Reclassification of *Paenibacillus durum* (Formerly *Clostridium durum*) Smith and Cato 1974) Collins et al. 1994 as a Member of the Species *P. azotofixans* (Formerly *Bacillus azotofixans*) Seldin et al. 1984) Ash et al. 1994

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Phenotypic studies, as well as the reaction of *Paenibacillus durum* genomic DNA with a 16s ribosomal DNA (sequence of variable regions V1 to V4)-based *Paenibacillus azotofixans*-specific PCR system and oligonucleotide probe, the presence of sequences homologous to Klebsiella pneumoniae nifKDH in both *P. durum* and *P. azotofixans*, and the results of DNA-DNA hybridization experiments performed with the *P. durum* and *P. azotofixans* type strains and one additional *P. durum* strain, showed that these two species form a homogeneous group. In addition, evidence was found for the presence of nif genes in *P. durum*, and *P. durum* was shown to fix atmospheric nitrogen. Therefore, the names *P. durum* and *P. azotofixans* should be considered synonyms. As *P. durum* was capable of fixing nitrogen and fixation without inhibition by nitrate is a major characteristic of the group, we propose that *P. durum* be included in the species *P. azotofixans*.

In 1974, a new species named *Clostridium durum* was described by Smith and Cato (11) as the dominant organism in a sediment core from the Black Sea. All of the isolates in this study formed a homogeneous group and had similar properties, and strain VPI 6563 was deposited as the type strain in the American Type Culture Collection as strain ATCC 27763. These organisms have characteristics which are atypical for clostridia, such as, for example, their inability to grow on egg yolk agar, their production of acetic and formic acids, and their inability to ferment lactose. Moreover, the guanine-plus-cytosine (G+C) content of the DNA of *C. durum* (50 mol%) was higher than the G+C contents of other clostridia (11). Actually, the strains were considered members of the genus *Clostridium* because of their ability to form spores under anaerobic conditions. Smith and Cato considered this criterion the only reliable criterion for separating the genus *Clostridium* from the genus *Bacillus*.

In 1994, Collins et al. (3), after studying the phylogeny of the genus *Clostridium*, proposed five new genera and 11 new species. It was evident from the data of these authors that *C. durum* is only remotely related to other clostridia. Phylogenetically, *C. durum* was considered a member of the newly formed genus *Paenibacillus* (1). This genus comprises rRNA group 3 of the *Clostridium* because of their ability to form spores under anaerobic conditions. Smith and Cato considered this criterion the only reliable criterion for separating the genus *Clostridium* from the genus *Bacillus*.

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The taxon *P. azotofixans* includes strains that are often isolated from the rhizospheres of different grasses (6, 8, 10) and consistently show a great capacity to fix atmospheric nitrogen in vitro (6). These strains are facultatively anaerobic, catalase positive, oxidase negative, and positive for acetoin fermenta-
system, and DNA-DNA hybridization to determine the levels of homology between the genomes of the *P. azotofixans* and *P. durum* type strains, as well as the genome of *P. durum* ATCC 27812.

All of the strains used in this study are listed in Table 1. Maintenance and propagation of cultures have been described previously (6, 7, 9). Analyses of phenotypic features other than those analyzed by the API system were performed as described by Gordon et al. (4). Much like *P. azotofixans*, both *P. durum* strains sporulated under aerobic and anaerobic conditions, producing oval to ellipsoidal spores that were located centrally to subterminally and distended the sporangia (6). As proposed by Smith and Cato (11), *P. durum* might form spores at the bottom of colonies, where anaerobic conditions prevail; all *P. azotofixans* strains tested (6) also formed spores when they were incubated under anaerobic conditions (GasPak jars filled with 80% N₂-10% H₂-10% CO₂). Hence, this criterion for *P. durum* was observed in both species. The following tests were negative for both species: gelatin, casein, urease, and indole. Catalase production was also observed in both *P. durum* and *P. azotofixans*. Furthermore, both *P. durum* strains tested (6) also formed spores when they were incubated under anaerobic conditions (GasPak jars filled with 80% N₂-10% H₂-10% CO₂). Hence, this criterion for separating aerobic spore-forming rods from anaerobic spore-forming rods is questionable. Moreover, acetoin fermentation was observed in both species. The following tests were negative for both species: gelatin, casein, urease, and indole. Catalase production was also observed in both *P. durum* and *P. azotofixans*. The remaining carbohydrates produced the same pattern of fermentation as that described for *P. azotofixans* (8).

Three genetic approaches were used to confirm the similarity between *P. durum* and *P. azotofixans*. Rosado et al. (5) described two specific primers (BAZO 1 and BAZO 2) and a specific probe (BAZOP) based on three variable regions of the 16S rRNA sequence of *P. azotofixans*, as determined by Ash et al. (1). The PCR product generated with the target strain, *P. azotofixans* ATCC 35681T, as well as with all of the other *P. azotofixans* strains tested, was 565 bp long and could be specifically detected by an 18-mer probe (5). Figure 1 shows the products generated by primers BAZO 1 and BAZO 2 and specific hybridization of a Southern blot of the PCR products (5) with probe BAZOP. Figure 1 shows that the *P. durum* type strain sequence was recognized by the primers and the probe, much like a *P. azotofixans* strain. Moreover, a stronger hybridization signal with probe BAZOP was obtained for the *P. durum* PCR product than for well-characterized *P. azotofixans* strains C3L4 and 2RC4 (Fig. 1). A similar PCR product was obtained with the other *P. durum* strain, ATCC 27812 (data not shown).

The second genetic aspect considered was the presence of sequences homologous to the prototype *nifKH* genes (cloned from *K. pneumoniae* on plasmid pSA30). All *P. azotofixans* strains studied so far show homology at high stringency to *nifKH*, and more than one hybridizing fragment is always obtained (9). Genomic DNA was isolated and digested with EcoRI as previously described (9). Figure 2 shows the hybridization pattern obtained with specific probe BAZOP.
ization patterns observed with DNAs from *P. azotofixans* ATCC 35681<sup>T</sup> and *P. durum* ATCC 27763<sup>T</sup> with a *K. pneumoniae* nifRDM (pSA30) probe. Both DNAs were digested with EcoRI prior to agarose gel electrophoresis and Southern hybridization with digoxigenin-labelled pSA30 DNA. Lane A, *P. azotofixans* ATCC 35681<sup>T</sup>; lane B, *P. durum* ATCC 27763<sup>T</sup>; lane C, lambda HindIII digoxigenin-labelled ladder (BMB). The vector sequences present in probe pSA30 did not hybridize with *P. azotofixans* genomic DNA (9).

**TABLE 2. Levels of DNA relatedness of *P. azotofixans*, *P. durum*, and *P. polymyxa* strains**

<table>
<thead>
<tr>
<th>Strain (target DNA)</th>
<th>% Homology with DNAs from the following probes&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td><em>P. azotofixans</em> ATCC 35681&lt;sup&gt;T&lt;/sup&gt;</td>
<td>100</td>
</tr>
<tr>
<td><em>P. durum</em> strains (formerly <em>Bacillus azotofixans</em>)</td>
<td>69</td>
</tr>
<tr>
<td>ATCC 27812</td>
<td>78.3</td>
</tr>
<tr>
<td><em>P. polymyxa</em> strains</td>
<td></td>
</tr>
<tr>
<td>DSM 356</td>
<td>16</td>
</tr>
<tr>
<td>LMAU B58</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are the averages of two determinations. The maximum variation observed was 9%.

with any of the three probes used. There were no great differences between the levels of homology measured when the genomic DNAs were used as targets or as probes. The levels of homology were determined by scanning the dot intensities with an LKB-Ultroscan XL instrument (Pharmacia). The peak areas were integrated and analyzed by using the software Gel Scan XL. The self-hybridization values were set at 100%, and the values obtained with the other strains were compared to this standard.

As demonstrated in this paper, we could not find significant differences to justify separation of the *P. durum* strains used (including the type strain) from the *P. azotofixans* strains used. When the genus *Paenibacillus* was proposed (1), *P. azotofixans* strains were already in group 3 of Ash et al. (1), whereas *P. durum* (formerly *C. durum*) was included later in this genus (3). Many current isolates of *P. azotofixans* are from the rhizospheres of very different crop plants, and these strains invariably show a high capacity to fix atmospheric nitrogen (6, 8, 10). Therefore, we propose that the nitrogen-fixing species *P. durum* should be included in *P. azotofixans* and that *P. durum* should be eliminated as a separate species.

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REFERENCES

7. Seldin, L., and D. Durbau. 1985. Deoxyribonucleic acid homology among...


