**Pseudomonas aeruginosa**: Changes in Antibiotic Susceptibility, Enzymatic Activity, and Antigenicity Among Colonial Morphotypes

DANIEL J. SHEEHAN, J. MICHAEL JANDA,* AND EDWARD J. BOTTONE

Department of Microbiology, The Mount Sinai Hospital, New York, New York 10029

Received 18 August 1981/Accepted 5 January 1982

Colonial variants of *Pseudomonas aeruginosa* have received renewed interest because of their occurrence in sputum cultures of patients with cystic fibrosis. We encountered 11 strains of *P. aeruginosa* from various body sites of non-cystic fibrosis patients. The strains showed two to three colonial variants, including smooth, rough, and iridescent morphotypes that arose from subculture of a single colony of *P. aeruginosa* originating from a primary source. The colonial segregants differed in antibiotic susceptibility (resistance to gentamicin, carbenicillin, chloramphenicol, and tetracycline), presence or absence of exoenzymes (gelatinase and elastase), degree of proteolytic activity (caseinase), pigmentation, and antigenicity. These observations suggest that in vivo dissociation with concomitant changes in enzymatic and surface properties might greatly enhance invasiveness. Concurrent differences in antimicrobial susceptibility among the colonial variants could account in some instances for the failure of antibiotic treatment in *P. aeruginosa* infections in which one would anticipate a positive therapeutic response.

The dissociation of *Pseudomonas aeruginosa* strains into different colonial morphotypes was first observed more than 50 years ago. The isolation of mucoid and nonmucoid, smooth and rough, and iridescent versus noniridescent colonies from individual strains of *P. aeruginosa* has been reported (4). In 1964, Zierdt and Schmidt (10) reported that 77 of 116 (66%) primary cultures of *P. aeruginosa* on blood agar displayed colonial heterogeneity. Because this phenomenon had been noted on primary culture, this phenotypic variation was attributed to a refluxing lysogenic state in the bacterium.

Interest in this phenomenon has been rekindled, with particular emphasis being placed on colonial variants of *P. aeruginosa* arising from sputum cultures of cystic fibrosis patients (7, 9). Several investigators have reported multiple isolates, reverting segregants, or morphological heterogeneity in strains isolated from such subjects. These morphologically distinct varieties of *P. aeruginosa* have been observed to exhibit differences in antibiotic susceptibility, pigment production, and antigenicity.

At present, discrepancies exist as to whether these phenotypic differences represent those of multiple strains of *P. aeruginosa* or merely varieties of a single strain (2, 3, 7, 9). Also to be determined is the frequency of occurrence of colonial heterogeneity of *P. aeruginosa* isolates recovered from non-cystic fibrosis patients. We report here the isolation of a number of phenotypes from individual *P. aeruginosa* strains isolated from diverse clinical sources from non-cystic fibrosis patients. The potential clinical significance of in vivo dissociation of *P. aeruginosa* giving rise to segregants possessing altered exoenzyme activity and antibiotic susceptibility patterns is assessed.

**MATERIALS AND METHODS**

**Isolation of colonial morphotypes.** *P. aeruginosa* isolates were obtained from primary cultures. A loopful of growth from a pure confluent lawn of each strain was inoculated into physiological saline, and 100-fold dilutions in 0.85% saline were plated onto diazylized brain heart infusion-skim milk agar (DBHI-SMA; 8), which enhanced visualization of colonial morphotypes.

Dilutions were performed to obtain ca. 30 to 70 individual colonies per plate. After 48 h of incubation at 37°C, those colonies exhibiting morphological differences were subcultured to Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) containing 5% sheep blood (BPA) and to DBHI-SMA. After 24 to 48 h of growth at 37°C, colonial morphotypes which appeared to be morphologically distinct on these media were evaluated.

**Biochemical tests and enzyme assays.** The isolated colonial morphotypes were confirmed as *P. aeruginosa* by previously described criteria (5). Each morphotype was analyzed by a battery of biochemical tests by using API 20E (Analytab Products, Plainview, N.Y.) and oxidation-fermentation medium of Hugh and Leif-
son containing either dextrose, sucrose, maltose, man-
nitol, lactose, or xylose. Also assessed were urease
production (Christensen urea agar), nitrogen gas for-
mation from nitrate, and arginine dihydrolase activity.
Exoenzyme activity was determined (in duplicate) by
substrate tube or plate assays for the detection of
protease (caseinase), elastase, and gelatinase as previ-
ously described (6).

Serology. Eleven isolates and their 25 colonial disso-
ciants were subjected to serological typing with Difco
(Liu) antisera against the 17 known serotypes. In
preparation of these isolates for typing, a single colony
of each P. aeruginosa morphotype was streaked onto
blood agar plates and incubated for 18 to 24 h at 37°C.
Confluent growth was harvested in 0.85% saline, auto-
claved, and centrifuged to recover the sedimented
antigen. Serological typing was performed with glass
slides, and the mixture of antisera and antigen was
hand rotated for 1 min before the test results were
recorded. Definitive serological typing was evidenced
by a 25% (2+) or greater agglutination of the auto-
claved antigen. Rough strains that agglutinated in
many or all of the 17 antisera were designated nontyp-
able.

RESULTS

A number of morphological criteria, including
colonial pigmentation, size, shape, texture, and
degree of spreading, were used to distinguish
colonial varieties on DBHI-SMA (Fig. 1). Of the
90 clinical isolates screened by this procedure,
11 strains (12.2%) showed morphological heter-
erogeneity and were phenotypically distinct
when viewed on DBHI-SMA and BAP (Table 1).
By source, wound and ulcer isolates harbored
the highest frequency (23.5%) of these variants,
being almost 2 times higher than the overall
average encountered for all cultures screened.
Isolates from blood, bile, ears, and eyes did not
yield discernible varieties. Of 28 gentamicin-
resistant strains tested, 5 (17.8%) yielded multiple
morphotypes, similar to the overall observed
average.

Three morphological variants arising from a
single urinary isolate were phenotypically heter-
ogeneous (Fig. 2A, B, C). These smooth and
rough isolated morphotypes maintained their
morphologic integrity on numerous subcultures
and exhibited differences in antibiotic sensitiv-
ity, total proteolysis (caseinase), gelatinase, and
elastase production (Table 2). Smooth varieties
were enzymatically more active than their rough
counterparts. There did not appear to be an
absolute correlation between colonial dissoci-
ation and exoenzyme production among the 11
isolates investigated.

Serological analysis of 11 strains and 25 colo-
nial dissociants revealed antigenic identity
among the colonial morphotypes of 10 strains.
Only one sputum isolate displayed serological
nondentity among its colonial segregants (Table
2). This isolate (patient no. 4) showed three
colonial morphotypes. Two were serotype 11
and one was nontypable. The nontypable
morphotype was also distinct on the basis of resis-
tance to tetracycline and chloramphenicol and
moderate resistance to carbenicillin.

Epidemiological characteristics related to the
11 clinical specimens yielding different morpho-
types of P. aeruginosa are shown in Table 2.
Three cultures yielded three distinct morpho-
types, whereas eight others yielded two colonial
varieties. The majority of those patients from
whom P. aeruginosa morphotypes were derived
were not receiving antimicrobial therapy when
the cultures were submitted for bacteriologica-
al analysis. We noted 10 antibiotic susceptibility
differences among the morphotypes of 5 of the
11 strains. These included variable zone diam-
ters to gentamicin, carbenicillin, chlorampheni-
FIG. 2. Three distinct colonial dissociants arising from a single urinary isolate of *P. aeruginosa*. (A) Colonial morphotype showing a central streak line of blue-green pigmentation with spreading colonial growth. (B) Rough "crater-form" variety showing a slight zone of beta hemolysis. (C) Smooth, slightly pigmented morphotype with glistening surface and spreading colonial borders.

col, and tetracycline. Morphological varieties derived from a patient isolate gave inhibition zone diameters differing by up to as much as 6 mm (carbenicillin) within the same sensitivity range to a given antibiotic. Rough morphological varieties were usually more resistant to antibiotics than were smooth colonial variants.

**DISCUSSION**

The results of this study indicate that multiple varieties of *P. aeruginosa* can be recovered from single primary cultures originating from non-cystic fibrosis patients with a frequency somewhere in excess of 10%. Although this figure is far below the 38 to 73% frequency reported by several groups for cystic fibrosis patients, a number of factors suggest that our findings may represent an underestimate of the overall frequency of this phenomenon. First, the average DBHI-SMA plate contained 30 to 70 isolated colonies after 100-fold dilutions of primary isolates. Variants arising with a frequency of less than 1.4 to 3.3% would not have been detected by our assay procedures. For example, 6 of the 11 isolates diluted and subcultured from primary plates showed only one or two colonies of a particular colonial variant. Other factors which might affect the frequency of dissociation include stability of original colonial morphology (e.g., mucoid morphotype), serotypic propensity, antibiotic selection in vivo, and testing of individual isolates after numerous subcultures. Additionally, phenotypic variation could be unrelated to colonial morphology since some morphotypes did not display differences upon repeated testing.

Serological analysis of the 25 different colonial morphotypes of *P. aeruginosa* that arose from nine patient isolates revealed antigenic identity among the dissociants of each of eight strains. One sputum isolate (patient no. 4) showed serological nonidentity for one of the three morpho-
TABLE 2. Epidemiology, exoenzyme production and antibiogram of multiple morphotypes of \textit{P. aeruginosa}a

<table>
<thead>
<tr>
<th>Patient Source</th>
<th>Colonial Morphologya</th>
<th>Serotype</th>
<th>Exoenzyme production</th>
<th>Antibiogramb</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Proteolysis</td>
<td>Gelatinase</td>
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<tr>
<td>1 Urine</td>
<td>Smooth</td>
<td>1</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Rough</td>
<td>1</td>
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<td>2 Ulcerd</td>
<td>Smooth</td>
<td>3</td>
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<td>Smooth</td>
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<tr>
<td></td>
<td>Rough</td>
<td>3</td>
<td>+</td>
<td>+</td>
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<tr>
<td>3 Wound</td>
<td>Smooth</td>
<td>11</td>
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<td></td>
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<td>5 Sputum</td>
<td>Smooth</td>
<td>12</td>
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<tr>
<td></td>
<td>Rough</td>
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<td>Smooth</td>
<td>NA</td>
<td>+</td>
<td>-</td>
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<tr>
<td></td>
<td>Rough</td>
<td>NA</td>
<td>+</td>
<td>-</td>
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<tr>
<td>8 Urine</td>
<td>Dwarf</td>
<td>11</td>
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<td></td>
<td>Rough</td>
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\(a\) Abbreviations: genta, gentamicin; tobra, tobramycin; carb, carbenicillin; tetra, tetracycline; chloro, chloramphenicol; S, sensitive; R, resistant; MR, moderately resistant; NT, nontypable; ND, not done; NA, non-agglutinable.

b Morphology as determined on blood agar plate after 72-h incubation at 23°C.

c As determined by the disk susceptibility method of Bauer et al. (1).

d These ulcer specimens were received from the same patient on different days and yielded two and three distinct morphotypes, respectively.

e Caseinase activity on reisolated (pure) varieties.

types recovered from the initial strain. This nontypable variety may represent a separate coinfecting strain, thus accounting for this phenomenon (7). Our data, however, suggest that this morphotype could have resulted from multiple dissociations from a single progenitor strain, conceivably producing both morphologically distinct, serologically identical and morphologically and serologically distinct varieties.

An interesting observation was the recovery of colonial varieties from different body sites (wound, sputum) in one patient (no. 4) and the same body site (ulcer) on separate occasions in another patient (no. 2). For both patients, the morphologically identical varieties were also serologically identical, being serotype 11 for patient no. 4 and serotype 3 for patient no. 2. This observation suggests that certain strains may have a greater propensity for dissociation.

Many of the dissociants also differed in extracellular enzyme production and in antimicrobial susceptibility after in vitro isolation and testing. Sensitivity changes have previously been shown to occur in many individual strains and their dissociants (9). Enzymatic differences were noted in elastase activity, degree of overall proteolysis, and gelatinase production. Since several of these enzymes have been extensively implicated in the pathogenicity of \textit{P. aeruginosa}, the observation that colonial dissociants vary in enzymatic expression is of considerable importance. For instance, in vivo dissociation with concomitant changes in enzymatic properties might greatly enhance the potential invasiveness (virulence) of
a given strain, especially when present in localized sites such as wounds and ulcers or in sputum. However, loss of a given property might signify self attenuation by a strain. Changes in antimicrobial sensitivity could dramatically affect the outcome of chemotherapy and in part afford an explanation for the failure of drug treatment in cases where one would anticipate a positive therapeutic response. We did not observe dissociation of P. aeruginosa among blood isolates. Perhaps this observation may be attributable to selection by the host (or by antibiotic usage) of the more invasive phenotype. Indeed, serum factors may simultaneously exert an inhibitory and selective pressure on colonial dissociation.

This study supports the concept that colonial variants of P. aeruginosa arise by dissociation of a single strain and are probably not the result of coinfection with different P. aeruginosa strains. Larger surveys are needed to more precisely correlate these phenotypic changes (e.g., exoenzyme production, antibiotic sensitivity) associated with the dissociants and their potential role in virulence.

LITERATURE CITED