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# Differential Mucoid Exopolysaccharide Production by Members of the *Burkholderia cepacia* Complex▽

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**We demonstrate that all nine species of the *Burkholderia cepacia* complex can express the mucoid phenotype. A survey of clinical isolates showed that strains of *B. cenocepacia*, the most virulent species of the complex, are most frequently nonmucoid. Additionally, isolates from patients with chronic infections can convert from mucoid to nonmucoid.**

The *Burkholderia cepacia* complex (BCC) is a group of at least nine closely related species, each of which is capable of causing infection in cases of cystic fibrosis (CF) and chronic granulomatous disease (13). Patients infected with BCC are at a proportionally higher risk of death than those who are uninfected (6). Additionally, patients infected with BCC can follow a number of courses, ranging from chronic infection with little or no impact on lung function to rapid deterioration with bacteremia and death (a condition known as cepacia syndrome) (10). The factors mediating these different clinical courses are not understood. Furthermore, patients carrying identical CF genetic mutations and infected with the same clonal bacterial strains can experience highly different clinical outcomes.

*Pseudomonas aeruginosa* ultimately infects most people with CF; therefore, much research has focused on possible mechanisms of pathogenicity. Typically, patients are initially infected with nonmucoid strains that during the course of chronic infection convert to the grossly mucoid "CF phenotype" that is due to the elaboration of a viscous alginate-like exopolysaccharide (EPS). The isolation of mucoid *P. aeruginosa* from respiratory tract secretions in CF infections is so common that it has been described as a pathognomonic observation and is linked to increased morbidity and mortality (8). Whereas the mucoid phenotype in bacteria from the BCC has not been widely described, as it is not apparent on routine growth media, a few recent reports have suggested that this morphotype may be underrecognized (2, 7). BCC bacteria can produce at least four different EPSs, and the biosynthetic genes involved in the synthesis of cepacian (the most widely expressed EPS) have been identified previously (14). Data from liquid culture, in S liquid medium, have shown that EPS is produced upon the entry of *B. cepacia* into stationary phase, and EPS production appears to be a stable phenotype (15). The EPSs of bacteria from the BCC appear to affect the outcomes of experimental and human infections. Both shiny and mucoid variants of *B. cenocepacia* persist longer in animal models of infection than their nonmucoid isogenic variants (3). Herein we report the largest survey to date of the mucoid phenotype in BCC isolates from clinical and environmental sources.

Given that EPS production on standard laboratory media, including Columbia blood agar, *B. cepacia* selective agar, and Luria-Bertani agar, is not readily observable, we used yeast extract medium (YEM; 0.5 g of yeast extract liter$^{-1}$ and 4 g of mannitol liter$^{-1}$ supplemented with 15 g of agar liter$^{-1}$) to assess the capacities of BCC isolates to elaborate EPS (16). A simple scoring method was developed for describing the extent of EPS production by each isolate. Bacteria were subcultured from a frozen stock by using a cotton swab to inoculate one-third of a 25-ml YEM plate, streaked to yield individual colonies, and grown at 35°C for 48 h. When bacteria were grown on this medium, the capacities of isolates to elaborate EPS could be clearly observed by visual inspection as opposed to an examination of the appearance of the isolates on conventional diagnostic microbiological media, such as Luria-Bertani agar and blood agar. We scored EPS production as − to +++d, and the criteria for the scoring are described in the legend to Fig. 1. Mucoidy was defined as follows: nonmucoid (−) or partially mucoid (+) isolates showed no or little EPS production, and among frankly mucoid (++, +++, and +++d) isolates, EPS production was evident in all colonies.

We first tested this semiquantitative method for scoring mucoidy with samples of bacteria, the identities of which were concealed, from the BCC experimental strain panel (4, 12). These data (Table 1) demonstrated that all species of the BCC included mucoid strains. However, among strains of *B. cenocepacia* (apparently the most virulent species in CF), 8 of 10 were non- or only partially mucoid. This observation was mirrored in a survey of environmental isolates from LMG and American Type Culture Collection strain banks and other sources (Table 2). These findings are in contrast to those for *P. aeruginosa*, mucoid strains of which are rarely isolated from the environment. Our data suggest that the capacity to elaborate EPS may be critical for survival in the environment, the natural niche of BCC bacteria. However, as demonstrated with the

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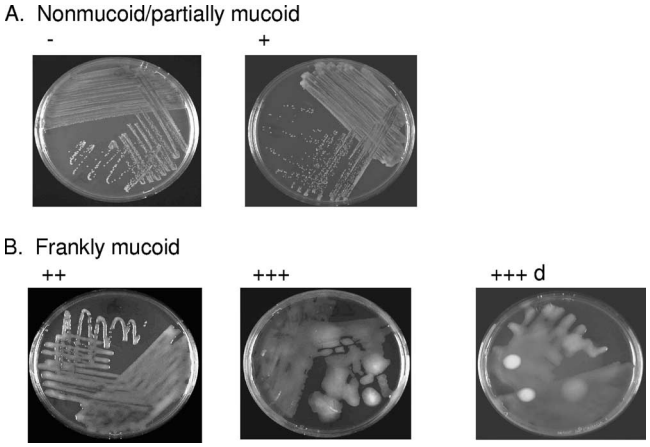


FIG. 1. *B. cenocepacia* mucoid phenotypes on YEM agar plates after 48 h of growth at 35°C. The scoring of nonmucoid and partially mucoid phenotypes was as follows: −, growth shows no evidence of EPS production and colonies are dry with a matte finish, and +, some evidence of EPS production in the confluent growth region is seen but the plate contains predominantly nonmucoid bacteria. The scoring of frankly mucoid phenotypes was as follows: ++, both the confluent area and the streaked-out region are mucoid in appearance and the growth is flat; +++, EPS production overwhelms the streaked-out area, making separate streaks hard to see, and instead of level growth across the plate there are raised areas; and +++d, same as +++ except that EPS drips onto the lid of the plate.

experimental strain panel, the proportion of frankly mucoid isolates among *B. cenocepacia* isolates, specifically among the *recA* IIIA lineage, was lower than those among the other species.

BCC isolates were collected from patients with CF attending either a pediatric clinic (British Columbia Children's Hospital) or an adult clinic (Shaughnessy or St. Paul's Hospital) between June 1981 and June 2007 and tested for mucoidy. A total of 560 isolates from 100 patients were obtained (Table 2). The largest number of clinical isolates tested were either *B. multivorans* or *B. cenocepacia*, reflecting the predominance of these species in CF patients infected with BCC; there were 45 cases of infection with one or both of these two species. All isolates of *B. cepacia* (four isolates in one case of infection) or *B. stabilis* (10 isolates in two cases of infection) were nonmucoid. The distributions of colony morphologies among *B. multivorans* and *B. cenocepacia* isolates were markedly different. Of the *B. multivorans* isolates, 173 (82.8%) of 209 were frankly mucoid, while only 102 (37%) of 276 *B. cenocepacia* isolates from the IIIA lineage were mucoid. Indeed, 146 (53%) of the *B. cenocepacia* isolates were absolutely nonmucoid (score, −). Of the isolates of the *B. cenocepacia* IIIB lineage, 100% were frankly mucoid; however, there were just 16 isolates in six cases of infection. Of the *B. vietnamiensis* isolates, 64% (23 of 36) were frankly mucoid. Typing was performed for each isolate by random amplified polymorphic DNA (RAPD) analysis as described previously (11). For *B. multivorans*, *B. cenocepacia*, and *B. vietnamiensis*, isolates were of multiple different genetic types, indicating that our observations were not due to strain-specific phenotypes. For *B. cenocepacia*, the common RAPD groups 1, 4, and 6 contained isolates corresponding to each of the five scores for mucoidy (−, +, ++, +++, and +++d); only *B. cenocepacia* RAPD group 2, of the ET-12, cable-piliated lineage, was uniformly nonmucoid (data not shown).

We also evaluated the frequency of phenotypic switching during chronic BCC infection of CF patients; no data on this topic have been published previously. A switch from the mucoid to the nonmucoid phenotype was defined by the isolation of a nonmucoid variant (score, −) from a patient from whom a frankly mucoid isolate (score, ++, +++, or +++d) of the same species and strain, as determined by RAPD typing, had previously been isolated on at least two separate occasions; a switch from nonmucoid to mucoid was defined vice versa. Phenotypic switches were observed in sequential isolates from 15 patients: nine mucoid-to-nonmucoid transitions of *B. mul-*

TABLE 1. Distribution of EPS production in the BCC experimental strain panel

| Species | Strain(s) with EPS score of: | | | |
|---|---|---|---|---|
| | Non- or partially mucoid | | Frankly mucoid | |
| | − | + | ++ | +++/+++d |
| *B. cepacia* | | LMG 17797 | CEP509 | ATCC 17759, ATCC 25416$^T$ |
| *B. multivorans* | LMG 13010$^T$ | 249-2 | | C5393, C1576, CF-A1-1, JTC, C1962, ATCC 17616 |
| *B. cenocepacia* | J2315, BC7, K56-2, C5424, C6433, CEP511 | C1394, PC184 | | J415, ATCC 17765 |
| *B. stabilis* | LMG 14294, C7322 | | LMG 14086, LMG 18888 | |
| *B. vietnamiensis* | | | PC259 | LMG 16232, FC441, FC0369 |
| *B. dolosa* | | CEP021 | E12 | AU0645, STM1441 |
| *B. ambifaria* | CEP0996 | | | AMMD$^T$, ATCC 53266 |
| *B. anthina* | | | AU1293 | W92$^T$, C1765, J2552 |
| *B. pyrrocinia* | | BC011 | | ATCC 15958$^T$, ATCC 39277 |

TABLE 2. Prevalence of nonmucoid and mucoid isolates from the environment and from cultures of respiratory specimens from CF patients at Vancouver children's and adult CF clinics from 1981 to 2007

| Species | No. of environmental isolates | No. of environmental isolates with score of: | | | | % Of frankly mucoid environmental isolates[a] | No. of clinical isolates[b] | No. of clinical isolates (no. of RAPD groups) with score of: | | | | % Of frankly mucoid clinical isolates[a] | No. of cases of infection[c] | No. of patients whose isolates converted from mucoid to nonmucoid | No. of patients whose isolates converted from nonmucoid to mucoid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | − | + | ++ | +++/+++d | | | − | + | ++ | +++/+++d | | | | |
| *B. cepacia* | 28 | | 1 | 3 | 24 | 96.4 | 4 | 4 (1) | | | | 0 | 1 | 0 | 0 |
| *B. multivorans* | 12 | | | 1 | 11 | 100 | 209 | 25 (12) | 11 (8) | 64 (24) | 109 (34) | 82.8 | 45 | 9 | 0 |
| *B. cenocepacia* | | | | | | | | | | | | | | | |
| IIIA | 5 | 2 | 1 | 1 | 1 | 40 | 276 | 146 (6) | 28 (3) | 61 (4) | 41 (6) | 37.0 | 45 | 3 | 1 |
| IIIB | 4 | | | | 4 | 100 | 16 | | | 2 (2) | 14 (5) | 100 | 6 | 0 | 0 |
| *B. stabilis* | 3 | | 1 | 1 | 1 | 66.6 | 10 | 10 (1) | | | | 0 | 2 | 0 | 0 |
| *B. vietnamiensis* | 10 | 1 | 1 | 6 | 2 | 80 | 36 | 8 (2) | 5 (3) | 19 (4) | 4 (3) | 64 | 9 | 1 | 1 |
| *B. dolosa* | 0 | | | | | | 0 | | | | | | 0 | | |
| *B. ambifaria* | 13 | | 1 | | 12 | 92.3 | 0 | | | | | | 0 | | |
| *B. anthina* | 15 | | | 3 | 12 | 100 | 0 | | | | | | 0 | | |
| *B. pyrrocinia* | 2 | | | | 2 | 100 | 0 | | | | | | 0 | | |

[a] Frankly mucoid isolates were those with a score of ++ or +++/+++d.
[b] Additionally, there were three *B. multivorans* and three *B. cenocepacia* isolates that did not grow on YEM agar and a further three *B. cenocepacia* isolates that appeared to have a mix of mucoid and nonmucoid colonies.
[c] Among 100 individuals, including seven that had both *B. cenocepacia* and *B. multivorans* isolates during their isolate history and one that had *B. cenocepacia* IIIA and *B. cenocepacia* IIIB isolates.

*tivorans* isolates, three transitions of *B. cenocepacia* IIIA isolates, and one transition of *B. vietnamiensis* isolates occurred; there were two nonmucoid-to-mucoid conversions, one each of *B. cenocepacia* IIIA and *B. vietnamiensis* isolates.

The data presented here show for the first time that all species of the BCC can express the mucoid phenotype when grown on YEM agar. Given that our previous studies demonstrated that both mucoid and shiny variants persist longer in mouse models and interact more poorly with components of the innate immune systems than their isogenic nonmucoid variants (3, 5), it is possible that the mucoid phenotype endows the bacteria with the tools for persistence during chronic infection in CF. This idea is consistent with the observation that *P. aeruginosa* converts to mucoidy during chronic infection. The role of BCC mucoid EPS in microbial persistence is further suggested by the capacity of EPS to scavenge reactive oxygen species, key components of the pulmonary host defense system, and its interference with neutrophil chemotaxis (1). Without chemical analysis for each isolate, we cannot specify the chemical composition of the EPS produced by each of the isolates in this study. A recent study demonstrated that the most common polysaccharide among the BCC, isolated from several different species, is cepacian; however, the researchers found an isolate of *B. multivorans* which produced another polysaccharide, PSI (9). Therefore, it is certainly conceivable that variations in the polysaccharides produced within the strains we studied may be significant.

The disproportionately high frequency of nonmucoid isolates among strains of *B. cenocepacia*, the most virulent species of the BCC, and the observation that phenotypic switching typically is from mucoid to nonmucoid are novel and intriguing, as they run counter to the observation that the conversion of *P. aeruginosa* to a mucoid phenotype is linked to an increased risk of morbidity and mortality. The mucoid-to-nonmucoid conversion in *B. cenocepacia* raises the possibility that nonmucoid isolates are associated with increased disease severity while the mucoid phenotype may be associated with persistence. There are a number of conceivable mechanisms for this situation; it is possible that without the metabolic burden of EPS production, nonmucoid bacteria are simply at a competitive advantage in the lung. Given that the mucoid phenotype of both *P. aeruginosa* and *B. cenocepacia* is associated with a reduction in virulence factor production (5, 17), it is also conceivable that the nonmucoid form is more invasive and capable of doing damage to the host. Additionally, it is possible that the lack of the EPS layer around the cells may facilitate interaction with the immune system, permitting invasion by members of the BCC. Therefore, in light of these observations, we will expand our bacterial and clinical databases to determine the significance of microbial phenotypes and phenotypic switching in the context of disease severity and the rate of clinical decline in patients with CF infected with bacteria from the BCC.

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