Chromosome Map of the Thermophilic Archaebacterium

*Thermococcus celer*

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A physical map for the chromosome of the thermophilic archaebacterium *Thermococcus celer* Vu13 has been constructed. Thirty-four restriction endonucleases were tested for their ability to generate large restriction fragments from the chromosome of *T. celer*. Of these, the enzymes *NheI*, *SpeI*, and *XbaI* yielded the fewest fragments when analyzed by pulsed-field electrophoresis. *NheI* and *SpeI* each gave 5 fragments, while *XbaI* gave 12. The size of the *T. celer* chromosome was determined from the sum of the apparent sizes of restriction fragments derived from single and double digests by using these enzymes and was found to be 1,890 ± 27 kilobase pairs. Partial and complete digests allowed the order of all but three small (less than 15 kilobase pairs) fragments to be deduced. These three fragments were assigned positions by using hybridization probes derived from these restriction fragments. The positions of the other fragments were confirmed by using hybridization probes derived in the same manner. The positions of the 5S, 16S, and 23S rRNA genes as well as the 7S RNA gene were located on this map by using cloned portions of these genes as hybridization probes. The 5S rRNA gene was localized 48 to 196 kilobases from the 5' end of the 16S gene. The 7S RNA gene was localized 190 to 504 kilobases from the 3' end of the 23S gene. These analyses demonstrated that the chromosome of *T. celer* is a single, circular DNA molecule. This is the first such demonstration of the structure of an archaebacterial chromosome.

The unique phylogenetic position and unusual physiology of the archaebacteria make them interesting subjects for genetic analyses. However, such analyses have been hampered by the paucity of genetic tools available for these organisms. A small number of genes have been cloned from various methanogens, extreme halophiles, and thermoacidophiles, but little is known about the overall structure of their chromosomes (for a recent review, see reference 4). The chromosomes of some methanogens are among the smallest known for free-living organisms: one-half to one-third the size of the chromosome of *Escherichia coli* (13). The chromosome of *Thermoplasma acidophilum*, a free-living, thermoacidophilic archaebacterium that lacks a cell wall, is reported to be approximately 1 x 10^8 daltons (6, 21). Such small chromosomes are also a feature of some facultatively anaerobic eubacteria (11).

This report describes the beginning of a study of chromosome structure among the archaebacteria, with the construction of a physical map of the chromosome of the thermophile *Thermococcus celer*. This sulfur-metabolizing archaebacterium is among the most slowly evolving of the archaebacteria, so its genome organization may retain features of the earliest genetic architecture (2). Genes encoding various stable ribosomal RNA species have been cloned from this organism (2, 7). *T. celer* has a relatively short generation time (50 min) and grows to a reasonable cell density, thus providing sufficient quantities of DNA for restriction digests. Their cell walls can be lysed with detergent and protease, making specific lytic enzymes unnecessary in the preparation of intact chromosomes.

Evidence which demonstrates that the genome of *T. celer* is composed of a single, circular DNA molecule approximately 1,890 kilobases (kb) in size will be presented. The size was determined by using three infrequently cutting restriction endonucleases (*NheI*, *SpeI*, and *XbaI*). Circularity was demonstrated by using partial digests with *SpeI*. These partial digests also allowed the order of the five *Spe* fragments to be determined. A partial digest with *NheI*, along with junction probe hybridizations, allowed alignment of the *Nhe* fragments, as well as independent confirmation of the shape of the genome. Digestion of the *Nhe* fragments with *SpeI* and hybridization analyses confirmed the proposed junctions of the *Spe* map and aligned the two maps relative to one another. Finally, the *Xba* fragments were arranged relative to this map by using double digests and hybridization probes. Hybridization probes derived from the cloned genes for 5S, 16S, and 23S ribosomal RNA and 7S RNA were used to locate these genes on the chromosome map. This is the first demonstration of the circularity of an archaebacterial genome and the first physical map of an archaebacterial chromosome.

**MATERIALS AND METHODS**

Preparation of inserts and digestion of chromosomal DNA.

A culture of *T. celer* Vu13 was kindly provided by W. Zillig. Cultures were grown on a mineral salts medium containing 0.2% yeast extract, 0.2% sucrose, 4% NaCl, and 0.5% sulfur under an atmosphere of hydrogen and carbon dioxide (80:20) with vigorous agitation at 88°C.

Cells of *T. celer* were prepared from a 20-ml culture (5% inoculum) that had been grown for 8 h. This yielded approximately 6 x 10^9 cells. Sulfur was removed from the culture by filtration through glass fiber filters, and the cells were harvested by centrifugation at 10,000 x g for 20 min at 4°C. The cell pellet was suspended in a 1-ml solution of molten 0.5% low-melting-point agarose in 0.5 M NaCl at 37°C. The suspended pellet was quickly dispensed into a mold to form inserts of 0.1 ml (2 by 5 by 10 mm). The embedded cells were digested in a lysis buffer consisting of 10 mM Tris (pH 9.5), 0.5 M EDTA, 1% sodium lauryl sarcosine, and 5 mg of...
pronase per ml. After a 24-h incubation at 37°C with gentle mixing, the liquid was decanted and 10 ml of fresh lysis buffer was added. After another 24-h incubation, the lysis buffer was decanted and the inserts were dialyzed twice with 10 ml of TE buffer (10 mM Tris, pH 8, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride at 4°C for 1 h each time. The dialysis buffer was removed, and the inserts were dialyzed twice with TE buffer alone at 4°C for 1 h each time. The inserts were then stored in fresh TE buffer at 4°C. For restriction digests, the inserts were routinely cut into four equal sections (herein referred to as plugs).

In a typical experiment, two plugs of embedded T. celer DNA were digested in a total volume of 0.2 ml (including the 0.05 ml contributed by the plugs) in the reaction buffer supplied by the manufacturer, supplemented with 1 mg of bovine serum albumin per ml and 1 mM dithiothreitol. For each digestion, 6 to 20 U of restriction endonuclease were used. Complete reaction mixtures were incubated on ice for 30 min to allow the mixture to penetrate the agarose. They were then incubated at 37°C for 6 to 20 h. At the end of the incubation, the plugs were dialyzed with TE buffer. The DNA fragments in both plugs were resolved in a single gel lane by using the GeneLine transverse alternating-field gel electrophoresis system developed by Beckman Instruments, Inc. (Pullerton, Calif.) (10, 23). The gel of 1% low electroendoosmosis agarose was subjected to electrophoresis at 13°C in buffer consisting of 10 mM Tris, 0.5 mM EDTA, and 4.35 mM acetic acid. Electrophoresis conditions were those described in the figure legends below. Resolved fragments were stained with ethidium bromide and photographed under short-wavelength ultraviolet illumination.

Digested isolated restriction fragments. Two plugs of T. celer chromosomal DNA were digested, and the resulting fragments were resolved in a gel consisting of either 1% low electroendoosmosis agarose or 1% low-melting-temperature agarose as described above. After staining with ethidium bromide, the bands were cut out of the gel while being viewed under long-wavelength ultraviolet illumination. The excised fragments were dialyzed against TE buffer and were digested with 12 U of another restriction endonuclease in a total volume of 0.45 ml (including the excised fragment, which was approximately 0.15 ml). The digested fragments were resolved as described above.

Hybridization analyses of resolved fragments. Restriction fragments resolved by transverse alternating-field gel electrophoresis were transferred to charged nylon membranes (Nytran; Schleicher & Schuell, Inc., Keene, N.H.) by capillary transfer after partial depurination and fragmentation of the DNA. DNA was fixed to the membranes by thoroughly drying the membranes at 68°C.

Hybridization probes for each Spe and Xba fragment were produced from excised individual restriction fragments. Preparative electrophoresis was carried out by using 26 plugs aligned across the top of a gel. After electrophoresis and staining, the bands were cut out of the gel while being viewed under long-wavelength ultraviolet illumination. DNA was released from the gel matrix by using either sodium iodide and glass beads (8) or phenol extractions (25). The extracted DNA was labeled with digoxigenin by using the Genius nonradioactive DNA labeling kit from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). After prehybridization for 4 h, these probes were hybridized to the resolved fragments in the aqueous hybridization solution recommended by the manufacturer at 68°C overnight. Posthybridization washes and color development were carried out according to the instructions of the manufacturer.

For reuse, these membranes were decolorized with warm dimethylformamide and the probe was removed by soaking them in 0.2 N NaOH-0.1% sodium dodecyl sulfate at 37°C for 30 min (1).

Materials. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), New England Biolabs, Inc. (Beverly, Mass.), or Pharmacia, Inc. (Piscataway, N.J.). Low electroendoosmosis agarose, low-melting-point agarose, concatemers of bacterial lambda DNA (43.7-kb monomer), and Saccharomyces cerevisiae 334 chromosomes were obtained from Beckman Instruments, Inc. (Palo Alto, Calif.). Lambda concatemers were also synthesized by the method of Waterbury and Lane (24). Lambda DNA was from Bethesda Research Laboratories.

RESULTS

Selection of endonucleases and determination of chromosome size. To prepare intact chromosomes, cells are embedded in agarose prior to their lysis (22). We found it was necessary to suspend and embed cells of T. celer in solutions containing at least 0.5 M NaCl to prevent premature lysis (26). Even with this precaution, some sheared DNA was still evident after electrophoresis, both with and without endonuclease treatment (data not shown).

A variety of restriction endonucleases were tested for their ability to digest the chromosome. Some were chosen because they contain potentially rare nucleotide combinations in their recognition sequences (16). The following enzymes were found to yield fragments with sizes less than 50 kb: ApaI, Aval, BamHI, BglII, EcoRI, EcoRV, HindIII, HinfI, HpaII, KpnI, NotI, PstI, PvuII, SacII, SalI, Sau3AI, ScaI, SphI, Styl, TagI, XhoI, and XmaI. Those yielding some fragments greater than 50 kb were Asel, BclI, ClaI, MboI, MluI, NheI, SfiI, SpeI, SspI, and XbaI (100 U) and SnaBI (10 U) did not digest the DNA properly, giving a smear of high-molecular-weight fragments. Of those that gave larger fragments, only NheI, SpeI, and XbaI gave fewer than 30 resolvable fragments. Digestion of chromosomal DNA with these enzymes gave rise to 5, 4, and 12 bands, respectively (Fig. 1).

The sizes of these bands, as well as those of double digests, were determined by comparison with size standards (Fig. 1 and Table 1). Because of measurement inaccuracies and the resolution limitation of the electrophoresis apparatus, these estimates have an error of approximately 3%. This caused some difficulty in aligning the fragments, since 3% of the size of the largest fragments was often larger than the sizes of the smallest fragments. Size estimates for fragments less than 300 kb were more reproducible than for larger fragments. The quantity of DNA loaded in each lane had an effect on the values obtained, particularly for the larger fragments (22). The lambda DNA concatemers used as size standards did not give distinct bands above about 900 kb. The published sizes for S. cerevisiae chromosomes vary, making these markers less reliable. This situation was especially bothersome for the largest Nhe fragment. The average of the six measurements of the size of the T. celer genome is 1,890 ± 27 kb.

Ordering of Spe fragments and demonstration of chromosome shape. The order of the Spe fragments can be deduced from the partial Spe digest shown in Fig. 2A. The fragment sizes discussed in the following paragraph were measured from this digestion alone and so differ slightly from those in Table 1. Given that SpeI digestion produces only the four
The placement of the last Spe fragment (S3) establishes the shape of the chromosome. Three configurations are possible: (i) S3 is independent of the other fragments, (ii) the chromosome is linear in the arrangement -S3-S2-S4a-S4b-S1-, (iii) the chromosome is linear in the arrangement -S2-S4a-S4b-S1-S3-, or (iii) S3 joins S1 to S2 in a circular chromosome. If S3 were an independent fraction of the chromosome, then one would expect no more than three bands greater than 1,000 kb in size, comprising S1-S2, S1-S2-S4a (and S4b-S1-S2), and S2-S4a-S4b-S1. If S3 were joined to either end of the S2-S4a-S4b-S1 fragment in a linear chromosome, no more than five bands above 1,000 kb would be observed. The fact that six bands are observed rules out both of these possibilities. The remaining possibility (the chromosome is circular with the arrangement -S1-S3-S2-S4a-S4b-) must therefore be the correct arrangement. The 10 bands expected in this case are apparently incompletely resolved. Note that the lowest band above 1,000 kb is brighter than the neighboring bands, suggesting comigration of fragments in this band.

The S2-S4a and S1-S3 joins can be directly demonstrated with junction probes. DNA fragments that contain rare restriction sites (22). Labeled fragment X2 was found to hybridize to itself, the two fragments generated after digestion with SpeI and XbaI, and both the S1 and S3 fragments (data not shown). This confirmed the S1-S3 junction and the placement of X2 at this site. The fact that the S3 fragment was labeled more intensely suggests X2 overlaps S3 more extensively. A junction probe was also found in a collection of the cloned 23S rRNA gene by L. Achenbach. A cloned portion of the 23S rRNA gene hybridized to both the S2 and S4a fragments as well as the X3 fragment. Analysis of the sequence of this region of the 23S rRNA gene revealed that it contains a single Spe site (L. Achenbach, personal communication). Other hybridization studies revealed no inconsistencies with the alignments of the Spe fragments described above.

**Confirmation of circularity and alignment of Nhe fragments.** Bands arising from complete digestion of the chromosome with NheI were observed at 48, 119, 291, 465, and 918 kb. The sum of the sizes of these fragments is close to the sum of the sizes of the five Spe fragments, suggesting that none of the four largest Nhe bands consist of multiple fragments. Most of the Nhe fragments were aligned by using partial digestion fragments (Fig. 2B). Intense partial-digest bands of 352, 583, 758, 817, 874, and 977 kb were observed. Bands larger than these were visible, but reasonable estimates of their sizes could not be made. The partial-digest bands were interpreted as follows (with actual and theoretical sizes, respectively, given in kb): N3-N5 (352; 339), N4-N2 (583; 584), N2-N3 (758; 756), N2-N3-N5 (817; 804), N4-N2-N3 (874; 875), and N4-N2-N3-N5 or N5-N1 (977; 923 or 966, respectively). These results yield the order N4-N2-N3-N5, with the placement of N1 relative to these unresolved.

The determination of the complete order of the Nhe fragments and confirmation of the shape of the chromosome were inferred largely from hybridization analyses. A probe derived from Xba fragment X2 hybridized to Nhe fragments N1 and N4, while a probe from X3 hybridized to N1, N5, and N3. This independently confirmed the circularity of the map and revealed the order of the Nhe fragments as -N1-N4-N2-N3-N5-.

The Nhe map was aligned relative to the Spe map as shown in Fig. 3 by using digests of individual Nhe fragments with SpeI (see Table 2) and hybridization analyses similar to

**FIG. 1.** (A) T. celer DNA digested with NheI (N), SpeI (S), XbaI (X), or combinations of these (NS, NX, SX, and NSX). Electrophoresis was carried out at 330 V for 30 min with a 4-s switching interval and at 250 V for 18 h with a 60-s switching interval. Size standards are yeast chromosomes (Y), bacteriophage lambda DNA concatemers (L), and a HindIII digest of lambda DNA (H). (B) The same digest as in panel A, except twice as much DNA was used to show the smaller fragments. Electrophoresis was carried out at 330 V for 30 min with a 4-s switching interval and at 250 V for 18 h with a 15-s switching interval.
Table 1. Sizes of restriction fragments derived from the chromosome of T. celer

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Size(^a) (kb)</th>
<th>Fragment</th>
<th>Size (kb)</th>
<th>Fragment</th>
<th>Size (kb)</th>
<th>Size of double-digest fragments (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>914 ± 6</td>
<td>S1</td>
<td>637 ± 10</td>
<td>X1</td>
<td>530 ± 19</td>
<td>Nhe-Spe(^b) (kb)</td>
</tr>
<tr>
<td>N2</td>
<td>484 ± 20</td>
<td>S2</td>
<td>578 ± 16</td>
<td>X2</td>
<td>367 ± 20</td>
<td>Nhe-Xba(^b) (kb)</td>
</tr>
<tr>
<td>N3</td>
<td>298 ± 7</td>
<td>S3</td>
<td>502 ± 12</td>
<td>X3</td>
<td>269 ± 11</td>
<td>Spe-Xba (kb)</td>
</tr>
<tr>
<td>N4</td>
<td>114 ± 5</td>
<td>S4a,b</td>
<td>95 ± 3</td>
<td>X4</td>
<td>235 ± 10</td>
<td></td>
</tr>
<tr>
<td>N5</td>
<td>48 ± 4</td>
<td></td>
<td></td>
<td>X5</td>
<td>134 ± 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>X6</td>
<td>118 ± 8</td>
<td>X7</td>
<td>101 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>X8</td>
<td>73 ± 3</td>
<td>X9</td>
<td>11 ± 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>X10</td>
<td>8 ± 1</td>
<td>X11</td>
<td>5,5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>X12</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>1,858</td>
<td>1,907</td>
<td>1,856.5</td>
<td>1,893</td>
<td>1,908.5</td>
<td>1,918.5</td>
</tr>
</tbody>
</table>

\(^a\) Sizes are averages from two to four determinations for each fragment and are given with standard deviations from the means. The measurement error for each fragment is less than 3%.

\(^b\) The restriction map suggests the presence of an additional small fragment in each of these two digests.

\(^c\) Band contained two comigrating fragments.

\(^d\) This fragment was not visible in gels, but its existence and size are indicated by the change in migration of X12 after digestion with SpeI, assuming X12 to be cut once by SpeI.

The three largest Xba fragments (X1, X2, and X3) were cut by SpeI, as were fragments X7 and X12. However, X4, X5, X6, X8, X9, X10, and X11 were not cut by SpeI, since bands of identical mobility corresponding to them were seen in both the Xba and Spe-Xba digests, and all five Spe sites were accounted for in other Xba fragments. The arrangement of the eight largest Xba fragments was determined by digestion of isolated fragments with a second enzyme (Table 2) and by hybridization analyses.

FIG. 2. Resolution of partial restriction fragments. Single plugs of embedded DNA were digested for 6 h with the indicated quantities of each restriction endonuclease. The resulting fragments were resolved by transverse alternating-field gel electrophoresis at 330 V for 30 min with a 4-s switching interval and 220 V for 24 h with a 90-s switching interval. The arrows indicate the locations of the bands resulting from complete digestion of the chromosome with each enzyme. Yeast chromosome (Y) and bacteriophage lambda DNA concatemers (L) are size markers. (A) Partial digest with SpeI. Lanes: 1, 0.05 U; 2, 0.02 U. (B) Partial digest with Nhel. Lanes: 1, 5 U; 2, 2.5 U; 3, 1.3 U; 4, 0.63 U.

FIG. 3. Chromosome map of T. celer. The 0-kb position was chosen as the SpeI site near the 5' end of the 23S rRNA gene. The map is drawn so that the direction of transcription of this gene is clockwise. Fragments X9, X10, and X11 are tentatively assigned the positions shown here.
Junction fragments for three important probes X3, chromosome the evolution of established blots (kindly provided by Achenbach). The work described here resulted in the first restriction map of an archaeabacterial chromosome. Although the resolution of this map is crude, it is significant in that it reveals three important features of an archaeabacterial genome. First, the chromosome is arranged in a circular form. Although this feature is taken for granted among the euabacteria, it had not been established for archaeabacteria, which are known to resemble eucaryotes in a number of ways. Interestingly, fragments of methanogen DNA that are capable of autonomous replication in yeast (17) have been found. Linear DNA is not a uniquely eucaryotic feature, since one instance was discovered recently in euabacteria (19). Second, the chromosome appears to be composed of a single DNA molecule; there are no other large chromosomal elements. Finally, the genome is relatively small. It is similar in size to the smallest methanogen chromosomes, the T. acidophillum chromosome, and those of some facultatively anaerobic euabacteria (11). The T. celer chromosome was previously reported to be 4,000 kb in size on the basis of measurements of the kinetics of hybridization of ribosomal RNA to chromosomal DNA (14). Estimates of genome sizes for methanogens in this same study, however, were two to three times greater than those determined by standard DNA renaturation kinetics measurements (13). It appears the size of the T. celer genome was overestimated by a similar amount.

The smallest Xba fragment, X12, contains an Spe site, as evidenced by its change in migration after digestion with SpeI. This, along with evidence from hybridization, allowed it to be placed at the junction of S3 and S2. The remaining small Xba fragments (X9 to -11) were tentatively placed by using probes derived from these fragments hybridized against blots of Xba partial digests. Because of their small size relative to the large fragments to which they are joined, their assigned positions are less certain.

**Location of RNA genes.** The genes encoding 5S, 16S, and 23S rRNA and 7S RNA were located by using hybridization probes constructed from cloned portions of these genes (kindly provided by L. Achenbach). As discussed previously, the 23S rRNA gene lies at the junction of fragments S2 and S4b. The 16S probe hybridized to fragments N5, S2, and X3, suggesting the 16S gene is in close proximity to the 23S gene. This result was expected, since the 16S and 23S rRNA genes are linked in T. celer (3, 18). The 5S probe hybridized to fragment X3 and to the 299-kb and 148-kb Nhe-Spe and Nhe-Xba double-digest fragments, respectively. These data localize the 5S rRNA gene to a region between the N3-N5 junction and the X4-X3 junction, thus placing it 48 to 196 kb from the 5' end of the 16S rRNA gene. T. celer was reported to have two 5S rRNA genes, one within 1.7 kb of the 3' end of the 23S rRNA gene and the other unlinked to either the 16S or 23S rRNA gene (18). There was no indication of a second gene in the hybridization results reported here. The 7S probe hybridized to fragments S1 and X1. This places the gene for the 7S RNA in a region from 190 to 504 kb from the 3' end of the 23S rRNA gene.

**DISCUSSION**

The work described here resulted in the first restriction map of an archaeabacterial chromosome. Although the resolution of this map is crude, it is significant in that it reveals three important features of an archaeabacterial genome. First, the chromosome is arranged in a circular form. Although this feature is taken for granted among the euabacteria, it had not been established for archaeabacteria, which are known to resemble eucaryotes in a number of ways. Interestingly, fragments of methanogen DNA that are capable of autonomous replication in yeast (17) have been found. Linear DNA

<table>
<thead>
<tr>
<th>Primary fragment</th>
<th>Secondary fragment(s) Xba</th>
<th>Secondary fragment(s) SpeI</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>620, 133, 94</td>
<td>X1 540, 20</td>
</tr>
<tr>
<td>N2</td>
<td>247, 236</td>
<td>X2 286, 110</td>
</tr>
<tr>
<td>N3</td>
<td>295</td>
<td>X3c 183, 75</td>
</tr>
<tr>
<td>N4</td>
<td>113</td>
<td>X4 248</td>
</tr>
<tr>
<td>N5</td>
<td>52</td>
<td>X5 150</td>
</tr>
<tr>
<td>S1</td>
<td>550, 105</td>
<td>X6 137</td>
</tr>
<tr>
<td>S2</td>
<td>465, 397, 262, 200, 152</td>
<td>X7d 75, 25</td>
</tr>
<tr>
<td>S3</td>
<td>424, 294, 220, 143, 87</td>
<td>X8 70</td>
</tr>
<tr>
<td>S4a,b</td>
<td>80 (doublet), 28, 17</td>
<td></td>
</tr>
</tbody>
</table>

* No bands observed.
* Fragments resulting from a partial digest of the primary fragment.
* Hybridization probe derived from X3 binds to fragment S2 and probably S4.
* Hybridization probe derived from X7 binds to either fragment S4a or S4b or to both.

The smallest Xba fragment, X12, contains an Spe site, as evidenced by its change in migration after digestion with SpeI. This, along with evidence from hybridization, allowed it to be placed at the junction of S3 and S2. The remaining small Xba fragments (X9 to -11) were tentatively placed by using probes derived from these fragments hybridized against blots of Xba partial digests. Because of their small size relative to the large fragments to which they are joined, their assigned positions are less certain.

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**Literature Cited**


