Aspergillus fumigatus is a saprobe fungus playing an essential role in recycling carbon and nitrogen. This fungal species is also an opportunistic pathogen in immunocompromised hosts. Besides its saprobic life, A. fumigatus has also become one of the most important human pathogens in industrialized countries. It causes different diseases like allergic bronchopulmonary aspergillosis, aspergillosis, and invasive aspergillosis depending on the underlying disease as well as the immunological status of the host (5, 32). A. fumigatus is today the most threatening fungal pathogen because nosocomially acquired invasive aspergillosis typically occurs in the treatment setting for hematological malignancy. The pathogenic behavior of this opportunistic fungus results from (i) specific biological features of the fungus, such as the release into the air of a high concentration of conidia of small size which are easily inhaled and able to germinate and grow at temperatures higher than 37°C without any specific nutritional requirement, and (ii) the increase in the use of immunosuppressive therapies which result in the lack of efficiency of the normal phagocytic host response (1, 15).

Recent epidemiological studies using molecular techniques have shown that, at least in some cases, infection occurs as a consequence of exogenous acquisition of the fungus (26). Two typing methods have been used until now. The method most currently used is the random amplification of polymorphic DNAs (4, 6, 16, 18, 30), although such amplified patterns are difficult to repeat or interpret (3, 20). In contrast, a restriction fragment length polymorphism method which uses a species-specific inactive retrotransposon-like repeated sequence as a marker has shown high reproducibility and reliability, allowing computer-aided analysis of a large population of A. fumigatus strains (11–13, 23). Previous epidemiological studies have investigated the nosocomial origin of aspergillosis cases in limited geographical areas and included fewer than 30 strains (6, 13, 16, 18). The present study includes over 800 isolates with different hosts and geographical origins. The main purpose of the study was to investigate the relatedness between clinical isolates and the known capacity to cause disease in order to test for the presence of a secondary adaptation of the clinical isolates to a parasitic condition.

MATERIALS AND METHODS

Isolates. The origins of the strains of A. fumigatus used in this study are given in Table 1. All strains from Denmark were isolated from cows with aspergillosis. Clinical isolates of A. fumigatus were from immunosuppressed patients suffering mainly from pulmonary or invasive aspergillosis with the exception of the strains from the Necker and Trousseau hospitals, which were from cystic fibrosis (CF) patients.

All isolates were kept on 2% malt agar and were eventually stored as a conidial suspension on dry silica gel (Prolabo) at room temperature (28). DNA isolation and characterization. Protocols for DNA extraction and EcoRI digestion were as previously described (11). Digested DNAs were loaded onto 20-cm long 0.8% agarose gels (SeaKem LE; FMC) at a ratio of 500 ng of DNA per well (4 by 4 mm). Electrophoresis and transfer were done on the same automated multiblottling device (Mark II; Bertin, France) at 14°C in 0.5× Tris-borate-EDTA buffer. After 20 min of preimmigration at 180 V, electrophoresis was performed for 15 h at 80 V. Transfer of DNA to Pall Biodyne B membranes lasted 40 min at 250 V. Eight to twelve gels of 15 slots each (12 restriction digests and 3 size markers) were run simultaneously. Membranes were treated with 0.4 M NaOH, neutralized in 0.5 M Tris-HCl (pH 7.5)–1.5 M NaCl, washed with water, and fixed at 80°C for 30 min. Southern blot hybridization was performed with the X3.9 probe containing the recently characterized (23) repeated sequence Afut1 isolated from A. fumigatus. Hybridization was performed as previously described (11) with 25 ng of probe DNA labelled with 2.5 μl of [32P]dCTP and two exposure times of 12 and 24 h with X-Omat film (Kodak) without an intensifying screen.

Analysis of fingerprints. Autoradiographs were scanned with an HP ScanJet IIcx/T (Hewlett-Packard) driven by a 466DX2/Sp personal computer (IBM). The resulting digital images (Tagged Image Format File) were converted and normalized with the GelCompar Software (Applied Maths BVBA, Kontich, Belgium). Each track corresponding to one slot was converted to a densitometric curve (700 points with values between 0 and 255, corresponding to the 256 gray levels). The conversion process included a background subtraction step and alignment on reference positions determined by size markers (corresponding to phage λ digested by XhoI, BstEII, and BsaHI). In addition, an undigested phage λ giving a band of 45 kb was added to each A. fumigatus DNA sample, allowing for the best alignment at the highest molecular weight bands. An example of a blot and its computer treatment are shown in Fig. 1. Densitometric profiles were clustered by the software with a matrix of similarity based on the Pearson product-moment correlation coefficient (2). As this method compares curves as a whole, it is independent of band definition and is not troubled by peak shoulders mismatches. The clustering method used was the unweighted pair group method with arithmetic averages.

Groups of genotype patterns were compared pairwise by principal component analysis (PCA). Definition of the groups was made a priori on the geographical location (ranging from a localized area, e.g., one Parisian hospital
to a more general origin, e.g., Europe) and host origin (human versus nonhuman and clinical versus environmental) of the isolates. PCA is a nonhierarchical method which allows densitometric data to be used directly without the application of any similarity coefficient (29, 31). The arrays of normalized densitometric values were used directly as input data for PCA. The three principal axes were used to produce a three-dimensional representation in which each single genotype occupied a separate place, shown as a dot. The degree of discrimination between the groups defined as indicated above was statistically evaluated with the software. Between each pair of groups, two values were calculated: the mean percentage relatedness (MPR) of the groups and the ratio of divergence (RD) (2). MPR is the sum of all Pearson product-moment correlation values ($r_{ij}$) of the entries of the first group (A) with the entries of the second group (B) divided by the total number of correlation values calculated:

$$\text{MPR} = \frac{\sum r_{ij}}{n_A n_B},$$

where $r_{ij}$ is the product-moment correlation between the $i$th and $j$th entries of groups A and B, respectively, and $n_A$ and $n_B$ are the number of entries in groups A and B, respectively. Within a group (A), this value ($r_{AA}$) is reduced to a measure of internal homogeneity:

$$\text{MPR} = \frac{\sum r_{ij}}{n_A^2}.$$

The ratio of divergence (RD) is calculated as the distance $1 - r_{AB}$ between two groups divided by the weighted internal distances $1 - r_{AA}$ and $1 - r_{BB}$.

$$\text{RD} = \frac{(n_A + n_B)(1 - r_{AA})}{n_A^2 (1 - r_{AA}) + n_B^2 (1 - r_{BB})}.$$

The MPR values provide information about the internal homogeneity of groups and the overall relatedness between groups. Values of MPR higher than 90 indicate very compact groups. In the case of an intragroup analysis, such a value would indicate that a specific hybridization profile can be associated with all of the isolates of the group. Values between 70 and 80 indicate an even scattering of the patterns analyzed inside a group or between groups. MPR data do not give information about the degree of discrimination between groups. The relative distance between groups, as revealed by the RD, gives an estimate of the degree of discrimination between two groups, taking into account their internal homogeneity. RD values of 1 to 1.5 indicate that the two clusters of points from the two groups are superimposed. RD values of greater than 2 (and up to 10 depending on how compact the groups being compared are) indicate that the cloud of points belongs to two statistically different groups.

Preliminary experiments on a random sample totalling 5% of the isolates showed that the same pattern was obtained from 10 single-spore strains isolated

![Figure 1](http://iai.asm.org/)

**FIG. 1.** (A) Southern blot hybridization patterns in a blot with 12 *A. fumigatus* isolates hybridized with the $\lambda 3.9$ probe. The first, eighth, and fifteenth lanes contain molecular size markers. Lanes A and B contain identical isolates (99% homology), the isolate in lane C has 90% homology with the isolates in lanes A and B, and the isolate in lane D has 56% homology with those in lanes A and B. (B and C) Densitometric curves obtained from digitalized images of the blot shown in panel A. Lanes are as defined above.
from the original slant, justifying our decision to consider every slant received as a single strain. In addition, on >20% of the strains, repeated extraction and hybridization of DNA samples from the same strain have shown the reproducibility of the method, with a coefficient of similarity of >95% between two replicates. Similarly, analysis of 75 identical strains isolated over a 3-year period from the same CF patient gave a 94.3% similarity coefficient. As a result of this analysis, genotypic profiles presenting a similarity coefficient of ≥92% were always compared individually by superimposition of densitometric curves and by direct visual examination of the autoradiograms to validate the software analysis and to confirm the identicalness of strains. Different exposure times were used to match the intensity levels of the bands and the background and to make sure that missing bands were not due to too little target DNA. For better accuracy in pattern comparison, profiles with fuzzy bands were always discarded and DNA extraction and hybridization were repeated. (Data are not presented in their entirety due to the considerable number of hybridization patterns analyzed but are available upon request.)

**RESULTS**

From the 879 isolates included in this study, clustering analysis of the Southern blot patterns obtained with the 3.9 probe gave distinct and unique genotypes for 424 strains (Fig. 2).

The presence of identical genotypes among isolates was due to our nonrandomized sampling method and corresponded to particular epidemiological situations: (i) isolates from the same patients (see below), (ii) environmental isolates from the same location, and (iii) environmental and clinical isolates obtained during nosocomial invasive aspergillosis outbreaks (8). For PCA analysis, only one representative of the genotype of each series of identical isolates was kept. Among the 424 strains studied, only 14 identities (14 couples) were found between strains of different origins (for example, a sample from a patient in Frankfurt, Germany, and a sample from a U.S. environment). In some cases, the possibility of laboratory contamination could not be ruled out; in other cases, contamination could be eliminated when, for example, DNA samples were prepared separately in the United States and in France.

Examination of the dendrogram in Fig. 2 shows that fingerprints are not grouped in very distinct groups but are in a number of small groups, indicating a gradual variability between all strains.

**Comparison of clinical and environmental strains.** Several (2 to 15) isolates were obtained per patient for 56 of the 140 patients from which *Aspergillus* was isolated (Table 2). The interval between the first and the last sampling dates varied from 1 day to 6 months for patients with aspergillosis and from 12 to 32 months for patients with CF. The number of strains isolated from CF patients was much higher than that from aspergillosis patients. In the CF patient group, no one patient was colonized by a single strain. Colonization of every CF patient was due to several genotypes which were repeatedly isolated from the same patient (data not shown). In contrast, in the aspergillosis group, the number of genotypes isolated per patient was close to 1. The recovery of a single genotype per patient among 18 of the 34 patients studied indicated that in more than 50% of the patients, infection was due to a single strain (Table 2).

PCA analysis of clinical and environmental isolates included 326 genotypes from among three groups: (i) 136 strains isolated from 115 neutropenic patients with evidence of *Aspergillus* infection, (ii) 97 strains obtained from 26 patients with CF and which apparently were not implicated in an infectious process, and (iii) 155 randomly chosen environmental strains, mainly isolated in hospitals.

PCA comparison showed no differences among the three groups of strains. Figure 3 shows a two-dimensional representation of the three-dimensional analysis. The absence of clustering was seen at all rotating angles. Pairwise comparisons gave values of MPR and RD which were not statistically different (Fig. 3, inset). In conclusion, although the clinical isolates were indeed the infective strains, no difference was seen between the saprobic and clinical isolates. This result means that every strain present in the environment can become pathogenic, if it encounters the appropriate host.

**Comparison of strains based on their geographical origin.**

The 12 groups of strains (a total of 424 strains) gathered on the basis of their common geographical origin were compared by the different clustering methods available with the software (dendrograms not shown) and by PCA. The PCA results showed that the genetic variability encountered in the species *A. fumigatus* was extremely high and that no discrimination between strains was possible on the basis of their geographical origin. Figure 4 gives an example of a comparison of strains from (i) a Parisian hospital, (ii) the Paris area, (iii) Europe, and (iv) the United States. Similar MPRs and RDs were found for comparisons between 56 strains from one Parisian hospital and either a random selection of 244 strains from the rest of the U.S. environment. In some cases, the possibility of laboratory contamination could not be ruled out; in other cases, contamination could be eliminated when, for example, DNA samples were prepared separately in the United States and in France.

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**TABLE 2.** *A. fumigatus* isolates obtained from patients by repeated sampling

<table>
<thead>
<tr>
<th>Patient status</th>
<th>No. of patients</th>
<th>No. of isolates per patient</th>
<th>No. of genotypes</th>
<th>Strain/patient ratio</th>
<th>PI/PT ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus</em> infection</td>
<td>34</td>
<td>2.20</td>
<td>46</td>
<td>1.35</td>
<td>18/34</td>
</tr>
<tr>
<td>CF</td>
<td>22</td>
<td>17.13</td>
<td>85</td>
<td>4.58</td>
<td>0/26</td>
</tr>
</tbody>
</table>

*PI, number of patients from whom only one genotype of *A. fumigatus* was isolated; PT, total number of patients.*
the world (MPR, 74.9; RD, 1.3) or 140 strains from France and 160 strains from outside of France (MPR, 73.4; RD, 1.1).

DISCUSSION

This is the first large-scale study of the genetic variability occurring in the opportunistic fungal pathogen *A. fumigatus*

Although this species has been shown to display some phenotypic variations (17, 19, 25), *A. fumigatus* looks taxonomically homogeneous on the basis of its morphology (27). In contrast, the intraspecies variability seen at the genomic level looks very high. As a result, comparison of different genotypes with either a clinical or an environmental origin showed no difference between the strains from either origin, suggesting that any

**FIG. 3.** Comparison by PCA of *A. fumigatus* strains isolated from patients with *Aspergillus* infections (PA) (■) or CF (□) or from the environment (EN) (□). The MPR and RD values between the groups are shown in the inset.

**FIG. 4.** PCA of *A. fumigatus* strains from different geographical locations. (A) A total of 62 strains from Hôtel-Dieu, Paris, France (■), versus 200 randomly chosen strains from other Parisian hospitals (□) (MPR, 76.1; RD, 1.3). (B) A total of 150 randomly chosen strains from Paris (■) versus 124 strains from other origins in Europe (□) (MPR, 78.9; RD, 1.0). (C) A total of 36 isolates from the United States (■) versus 200 randomly chosen strains from Europe (□) (MPR, 76.4; RD, 1.0).
environmental strain of *A. fumigatus* is a putative infectious strain. This conclusion has some practical implications in that it indicates that prevention measures should be applied to any environmental *Aspergillus* conidia. The absence of strains of *A. fumigatus* with different pathogenicity potentials is in agreement with the results of previous biochemical, molecular, and immunological studies which were unable to identify a key factor responsible for the pathogenicity of *A. fumigatus* (15). For example, until now, no avirulent or hypervirulent mutant has been found or constructed by genetic manipulation. However, the failure of this molecular approach may just reflect the very few proteins purified from *A. fumigatus* and the low number of disrupted genes.

The ability of any strain of *A. fumigatus* to become pathogenic in contact with an appropriate host can also be linked to the absence of host specificity. No marked difference was seen between strains isolated from cows or humans. MPR and RD values between the two groups of strains were 78.3 and 1.2, respectively, whereas the MPR values for the intragroup variability of the 98 strains isolated from humans and the 14 strains isolated from cow strains were 81.7 and 78.2, respectively. This is in contrast with true phytopathogenic fungi where pathotypes are genetically different and host-associated pathotypes can be separated through diverse typing methods on the basis of their host or geographical origin (21, 22).

All these studies suggest that virulence in *A. fumigatus* is multifactorial and that in vivo development requires the activation of multiple genes. Questions regarding the identity of the genes expressed and their differential levels of expression in vivo and in vitro have never been asked seriously until now. The potential pathogenicity of strains with very different genetic backgrounds is in agreement with the absence of any selective pressure for this fungus resulting from the accidental and rare development of *A. fumigatus* in a human being. This fungus does not need a human host to complete its biological cycle, and encountering an immunocompetent human host is indeed a deadly cul-de-sac for it. *A. fumigatus* is a true saprobiotic fungus which becomes pathogenic only when the human defense reactions are very weakened (for example, in the terminal stage of an AIDS infection or as a result of heavy immunosuppressive therapies). Virulence would seem to represent more a lack of resistance of the human host than the expression of specific disease-related proteins. Experimental intranasal infection of mice never succeeds even at the maximal possible dose (10^8 conidia per mouse) unless a mouse is immunosuppressed (unpublished data).

The question of the origin of the variability encountered in this species remains open. No teleomorph has been found, and the possibility that *Neosartorya fischeri* represents the sexual stage of *A. fumigatus* has been definitely discarded (14, 24). Three hypotheses can be put forward. (i) Continuous genetic exchange can occur through the parasexual cycle. *A. fumigatus* remains today a continuously evolving species. Vegetative compatible groups have been found in *A. fumigatus* (9), indicating the possibility of a parasexual cycle in this species. However, it has never been shown that nonmeiotic parasexual recombination can account for recombination in a natural population of any fungus (7). (ii) The variability could have been fixed a long time ago through meiotic exchange at a time when *A. fumigatus* had a sexual stage. Continuous exchange of conidia through air currents all around the world, in addition to the absence of any adaptation to a parasitic condition, would explain the lack of recovery of identical multilocus genotypes from geographically and temporally nonassociated hosts (6). (iii) The third hypothesis concerns the presence of a sexual stage in *A. fumigatus* which has gone undetected. Recent population genetic studies have suggested that such a cryptic sexual stage exists in the case of another human pathogen, *Coccidioides imites*, which has been found to be almost completely recombining (7). The presence of such a sexual stage would be correlated with variations occurring through meiotic recombination. In a study with different heterokaryon compatibility groups of *Aspergillus nidulans* (thus incapable of mitotic recombination), genetic variations were found indicating that heterokaryon groups represent recently diverged lineages that arose via meiotic recombination (10).

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