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Subgrouping of *Pseudomonas cepacia* by Cellular Fatty Acid Composition

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The cellular fatty acid compositions were determined for 42 strains of *Pseudomonas cepacia* from five cystic fibrosis centers in North America. All isolates contained significant (20%) amounts of hexadecanoic ($C_{16:0}$), and cis-9 hexadecenoic ($C_{16:1}$ cis9) acids and an isomer of octadecenoic acid ($C_{18:1}$). None had hydroxy acids containing fewer than 14 carbon atoms. The quantitative data from the fatty acid analysis were highly reproducible and provided a basis for numerical analysis. Five subgroups comprising all the strains were obtained by cluster analysis and further characterized by principal-component analysis. With minor exceptions, the predominant subgroup identified in each center was different from that identified in other centers and accounted for one-half of the isolates within each center. Cellular fatty acid composition is a useful adjunct to biochemical characterization for the identification of *P. cepacia* isolated from cystic fibrosis patients. Numerical analysis of the fatty acid data can separate *P. cepacia* into subgroups, which may provide useful epidemiologic information or a basis for further analysis by more complex techniques such as DNA probe analysis.

In recent years several cystic fibrosis (CF) treatment centers have reported an increased incidence and prevalence of colonization by *Pseudomonas cepacia* among CF patients (17, 29, 30). Some patients are apparently colonized with *P. cepacia* in the absence of worsened respiratory symptoms (17, 31), whereas other patients with *P. cepacia* rapidly progress to respiratory failure and death (31). Isolates of *P. cepacia* isolated from CF patients tend to be more resistant than non-CF isolates to commonly used antipseudomonal antibiotics (1, 2). It is the increased prevalence of this multiply-resistant pathogen capable of causing significant morbidity among patients with advanced pulmonary disease which has made *P. cepacia* important in CF patients (9, 10, 17).

The epidemiology of *P. cepacia* remains to be defined. However, the clustering of colonization at some CF centers and the increased colonization rate among siblings with CF suggest a common environmental source or person-to-person transmission (29, 31). Epidemiologic investigations have been hampered by a lack of suitable typing methods for *P. cepacia*, although several have been studied. These include bacteriocin susceptibility and production (11), serotyping (15, 22), and ribotype analysis (19). Many of these methods are not easy to perform and have demonstrated limitations in terms of sensitivity, specificity, and general applicability (19).

Gas-liquid chromatography of bacterial cellular fatty acid methyl esters (FAME) has been extensively used in clinical microbiology as either a primary or an adjunctive means for identification of many medically important gram-negative bacteria (6, 18). With gas-liquid chromatography, characteristic FAME profiles of *Pseudomonas* spp., including *P. cepacia* (21) and the closely related *P. gladioli* (4), have been described. It has been well established that the total fatty acid composition of a microorganism is an important taxonomic character (23) and that fatty acid data can be analyzed quantitatively to provide useful taxonomic information at the species level and, in some cases, the subspecies level (24). The purpose of this study was to determine whether numerical analysis of the quantitative fatty acid data obtained by gas-liquid chromatography could be used to differentiate strains of *P. cepacia* isolated from CF patients. Secondly, since conventional biochemical tests used to identify *P. cepacia* may produce equivocal results, we assessed the utility of FAME profiles as an adjunct to biochemical characterization. The distribution of FAME profile patterns in five different CF centers is also described.

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MATERIALS AND METHODS

Bacterial strains and cultivation. The source of the strains has previously been described (32). In short, *P. cepacia* was isolated from respiratory tract specimens of CF patients during a multicenter clinical microbiological evaluation of a selective and differential medium containing an oxidation-fermentation base, polymyxin B, bacitracin, lactose, and agar. The centers were located in Calgary, Alberta, Canada, Salt Lake City, Utah, Columbus, Ohio, Mobile, Ala., and Shreveport, La. Cells used to inoculate biochemical tests were grown on 5% sheep blood agar incubated at 30°C. Cells for fatty acid analysis were harvested from 24-h cultures grown at 30°C on plates containing Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) solidified with 1.5% agar. Identification of isolates was accomplished primarily by conventional methods (8), with the aid of supplementary commercial (Uni-N/F-Tek, from Flow Laboratories, Inc., McLean, Va.; and Autobac, from General Diagnostics, Div. Warner-Lambert Co., Morris Plains, N.J.) identification systems.

Analysis of cellular fatty acids. Whole-cell fatty acids were extracted and analyzed as methyl ester derivatives as previously described (20). Recognition of the fatty acid profiles was performed with a model 5898A microbial identification system (Hewlett-Packard Co., Avondale, Pa.) consisting of a model 5890 gas chromatograph equipped with a 5% phenylmethyl silicone capillary column (0.2 mm by 25 m), a flame ionization detector, a model 3392A integrator, a model 7673A automatic sampler, and a model 310 computer. Peaks

were automatically integrated, and fatty acid identities and percentages were calculated.

Reproducibility of fatty acid analysis. The reproducibility of the chromatographic technique was determined by repeated analyses of a standard quantitative FAME mixture (Microbial ID Inc., Newark, Del.). The reproducibility of the FAME profiles of *P. cepacia* isolates was determined by analyzing each isolate on three or more separate occasions under the standardized growth conditions. Each analysis was separated in time by serial passages on the culture media. The coefficient of variation, measured as (standard deviation/mean) × 100 was calculated for each peak in the chromatogram.

Numerical analysis of fatty acid data. The quantitative data obtained from the FAME profiles were used as the basis for numerical analysis. Peak area values for each fatty acid were calculated as percentages of the total peak area to eliminate the effect of inoculum size variation. Similarities were calculated with the generalized similarity coefficient of Gower (12) and the coefficient based on the Euclidean distance between pairs of bacteria (3). Clustering of strains was achieved by the unweighted pair group method for arithmetic averages (28) with a program provided in the HP Library Generation Software (Hewlett-Packard Co.), resulting in a dendrogram. Principal-component analysis (13) of the quantitative fatty acid data was performed with the above-mentioned statistical program, and the results were plotted graphically in two dimensions.

RESULTS

Identification of strains. The characteristics used for the identification of *P. cepacia* by conventional methods were oxidase production (positive); nitrate reduction (positive or negative) and nitrogen gas production (negative); indole (negative), lysine decarboxylase (positive), and arginine dihydrolase (negative); acidification of oxidative low-peptone medium containing glucose, lactose, maltose, mannitol, sucrose, or xylose; *o*-nitrophenyl-β-D-galactopyranosidase production (positive); polymyxin B resistance; and flagellar arrangement (at least two polar flagella). All isolates of *P. cepacia* from one CF treatment center (center 2) were lysine decarboxylase negative.

Cellular fatty acid composition. Under standardized growth conditions all of the strains displayed qualitatively similar profiles. Fatty acids common to all of the isolates were saturated tetradecanoic ($C_{14:0}$), hexadecanoic ($C_{16:0}$), and octadecanoic ($C_{18:0}$) FAME; unsaturated cis-9 hexadecenoic acid ($C_{16:1}$ cis9) and an isomer of octadecenoic acid ($C_{18:1}$) (summed feature 7); the cyclopropane acids, which were cis-9-10 methylene hexadecanoic ($C_{17:0}$ cyc) and cis-11-12 methylene octadecanoic ($C_{19:0}$ cyc); and the hydroxy acids, which were 2-hydroxyhexadecanoic (2-OH $C_{16:0}$), 3-hydroxyhexadecanoic (3-OH $C_{16:0}$), 2-hydroxyhexadecenoic (2-OH $C_{16:1}$), and 2-hydroxyoctadecenoic (2-OH $C_{18:1}$) (Table 1). There were three predominant fatty acids, each accounting for ≥20% of the total fatty acid composition: $C_{16:1}$ cis9, $C_{16:0}$, and an isomer of $C_{18:1}$ (summed feature 7) (Table 1).

Reproducibility of fatty acid analysis. When the same strain was cultured under standardized conditions and analyzed repeatedly, there was a high reproducibility of the FAME profiles both qualitatively and quantitatively. The coefficient of variation, calculated as the (standard deviation/mean) × 100 for each fatty acid representing ≥4% of the total fatty acid content within each cluster, was ≤13%, except for $C_{17:0}$

TABLE 1. Cellular fatty acid compositions of 42 strains of *P. cepacia*

Sub-group	No. of strains	% Mean ± SD (range) [coefficient of variation] for the following acid[a]:												
		Saturated			Unsaturated, $C_{16:1}$ cis9	Cyclopropane		Hydroxy				Summed feature[b]		$C_{16:1}$ cis9 + $C_{17:0}$ cyc	Summed 7 + $C_{19:0}$ cyc
		$C_{14:0}$	$C_{16:0}$	$C_{18:0}$		$C_{17:0}$ cyc	$C_{19:0}$ cyc	2-OH $C_{16:0}$	3-OH $C_{16:0}$	2-OH $C_{16:1}$	2-OH $C_{18:1}$	3	7[c]		
A	14	4.0 ± 0.19 (3.5–4.5) [5]	21.1 ± 0.8 (19.3–23.9) [4]	1.2 ± 0.31 (0.8–2.2) [26]	19.5 ± 2.19 (13.3–23.1) [11]	3.0 ± 1.44 (0.8–6.6) [47]	1.5 ± 0.8 (0.3–3.5) [55]	1.2 ± 0.35 (0.5–2.0) [30]	4.4 ± 0.2 (3.9–4.8) [5]	1.0 ± 0.28 (0.3–1.5) [30]	1.5 ± 0.39 (0.4–2.2) [27]	5.2 ± 0.25 (4.4–5.8) [5]	36.1 ± 1.5 (32.8–39.7) [4]	22.6 (14.1–29.7)	37.6 (33.1–43.2)
B	10	4.0 ± 0.17 (3.7–4.4) [4]	24.3 ± 1.68 (21.0–26.9) [7]	1.4 ± 0.31 (0.5–2.0) [22]	18.7 ± 2.52 (13.6–23.9) [13]	4.3 ± 1.65 (1.2–9.7) [38]	2.4 ± 1.18 (0.3–5.4) [50]	1.0 ± 0.23 (0.5–1.3) [24]	4.5 ± 0.26 (3.9–4.9) [6]	0.8 ± 0.19 (0.3–1.2) [22]	1.2 ± 0.53 (0.4–2.6) [43]	5.1 ± 0.35 (4.6–5.9) [7]	31.8 ± 2.0 (27.1–36.1) [6]	23.0 (14.8–33.6)	34.2 (27.4–41.5)
C	7	4.3 ± 0.21 (3.9–4.7) [5]	22.2 ± 2.23 (17.9–25.8) [10]	1.6 ± 1.63 (0.4–5.4) [105]	23.1 ± 2.69 (17.9–27.8) [12]	5.0 ± 2.09 (2.0–8.9) [42]	1.7 ± 0.98 (0.5–3.9) [58]	0.7 ± 0.26 (0.3–1.2) [35]	4.9 ± 0.51 (4.1–5.9) [11]	1.0 ± 0.36 (0.5–1.8) [37]	1.6 ± 0.37 (0.6–2.0) [24]	5.3 ± 0.33 (4.7–6.0) [6]	27.6 ± 1.47 (25.3–29.4) [5]	28.1 (20.0–36.8)	29.3 (25.8–33.3)
D	9	4.2 ± 0.2 (3.8–4.7) [5]	24.9 ± 2.42 (21.1–29.9) [10]	1.0 ± 0.64 (0.2–2.3) [67]	19.6 ± 4.25 (13.5–29.2) [22]	7.9 ± 3.34 (2.1–12.9) [42]	2.9 ± 1.44 (0.4–5.6) [49]	0.8 ± 0.25 (0.4–1.3) [31]	4.9 ± 0.37 (4.3–5.8) [8]	0.9 ± 0.31 (0.4–1.4) [34]	1.7 ± 0.78 (0.7–3.1) [47]	5.1 ± 0.39 (4.3–5.7) [8]	25.1 ± 3.19 (19.3–32.2) [13]	27.5 (15.7–42.2)	28.0 (19.7–37.9)
E	2	4.5 ± 0.16 (4.3–4.7) [4]	32.1 ± 0.98 (31.1–33.8) [3]	0.6 ± 0.19 (0.4–0.9) [29]	28.2 ± 1.44 (26.4–29.8) [5]	7.3 ± 1.80 (5.3–9.7) [24]	1.0 ± 0.20 (0.8–1.3) [21]	0.8 ± 0.17 (0.5–1.0) [22]	4.7 ± 0.17 (4.5–4.9) [4]	1.8 ± 0.54 (0.3–2.6) [30]	0.6 ± 0.13 (0.5–0.8) [21]	5.7 ± 0.26 (5.4–6.0) [5]	10.9 ± 2.20 (7.5–13.1) [20]	35.5 (31.7–39.6)	11.9 (8.2–14.3)

[a] Percentage of total cellular fatty acids. Each strain was analyzed on three occasions.
[b] Represents structurally similar fatty acids that cannot be resolved by the methods used.
[c] Includes $C_{18:1}$ cis-11 (vaccenic acid) FAME.

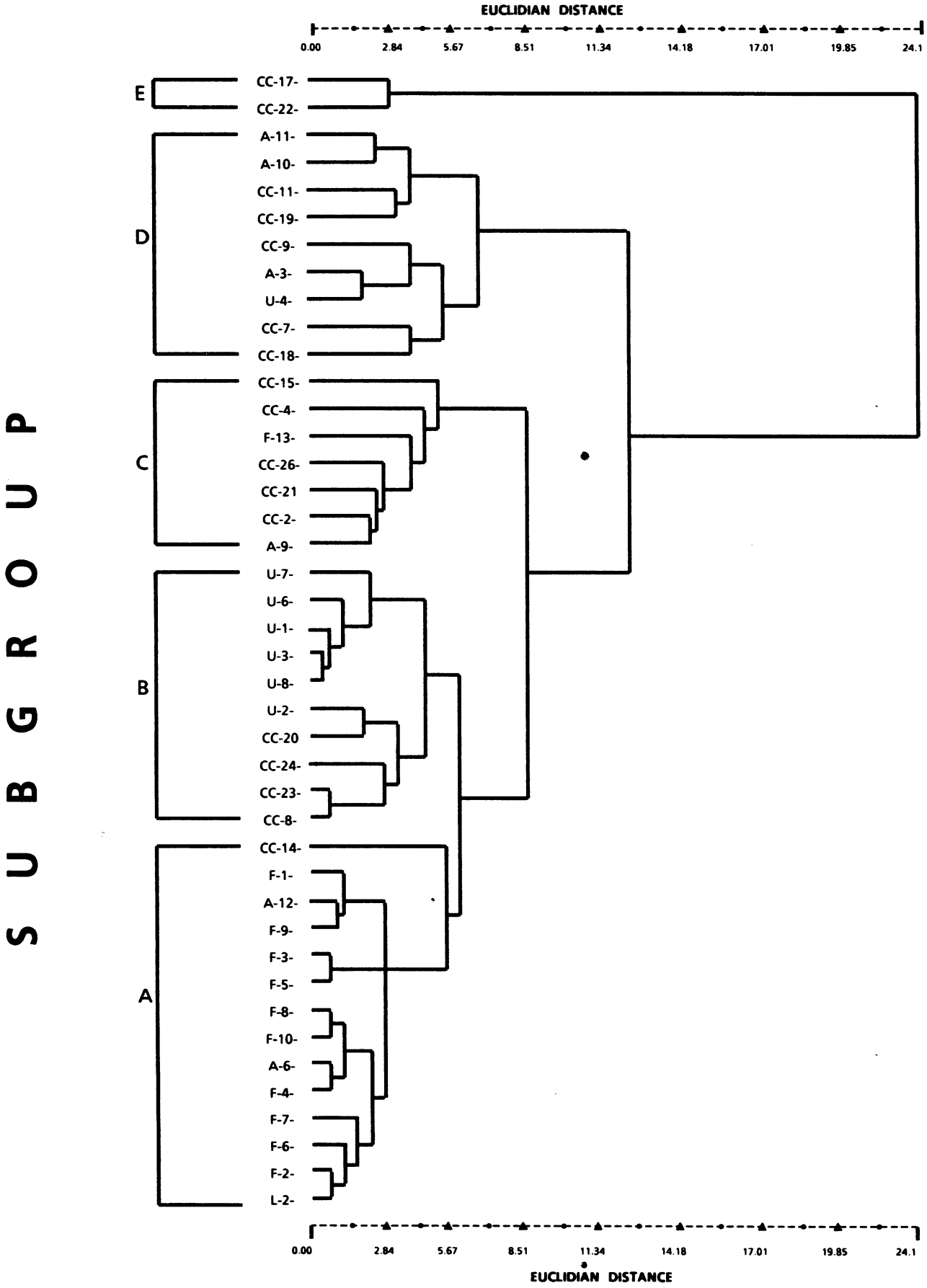

FIG. 1. Dendrogram of isolates generated by cluster analysis of FAME profiles showing subgroups of *P. cepacia*.

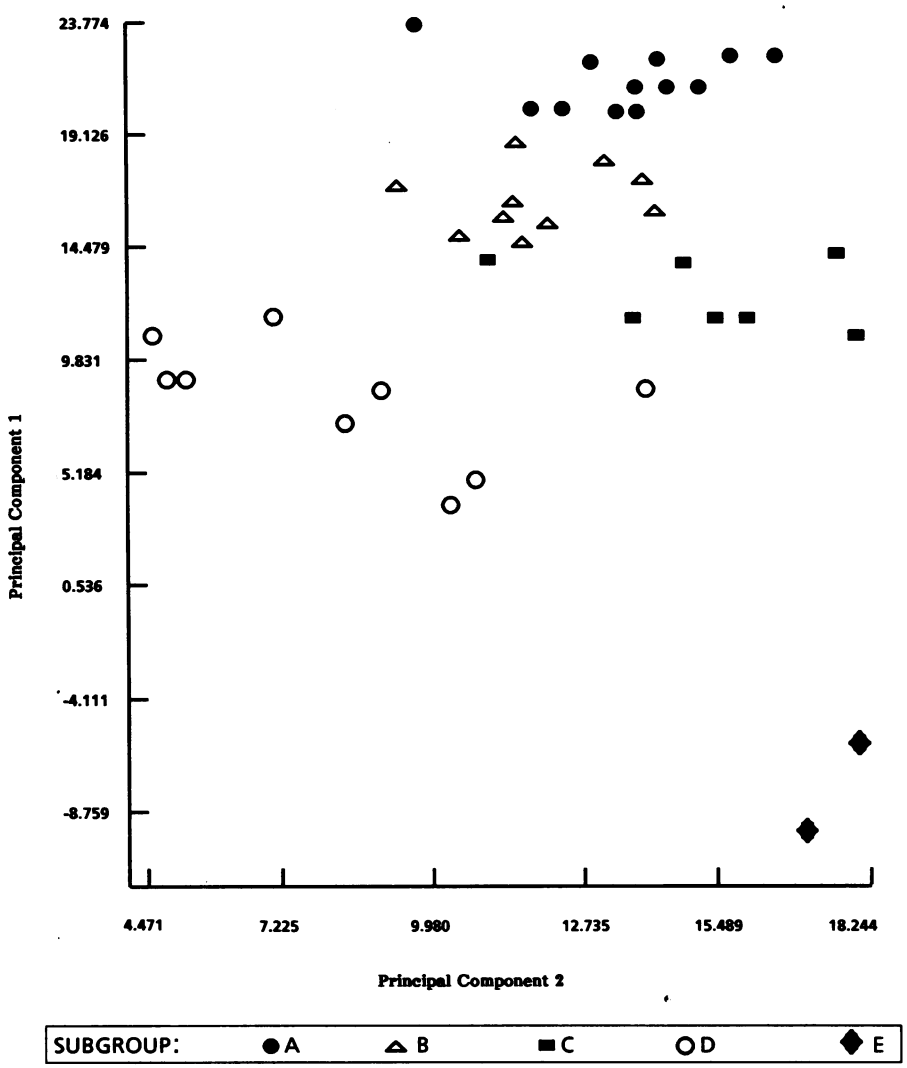

FIG. 2. Two-dimensional plot of isolates generated by principal-component analysis of FAME profiles showing distribution of subgroups of *P. cepacia*.

cyc, $C_{19:0}$ cyc, and their respective unsaturated fatty acid precursors, $C_{16:1}$ cis9 and the isomer of $C_{18:1}$ (summed feature 7) (Table 1).

Numerical analysis. Examination of the quantitative fatty acid data by cluster analysis (28) revealed that the 42 strains of *P. cepacia* could be placed into five different subgroups (A to E [Fig. 1]) based mainly on the relative amounts of $C_{16:0}$, $C_{16:1}$ cis9, $C_{17:0}$ cyc, $C_{19:0}$ cyc, and the isomer of $C_{18:1}$ (summed feature 7) (Table 1). For purposes of differentiation of the *P. cepacia* subtypes, the amounts of the unsaturated fatty acid precursors $C_{16:1}$ cis9 and $C_{18:1}$ were added to the amounts of the respective cycloacids $C_{17:0}$ cyc and $C_{19:0}$ cyc (Table 1). The relationships of the FAME profiles of the *P. cepacia* isolates to each other are depicted in the dendrogram (Fig. 1) based on the coefficient of similarity generated by the Euclidean distance between pairs of bacteria. At a Euclidean distance of about 24.1, all strains of *P. cepacia* were grouped together as a single cluster. At a distance of about 6.3, the five different clusters were well separated, with subgroups B and D showing two further subdivisions. This relationship of the FAME profiles is also illustrated by principal-components analysis (13) of the data in two dimensions (Fig. 2). The strains from subgroups A and B were closely related to each other but were still distinguishable from each other. Most of the strains from subgroup C were well separated. There was some diversity among the strains in subgroup D, again illustrating two major subdivisions within this group. The two strains in subgroup E were placed into a more distantly related fifth cluster.

Distribution of subgroups among CF treatment centers. The distribution of FAME subgroups among isolates obtained from four CF treatment centers is shown in Fig. 3. A fifth center had only one isolate, which belonged to subgroup A. In center 1, analysis revealed that 10 of 11 isolates belonged to the same subgroup, A. Similarly, in center 2, six of the seven isolates (all of which had an unusual biotype, lysine decarboxylase negative) belonged to a single but different subgroup, B. Five different subgroups were present in center 3, while three subgroups accounted for the six isolates in center 4. With one exception (center 4), the predominant subgroup identified in each center was different from the predominant subgroup found in other centers. A predominant subgroup accounted for one-half of the isolates within

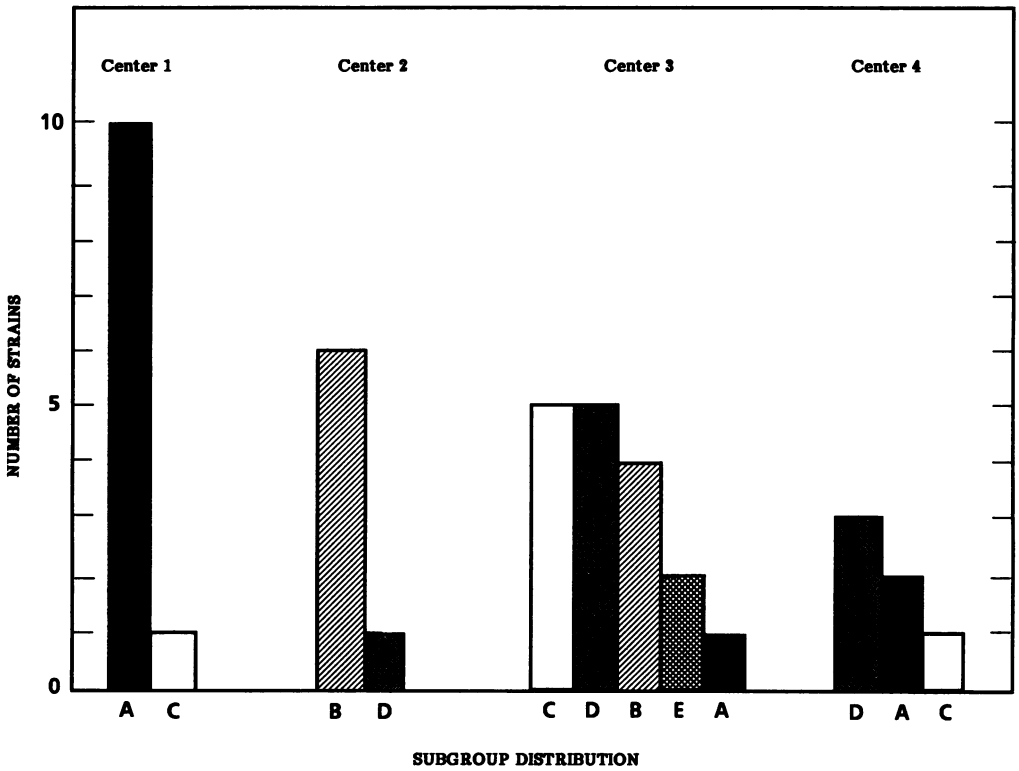

FIG. 3. Bar graph showing distribution of subgroups of *P. cepacia* among four CF centers.

each center, except for center 3, in which the predominant subgroup accounted for one-third of the isolates.

DISCUSSION

Some CF treatment centers (17, 29) but not others (32) have reported an increase in the incidence and prevalence of *P. cepacia* colonization among CF patients. The spectrum of clinical response observed has ranged from asymptomatic carriage through gradual deterioration to rapid and usually fatal deterioration (17, 31). These observations raise the question of how *P. cepacia* is acquired and whether different subgroups or closely related species such as *P. gladioli* (4) are responsible for the variation in clinical outcome. *P. cepacia* is ubiquitous, being found in a variety of natural aquatic and soil environments as well as in hostile environments such as aerosol antibiotics, topical anesthetics, and disinfectants (10, 14). Although studies have suggested a possible role for patient contact with other patients or siblings colonized as a risk factor for acquiring *P. cepacia* (14, 29, 30), this has not been demonstrated unequivocally. Similarly, the role of the inanimate environment during hospitalization (i.e., pulmonary function equipment, aerosol solutions, stethoscopes, etc.) in the acquisition and transmission of *P. cepacia* among CF patients remains undetermined (14, 17, 29–31). It was to elucidate these epidemiologic and clinical factors for *P. cepacia* in CF patients that we assessed the utility of numerical analysis of FAME profiles for identifying and typing *P. cepacia*.

Cellular fatty acid compositions are widely used as a basis for the characterization of bacteria (7). However, despite the potential of quantitative analyses of fatty acid profiles to enhance differentiation, most studies still rely on a qualitative interpretation of the data, resulting in reliable differentiation only at the genus level. The development of simpler extraction and refined analytic systems (20), together with the ability to obtain reproducible profiles (7, 26), may offer a readily available but underexploited source of taxonomic information at species and subspecies levels (24, 27).

The overall fatty acid compositions of the 42 strains of *P. cepacia* in this study were qualitatively similar to those of other members of the pseudomallei group of pseudomonads. Other species in this group include *P. pseudomallei*, *P. mallei*, *P. pickettii*, and *P. gladioli* (4). Characteristic cellular fatty acids found in this group by other investigators (5, 21) and reported in this and our earlier study (4) include relatively high (>20% of total fatty acids) amounts of $C_{16:0}$ and small amounts (<5%) of $C_{14:0}$, 3-OH $C_{14:0}$, and 3-OH $C_{16:0}$. The distinguishing features of *P. cepacia* were the absence of hydroxy acids with less than 14 carbon atoms and the relatively small amounts (<13%) of $C_{17:0}$ cyc and $C_{19:0}$ cyc. Although it has been felt that these two cycloacids must be considered in relation to their respective unsaturated fatty acid precursors ($C_{16:1}$ cis9 and $C_{18:1}$), under the growth and chromatographic conditions we used, the amounts of these two cycloacids were consistently lower than those found in other members of the pseudomallei group, such as *P. gladioli*.

An assessment of the efficiency of a typing method for bacterial strains must take into account three factors: typeability, reproducibility, and discrimination. Since all *P. cepacia* strains possess a FAME profile, they are all potentially typeable by quantitative analysis of the fatty acid data. For a typing system based on a comparison of FAME profiles to be effective, all conditions leading to the preparation of samples for the analysis should be as uniform as possible (26). Culture media, incubation times, and temper-

atures should be standardized to avoid variations in the cellular fatty acid compositions. Our results support the work of others (7) in that if standardized conditions are adhered to, a single strain cultured and analyzed repeatedly will provide highly reproducible FAME profiles. We observed no alteration in FAME profiles after multiple subcultures of a particular strain.

The discriminating ability of our proposed typing scheme, according to a recently described numerical index of discrimination (16), was 0.775. This index is based on the probability that two unrelated strains sampled from the test population will be placed into different typing groups. The index for our results indicates that if two strains were sampled randomly from the population, then on 77.5% of occasions they would fall into different groups. The relatively low discrimination index obtained in our study is partly due to the small population of strains studied but can also be attributed to the high similarity of their FAME profiles, as reflected by tight linkage in the cluster analysis of FAME profiles (Fig. 1). Consequently, a validation of this typing method requires analysis of a larger number of strains isolated from well-documented CF and non-CF patients as well as environmental sources.

Numerical analysis of the quantitative fatty acid data is easy to perform and reproducible. It may provide a basis for further epidemiologic studies by more complex procedures such as DNA probe analysis, similar to that which has been developed for *P. aeruginosa* (25). One major advantage of this method is its ability to store information on numerous isolates and compare complicated FAME profiles with the aid of a computer, greatly reducing subjective errors and increasing the accuracy of subgroup discrimination.

The results showing the distribution of clusters among the CF treatment centers demonstrated that the majority of patients colonized with *P. cepacia* within these centers harbored strains of the same FAME profile. Furthermore, strains with different FAME profiles tended to predominate at different CF centers. These data probably reflect regional variations in the distribution of *P. cepacia* within the environment, although the data are also suggestive of nosocomial acquisition of this pathogen. Studies designed to compare the distribution of FAME profiles among *P. cepacia* isolates from CF treatment centers in close proximity, those from nosocomial outbreaks, and those from environmental sources are necessary for a fine analysis of the utility of this approach in elucidating the epidemiology of *P. cepacia*.

In conclusion, numerical analysis of the quantitative data obtained by FAME analysis of *P. cepacia* strains isolated from CF patients is a useful adjunct to conventional methods for the characterization of *P. cepacia*. As a typing scheme the method is easy to perform, is highly reproducible, has universal typeability, and has the capacity to store information on numerous isolates. Its discriminating ability needs to be further validated by analysis of additional isolates of *P. cepacia*.

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