, levofloxacin Etest results under the same test conditions yielded susceptible MICs of 0.25 to 3 μg/ml for four of the five isolates, suggesting that levofloxacin activity may be retained against some of the SCV *S. maltophilia* isolates. While minocycline also appeared to have in vitro activity, quality control was out of range on chocolate agar at all time points. It must be stressed that these MIC results were generated under nonstandard test conditions and thus cannot be directly compared to MICs obtained under standard conditions. Furthermore, the in vitro activities of the antibiotics need to be confirmed by in vivo efficacy studies.

Evaluation of multiple *S. maltophilia* isolates from a single patient (patient A) demonstrated three distinct patterns. Two independent genotypes of wild-type *S. maltophilia* were present, unique to two separate specimens, while all 15 SCV *S. maltophilia* isolates had principally the same genotypic fingerprinting pattern. This raises the possibility that wild-type *S. maltophilia* isolates may be transient colonizers while SCV *S. maltophilia* strains may be able to persist in the CF airway, perhaps because of slower growth and increased antibiotic resistance (16). The finding of multiple morphologically variant but genotypically similar SCV *S. maltophilia* strains from individual sputum cultures suggests a variable degree of down-regulation or alterations in one or more related biochemical pathways. Future studies should include longitudinal evaluations of all of the infected patients so that the process of phenotypic switching can be sampled and defined at the molecular level.

Genotyping studies also revealed that a single genotype of SCV *S. maltophilia* was carried in the lungs of one patient for nearly 3 years. Long-term airway infection with *S. maltophilia*, along with frequent antibiotic administration, prompted us to hypothesize that the switch to the small-colony morphology is likely an adaptation mechanism to the CF lung, involving down-regulation of the bacterial DHFR and/or electron transport pathway (16). This adaptation to the presence of antibiotics has been described previously for *S. aureus* (12, 13, 15); in fact, this patient also harbored SCV *S. aureus* in many of the same sputum samples. The nutritional requirements of the SCV *S. maltophilia* isolates were also consistent with this hypothesis, as DHFR impairment results in thymidine and methionine auxotrophy. Interestingly, methionine auxotrophy has long been noted as the most common amino acid deficiency associated with *P. aeruginosa* isolates in CF patients (2); thus, its association with antibiotic resistance deserves further investigation. Additional support for the hypothesis of SCV *S. maltophilia* association with antibiotic pressure was provided by the recovery of phenotypic small colonies from a wild-type *S. maltophilia* isolate through in vitro passages in broth medium containing sub-MIC levels of SXT.

Over the past decade, there has been an evolving perception of *S. maltophilia* as a potential CF respiratory pathogen (21). Along with this awareness has come increased use of antibiotics directed specifically against this organism. This increased antibiotic pressure may explain why we are now observing the emergence of the novel SCV phenotype among CF patients. The SCV *S. maltophilia* phenotype is a potential problem for CF microbiologists and caregivers for several reasons: (i) it grows poorly on basic sputum culture agar medium, making initial detection, recognition, and identification difficult; (ii) susceptibility results cannot be reliably generated by conventional susceptibility testing methods; and (iii) it is potentially resistant to SXT, considered first-line antimicrobial therapy against *S. maltophilia*. We do not yet fully understand the mechanism or significance of the SCV *S. maltophilia* phenotypic switch within the context of CF microbiology or clinical management. Future studies employing clinical data, treatment history, and molecular characterization may help to address both the prevention of the selection of small-colony variants and the choice of antibiotic therapy against these pathogens.

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