Deoxyribonucleic Acid Relatedness between Selected Members of the Genus *Aquaspirillum* by Slot Blot Hybridization: *Aquaspirillum serpens* (Mueller 1786) Hylemon, Wells, Krieg, and Jannasch 1973 Emended to Include *Aquaspirillum bengal* as a Subjective Synonym

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Deoxyribonucleic acid relatedness of five strains of *Aquaspirillum serpens* and also four other species of this genus was assessed by slot blot hybridization. A high degree of genetic relatedness was evident among the strains within the species *A. serpens*, but a low degree of relatedness was shown between the species *A. serpens*, *A. sinuosum*, *A. itersonii*, and *A. putridiiconchilyllum*. Hybridization homology indicated that *A. bengal* is a subjective synonym for *A. serpens* and should not have separate species status; therefore, the description of *A. serpens* is emended accordingly. The strains that grow optimally at higher temperatures (41°C) and produce brown pigments in the presence of some amino acids may be considered varietal and are referred to as *A. serpens* biovar bengal. This study demonstrates the usefulness of slot blot hybridization for quickly assessing the relatedness of different taxonomic groups of bacteria.

The application of molecular genetic techniques to bacterial systematics has initiated an extensive reordering of the taxonomic groupings of many bacteria which had previously been grouped according to phenotypic data. The goal is to provide means for classifying bacteria into phylogenetic ribonucleic acid (DNA) and ribonucleic acid (RNA) homology studies that are suitable for less sophisticated laboratories.

The helically curved spirilla have undergone extensive taxonomic regrouping in the past decade because genetic information modifies the previous phenotypic arrangements. The first edition of Bergey's Manual of Systematic Bacteriology (13) now places members of this group into three genera (*Aquaspirillum*, *Oceanospirillum*, and *Spirillum*) according to Hylemon et al. (9). The wide range of DNA base compositions indicated that a single genus was inappropriate for these organisms. The genus *Aquaspirillum* (mol% G+C range of 49 to 66) is still based largely on phenotype. DNA/DNA hybridization studies have not been done to support or reject all 17 species presently recognized within this genus, except in the case of *A. autotrophicum* versus *A. dispar* (1).

Slot blot hybridization, a modification of dot blot hybridization (10), appears to be an ideal candidate as a rapid method for comparing multiple DNA samples from different strains of bacteria. Dot blot hybridization has already been tested against a spectrophotometric DNA hybridization assay for examining the relatedness between *Mycobacterium leprae* or *Mycobacterium leprae marium* and other selected bacteria (2).

In this study, we have used slot blot hybridization to examine the DNA relatedness between representative strains of five different species of the genus *Aquaspirillum*, as compared to an *Escherichia coli* strain and a *Campylobacter jejuni* strain as distantly related controls. We have also examined five selected strains from the species *Aquaspirillum serpens* to determine whether they do in fact belong within the same species group, because these strains are of similar phenotype (Table 1). Three of them are variants of the *A. serpens* VH strain (9), and they differ in minor ways. Strain VHL has lost the hexagonal protein layer on the surface of the cells, while the Straight Rhodes strain (SRS) is not helical; the *A. serpens* ATCC 12638T (T = type strain) does not normally possess a surface array, and strains VHA and MW5 possess single or double-layered surface arrays, respectively (11, 12). We needed the assurance provided by genetic data that the strains whose surface arrays we were studying did belong in the species *A. serpens*.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The strains used in the homology experiments and their sources are listed in Table 2. All *A. serpens* strains were maintained at 30°C on peptone-succinate-salts agar slants in screw-capped tubes (9) and were subcultured every 2 weeks. Other *Aquaspirillum* species were maintained on peptone-succinate-yeast extract medium containing 0.15% agar in screw-capped tubes (3). These cultures were maintained at 30°C, with the exception of *A. bengal* which was maintained at 41°C. KOH was used to adjust the pH in all of the above media, since spirilla have been reported to be sensitive to low concentrations of Na+ (19). The *C. jejuni* strain was maintained at 42°C on brucella agar slants (Difco Laboratories) with weekly subculturing. The *E. coli* B strain was maintained at 37°C on nutrient agar slants (Difco).

**DNA isolation and preparation.** Overnight 100-ml broth cultures from a second 24-h serial transfer were used for DNA isolations from each of the strains. Cells were washed once with 1× SSC (0.15 M NaCl, 0.015 M trisodium citrate [pH 7.0]) and suspended in 3 ml of STE buffer (0.1 M NaCl, 0.02 M Tris, 0.01 M EDTA [pH 7.4]). DNA samples were prepared according to a modified method of Cohen et al. (7).
followed by phenol-chloroform extractions and dialysis. The DNA samples were subjected to agarose gel electrophoresis as described by MacInnes et al. (15). This involved digestion of DNA at 37°C overnight. Phenol-chloroform extractions were done as follows: samples were prepared in 1 M ammonium acetate after having been denatured for 30 min in 0.3 M NaOH at room temperature. Samples (50 µl) were applied to a pre-equilibrated nitrocellulose sheet placed on a slot blot apparatus (Schleicher & Schuell Inc.). The nitrocellulose sheet was soaked for 10 min in 4 x SSC and then baked for 2 h in a vacuum oven at 80°C. Sheets were preannealed for 24 h at 42°C with preannealing buffer (3 x SSC, 50% formamide, 200 µg of yeast RNA per ml, 20 µg of denatured salmon sperm DNA per ml, 1 x Denhardt buffer [3% Ficoll, 3% polyvinyl pyrrolidone, 3% bovine serum albumin]). 32P-labeled DNA probes were prepared from 1-µg DNA samples by nick translation (16, 18). Before hybridization, each of the 32P-labeled DNA probes was boiled for 10 min.

Water soluble pigment formed in the presence of 0.1% tyrosine

- nt, Not tested.

**RESULTS**

The DNAs from five strains of *A. serpens* were spotted in twofold dilutions on a nitrocellulose filter and hybridized with *A. serpens* ATCC 12638 DNA as the probe. The degree of hybridization among all five strains was similar to Fig. 1, lane A, rows 1 to 8, which contained the homologous *A. serpens* ATCC 12638 DNA. Taking into account the dilution factor, it can be visually estimated that the strains share at least 50% homology.

The DNAs from five different species of the genus *Aquispirillum* were compared in a series of five blots. The DNAs from each species were spotted in 10-fold dilutions in an identical order on each nitrocellulose filter. In a similar manner, DNAs from the *C. jejuni* and *E. coli* B strains were also included on each filter. Each filter was then hybridized with a different probe made from one of the *Aquispirillum* species. In this manner, reciprocal hybridizations were done between all of the *Aquispirillum* species examined.

In Fig. 2a, *A. sinusomum* ATCC 9786 DNA was used as the probe, and it can be seen that the greatest amount of hybridization occurred with the homologous *A. sinusomum* DNA in lane B, rows 1 to 5. Only low levels of hybridization could be observed with the other *Aquispirillum* species when compared to that occurring between *E. coli* B (Fig. 2a, lane A, rows 11 to 15) and *A. sinusomum*.

In Fig. 2b, *A. putridiconchylium* ATCC 15279 DNA was used as the probe, and the greatest homology can be seen with the *A. putridiconchylium* DNA spotted in lane B, rows 6 to 10. Again, no marked degree of homology was observed with any of the other species, although a slight amount occurred with the *A. serpens* and *A. bengal* DNAs.

A probe made from *A. itersonii* ATCC 11331 DNA was used in Fig. 2c. Again the only appreciable amount of hybridization observed was with the homologous *A. itersonii* DNA in lane C, rows 1 to 5.

In Fig. 2d, a probe made from *A. serpens* ATCC 12638 DNA was used. In this case, the degree of hybridization observed between *A. serpens* DNA (lane A, rows 1 to 5) and

### DNA RELATEDNESS OF AQUASPIRMILLUM SPP.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ATCC 12638</th>
<th>MW5</th>
<th>VHA</th>
<th>VHL</th>
<th>SRS</th>
<th>ATCC 27641</th>
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<tr>
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<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
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<tr>
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<td>+</td>
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</tr>
<tr>
<td>MacConkey</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>TSI</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
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<td>-</td>
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<tr>
<td>Water soluble pigment</td>
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<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>+</td>
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<tr>
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<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>+</td>
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<td></td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>+</td>
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</table>

* 2+, Double-layered surface array.

nt, Not tested.

Hybridization temperatures between 40 and 70°C were tested at 10°C intervals; 40 to 42°C was found to be optimum for *Aquispirillum* DNAs. Each DNA probe was then hybridized separately to a single filter at approximately 2 x 10⁴ cpm/cm² of filter and incubated at 42°C for 48 h in annealing buffer. Blots were washed three times with 2 x SSC at room temperature and then washed twice at 50°C with 0.1 x SSC + 0.1% sodium dodecyl sulfate. The blebs were then rinsed consecutively through two baths of 0.1 x SSC + 0.1% sodium dodecyl sulfate and then through five baths of 0.1% SSC. The resulting slot blots were visualized by autoradiography as described by Swanstrom and Shank (20).

**Densitometric scanning.** Densitometric scanning of autoradiographs was done using a Beckman UV-visible spectrophotometer with a DU-8 gel-scanning system.

**Controls.** (i) Salmon sperm DNA was included in all experiments as a nonhomologous control. (ii) In the DNA trapping experiment the effect of various concentrations of nonhomologous DNA upon the signal being received from homologous DNA annealed to a 32P-labeled probe was assessed. In this experiment, DNA from *A. serpens* ATCC 12638 was spotted in 10-fold dilutions on a nitrocellulose filter. Each dilution of this DNA was then mixed with 1-, 2-, and 3-µg amounts of salmon sperm DNA and spotted in separate columns on the filter. The filter was hybridized with a probe made from *A. serpens* ATCC 12638 DNA.

### TABLE 1. Observed characteristics of various strains of *A. serpens* and of *A. bengal* ATCC 27641

<table>
<thead>
<tr>
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<td>nt</td>
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</table>
A. bengal DNA (lane A, rows 6 to 10) was similar to the last dilution level. Since 10-fold dilutions were used, this indicates that A. bengal and A. serpens share at least 10% homology. When the reciprocal hybridization experiment was done, using A. bengal ATCC 27641 DNA as the probe, again there was a similar degree of hybridization occurring between the two species A. bengal and A. serpens (Fig. 2e). Densitometric scanning of the autoradiographs was done to more closely determine the degree of homology between A. bengal and A. serpens. At least 60% homology was evident, because the unsaturated bands on both autoradiographs showed values ranging from 59 to 73% homology. With the other Aquaspirillum species, there was at least a 100-fold difference in the level of hybridization against either probe, indicating less than 1% homology.

The results of the DNA trapping experiment are shown in Fig. 3. The intensities of the signals obtained from the mixtures of homologous and nonhomologous DNAs, were identical to the signal seen in the corresponding dilutions of completely homologous DNA. This demonstrates that, in the range of dilutions used in our experiments, there was no nonspecific trapping of the probe onto the filter nor any quenching of the $^{32}$P signal by excess amounts of nonhomologous DNA.

**DISCUSSION**

Comparison of the A. serpens strains ATCC 12638, MW5, VHA, VHL, and SRS, against the type strain probe, showed that there were no obvious genotypic differences to reflect the differences in their phenotypes. These strains can therefore be considered as a single genetic species. The limit of resolution of slot blot hybridization is not sufficient to examine the genetic nature of the different phenotypic characters expressed in these strains; therefore, future studies will likely have to resort to restriction endonuclease analysis (4) to do this.

Comparison of the five Aquaspirillum species by slot blot hybridization, using probes involving the whole genome, provides information on the relatedness of these organisms. It is clear the the DNA homology of the species A. serpens and A. bengal, represented by their type strains, does not warrant separate species status. Furthermore, Woese et al. (22) compared 16S ribosomal RNA oligonucleotide sequences from a wide range of gram-negative bacteria, including a number of Aquaspirillum species in particular. A. serpens and A. bengal. They found these latter two to be most closely related among the aquaspirilla studied, with an $S_{AB}$ value of 0.93. Woese and his colleagues state that this is high enough for A. serpens and A. bengal to be considered strains of the same species. Therefore, we propose that A. bengal be declared a subjective synonym for A. serpens.

The genus Aquaspirillum appears to be quite broad considering that the levels of hybridization observed between these species was often no greater than or even less than the level of hybridization which occurred with E. coli B. Woese et al. found a great degree of phylogenetic heterogeneity among the Aquaspirillum species used in their studies in-
FIG. 2. Comparison of five *Aquaspirillum* species by a series of slot blot hybridizations. DNA samples were spotted identically on each nitrocellulose filter in 10-fold dilutions starting with 1 μg of DNA each. Lane A, rows 1 to 5, *A. serpens* ATCC 12638; lane B, rows 1 to 5, *A. sinuosum* ATCC 9876; lane C, rows 1 to 5, *A. itersonii* ATCC 11331; lane A, rows 6 to 10, *A. bengal* ATCC 27641; lane B, rows 6 to 10, *A. putridiconchylium* ATCC 15279; lane C, rows 6 to 10, *C. jejuni* UWO 1210; lane A, rows 11 to 15, *E. coli* B ATCC 15669; lane B, rows 11 to 15, salmon sperm DNA; the X-ray film was exposed for 3 days. (a) *A. sinuosum* ATCC 9876 DNA was used as a probe. (b) *A. putridiconchylium* ATCC 15279 DNA was used as a probe. (c) *A. itersonii* ATCC 11331 DNA was used as a probe. (d) *A. serpens* ATCC 12638 DNA used as a probe. (e) *A. bengal* ATCC 27641 DNA used as a probe.

Volving 16S ribosomal RNA cataloging (21, 22). The relation of *A. itersonii* and *A. polymorphum* to the genus *Azospirillum* has also been indicated by ribosomal RNA/DNA hybridization studies (8).

We have shown that slot blot hybridization can provide reliable visual information about the degree of DNA/DNA homology between different bacteria as long as optimum conditions are employed. The amounts of DNA applied to the filter must be standardized, saturation of the filter must be avoided, and an optimum temperature of hybridization must be chosen. The DNA trapping experiment demonstrates the specificity of this method for homologous DNA even in mixtures containing low concentrations of that DNA. Indeed, Kafatos et al. (10) found that with dot blot hybridization they could obtain reproducible estimates of relative concentrations for sequences accounting for less than 1:10⁻⁴ of a mixture.

While the information gained from slot blot hybridization
is only semiquantitative, the technique is still extremely useful for taxonomically grouping bacteria into species within a genus. No previous genetic information needs to be known for the organisms being examined, and reciprocal hybridizations can be done relatively quickly. Depending upon whether 10-fold or 2-fold dilutions are used, one can visually estimate at least 10 or 50% homology between different DNA samples. This is sufficient for many taxonomic purposes and avoids the tedious chore of cutting out the slots from the nitrocellulose sheet to be counted in a densitometric scanning of autoradiographs.

Nomencclatural considerations. The only nomenclatural requirement is that A. serpens takes precedence over A. bengal; the phenotypic distinction is varietal and infrasubspecific. ATCC 27641, formerly the type strain for A. bengal, should now be recognized as representing A. serpens biovar bengal and distinguishable from strains of A. serpens biovar serpens by a high optimum temperature for growth (41°C) and by the formation of brown pigments in the presence of 0.1% of tyrosine and of tryptophan in media (Table 1). The apparent subjective synonymy of A. serpens (Muller 1786) Hylemon, Wells, Krieg, and Jannasch 1973 (9) and A. bengal Kumar, Banerjee, Bowdre, McElroy, and Krieg 1974 (14) requires emendation of the description of A. serpens to allow inclusion of the strains of the biovar bengal.

Description of the species, A. serpens (Muller 1786) Hylemon, Wells, Krieg, and Jannasch 1973 (9) should be emended to include: cell diameter, 0.6 to 1.2 μm; optimum growth temperature, 35 to 41°C; brown pigments may be produced in media containing tyrosine or tryptophan; and G+C values of the DNA range from 49 to 52 mol%.

FIG. 3. DNA trapping experiment for slot blot hybridization. A. serpens ATCC 12638 DNA was spotted alone or with salmon sperm DNA onto a nitrocellulose filter and hybridized with an A. serpens ATCC 12638 probe as described in the text. Lane A, rows 1 to 4, A. serpens DNA in 10⁻¹, 10⁻², 10⁻³, and 10⁻⁴ μg amounts, respectively; lane B, rows 1 to 3, A. serpens DNA (10⁻¹ μg) mixed with 1, 2, and 3 μg of salmon sperm DNA, respectively; lane C, rows 1 to 3, A. serpens DNA (10⁻² μg) mixed with 1, 2, and 3 μg of salmon sperm DNA; lane D, rows 6 to 8, A. serpens DNA (10⁻³ μg) mixed with 1, 2, and 3 μg of salmon sperm DNA; the x-ray film was exposed for 3 days.

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LITERATURE CITED


