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Physical and Genetic Mapping of *Candida albicans*: Several Genes Previously Assigned to Chromosome 1 Map to Chromosome R, the rDNA-Containing Linkage Group

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Analysis of the karyotypes of multiple *Candida albicans* isolates by pulsed-field electrophoresis confirms the observation by Lasker et al. of eight chromosomes. The genes previously assigned to chromosome 1 in fact fall into two groups, one (including *ADE1*, *SOR9*, and *CDC10*) is linked to the ribosomal DNA genes on a chromosome called R, whereas the others are found on chromosome 1. Chromosome R varies in electrophoretic mobility among strains, usually running equal to or faster than chromosome 1 but in rare cases running slower than chromosome 1. In strain 1012A, the decreased mobility of one homolog is associated with the very large majority of the rDNA genes being on that homolog; the second homolog, with only a few copies, migrates with chromosome 2. Linkage analysis by using spheroplast fusion confirms the gene assignments made by hybridization to blots of the electrophoretic karyotype. A newly cloned gene, *LYS2*, hybridizes to chromosome 1.

The dimorphic, asexual yeast *Candida albicans* is one of the most important human fungal pathogens. In the last 10 years, significant advances in the study of the genetics of this organism have been made, including the demonstration that virtually all isolates are diploid (19), the development of parasexual genetics (4, 10), and the demonstration of DNA-mediated genetic transformation (6). During the same period, important aspects of the biology of the organism were discovered, including the phenotypic transition, which involves reversible changes in colony morphology or cell shape (16, 17; for a review, see reference 13), and the discovery of the existence of a number of repeated DNA elements in the genome (15). The interest in and importance of *C. albicans* make it imperative that the tools used to study its genetic system continue to become more powerful.

With the advent of pulsed-field electrophoresis, determining the number of chromosomes in *C. albicans* has become feasible. To advance our knowledge of the genetic system, the genetic map must be aligned with the physical map (the electrophoretic karyotype). Early attempts to accomplish this included determining the location of cloned genes by DNA hybridization to the chromosomal bands of the electrophoretic karyotype (9). Unfortunately, none of these karyotypes, which gave an estimate of six to nine chromosomes (7, 9, 18), was definitive, since one could not be sure that all the chromosomes had been resolved. Adding the estimated size of all the chromosomes and comparing the sum with the calculated size of the genome was not definitive, since the probable chromosome number, seven, gives a DNA size (14 mb [9]) at the low end of the genome size estimates (15 to 18 mb, based on the DNA content of the cells [13]), whereas eight (assuming that the largest band contains two chromosomes) gives a DNA size at the high end of the estimates. The same arguments, however, make nine highly improbable as the chromosome number. Genetic studies point to at least seven linkage groups (12). Results reported herein, based on both advanced methods of pulsed-field electrophoresis and studies of genetic linkages, support eight as the basic chromosome number for *C. albicans*; previous counts of seven were the result of a failure to separate the two largest chromosomes, one of which carries the rDNA genes and varies in its mobility compared with that of chromosome 1, depending on the strain. We propose to call this large chromosome chromosome R, in recognition of the fact that its position in pulsed-field gels compared with those of the other chromosomes can vary within or between strains. The evidence that several of the genes previously assigned to the largest chromosomal band fall into two linkage groups is a further argument against nine as the chromosome number.

MATERIALS AND METHODS

Strains and media. The strains used are listed in Table 1. Cells were grown and maintained on YEPD medium (19). For selection of fusants, the cells were grown on minimal medium (6.7 g of yeast nitrogen base [Difco] per liter, 2% glucose), to which were added the appropriate supplements at standard concentrations of 20 to 30 mg/liter. YNZ medium contains 2% dextrose, 0.25% yeast extract, 1% peptone, 0.1% NaCl, 0.1% potassium phosphate, and 0.05% $MgSO_4$. Two percent agar was used for solid media.

Pulsed-field electrophoresis. Plugs were prepared for electrophoresis in one of two ways. Procedure A was essentially as described previously (9) with broth cultures containing 2×10^7 to 4×10^7 CFU/ml. These plugs were stored for up to 6 months at 4°C in 0.5 M EDTA (pH 9.0).

Procedure B (see Fig. 2) was identical to a previously described method used for *Candida stellatoidea* (20). Cells (3×10^8) from a 20-h Sabouraud agar plate grown at 30°C were washed twice with 1.0 ml of washing buffer (0.5 M EDTA, 0.1 M Tris [pH 7.5]) in a 2.0-ml tube (Sarstedt, Numbrecht, Germany). Cells were suspended in 100 mM

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TABLE 1. Strain list

Strain	Relevant genotype	Source or reference
C. albicans		
FC18		18
1012A	*RDN1-2*	7
B-4497		Type culture, ATCC 8804
B-311		ATCC 32354
3153a		17
A58	*lys2 ade*	5
hOG24	*ade1 pro suf2 RDN1-1*	11
983	*arg pro*	This study
1012M	*met RDN1-2*	This study
hOG676	*ade1 asn met*	10
1161	*MPA1 arg57 ser57 lys1 gal1 ura3*	This study
S. cerevisiae		
1039	α *ura3-52 ade1 trp1*	This study
1053	**a** *hpt1 ura3 Δ200 lys2-801 ade2*	This study
YPH98	**a** *ura3 lys2 ade2 Δ(trp1-ars1)*	J. Berman

Tris (pH 8.0)–5 mM EDTA (pH 8.00) containing 5.0% 2-mercaptoethanol and incubated in a 37°C water bath for 30 min. Suspensions were pelleted, washed once in 20 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.0)]–1 M Sorbitol, and suspended in SCE (1 M Sorbitol, 0.1 M sodium citrate [pH 5.8], 0.01 M EDTA [pH 8.0]); 50 μl of a 10-mg/ml stock spheroplasting solution (Zymolyase 20T; ICN Immunobiologicals, Lisle, Ill.) made up in SCE was added to the suspension, which was vortexed briefly. Then 300 μl of 1.0% low-melting-point agarose (Incert agarose; FMC, Rockland, Maine) made up in 0.125 M EDTA (pH 7.5) held at 37°C was immediately added to the tubes, which were mixed by inversion. Molten plugs were poured into a 96-well microtiter plate on ice. Plugs were removed to 2.0 ml of LET (0.45 M EDTA [pH 9.0], 10 mM Tris [pH 8.0]) containing 1.0% 2-mercaptoethanol in a 10.0-ml tube and incubated for 24 h at 37°C. LET was removed, LET–1.0% sarcosine (pH 9.0)–2 mg of proteinase K per ml was added, and then the mixture was incubated at 50°C for 20 h. Before loading, plugs were rinsed twice in 10 volumes of running buffer (0.5× TBE [9]) at 5°C for 1 h each. Rinsed plugs were removed to Eppendorf tubes and melted at 67°C. Samples were gently loaded into the wells of a 0.6% agarose gel (chromosomal grade agarose; Bio-Rad, Richmond, Calif.) made up in 0.5× TBE with a 1.0-ml syringe and a 19-gauge needle. Electrophoresis was performed in a Bio-Rad CHEF DRII unit at 70 V with a dual-ramp switch time of 180 to 300 s for 30 h followed by 420 to 900 s for 66 h. Gels were stained for 15 min in ethidium bromide, destained for 3 h in distilled water, and then photographed. This method works well with isolates that grow primarily as yeast cells but is particularly effective for isolates that tend to form abundant pseudohyphae.

Electrophoresis was carried out under the following regimen, with a Bio-Rad CHEF-DRII instrument: 30 h during which the switch time was ramped from 120 to 300 s; 66 h during which the switch time was ramped from 420 to 900 s; and, where indicated, 24 h with the switch time ramped from 900 to 1,200 s all at 65 V and 11°C. The gels were 0.4% Bio-Rad chromosomal-grade agarose made up in running buffer. Gels were stained with ethidium bromide and blotted to Zetaprobe (Bio-Rad) or Nytran (Schleicher & Schuell) filters, which were hybridized in 0.5 M sodium phosphate (pH 7.2)–1 mM EDTA–7% sodium dodecyl sulfate and subjected to radioautography as described by the manufacturers. Filters were stripped and reused several times (Fig. 1).

Crosses. Genetic crosses were carried out by spheroplast fusion as described by Poulter and Rikkerink (11). Heat shock (4) to induce chromosome loss was carried out by using conditions determined to be optimal for each fusion product, but temperatures were generally around 51°C and times were from 90 to 150 s. After the heat shock, the survivors were plated on YNZ (11) medium and replicated onto selective media to look for chromosome loss as indicated by segregation of one of the parental auxotrophs. Segregants were then scored for potentially linked markers. Alternatively, segregants were identified by the appearance of the recessive marker, 2-deoxygalactose resistance. Deoxygalactose resistance was scored on supplemented minimal medium containing 3% glycerol as the carbon source and 0.1% 2-deoxygalactose. Adenine auxotrophs were identified by the red color of the colonies on YNZ medium.

Southern analysis. Enzyme digestion and Southern analysis were carried out as described by Magee et al. (8). All probes were labeled by nick translation or oligonucleotide priming.

Gene isolation. The *ADE1* and *LYS2* genes from *C. albicans* were isolated by complementation of the appropriate defect in *Saccharomyces cerevisiae* with a library prepared in plasmid p1086 (3) or pEMBLY23 (1). Ura^+ transformants were tested for prototrophy for the desired markers. For *ADE1*, the recipient was strain 1039. For *LYS2*, the recipient was YPH98. Plasmids from the transformants were returned to *Escherichia coli* by standard means, and the reisolated plasmids were tested in *S. cerevisiae*. For *ADE1*, the identity of the sequence was proved by transformation of the *C. albicans* auxotrophs hOG24 and hOG676. For *LYS2*, the sequence was shown to complement the auxotrophy in A58, previously called *lys1* (5). *ADE1*, a subclone in pBR322 (plasmid 1055), was used as a DNA hybridization probe. For *LYS2*, the cloned insert was cut out and labeled for probing.

RESULTS

Lasker et al. (6) demonstrated that the electrophoretic karyotype of strain 1012A contained eight separable bands, rather than the seven previously reported in several strains. This extra band, which contained the rDNA genes, could be a homolog of one of the previously identified chromosomes that was altered in mobility by a translocation, or it could represent a new pair of homologs that were resolvable in strain 1012A but not found in previous electrophoretic separations. If it represents a newly resolved set of homologs, some but not all of the genes that were shown to hybridize to the largest band in previous studies ought to hybridize to it and not the second band in the karyotype. Figure 1A shows the electrophoretic karyotypes of strains 1012A and FC18. Figure 1B shows the hybridization of several genes to blots of these karyotypes. The genes previously assigned to the largest band fall into two groups, one that hybridizes to the rDNA-containing band and one that hybridizes to the next-largest band in 1012A (Fig. 1). It is therefore clear that previous separations failed to resolve the eighth pair of homologs in the strains examined, as suggested by Lasker et al. (7).

ADE1, *SOR9*, and *CDC10* show interesting hybridization behavior; they hybridize weakly in 1012A to the largest band, chromosome R, and strongly to a band that is indistinguishable from chromosome 2, although in all other

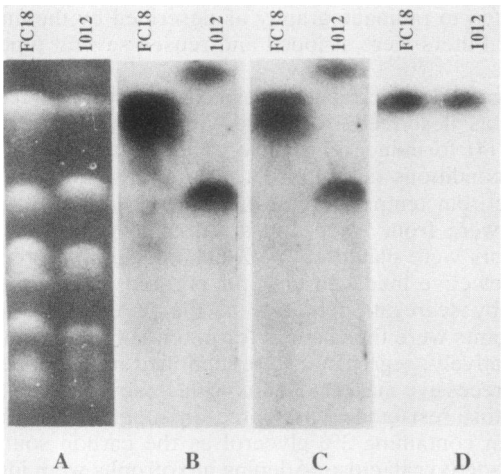

FIG. 1. Electrophoretic karyotypes and gene hybridization to strains 1012A and FC18. (A) Electrophoretic karyotype. The pulsed-field separations were run as described in Materials and Methods. (B) Karyotype in panel A probed with the *SOR9* clone. (C) Blot in panel B probed with the *ADE1* gene. (D) Similar karyotype blotted and probed with the *TRP1* clone.

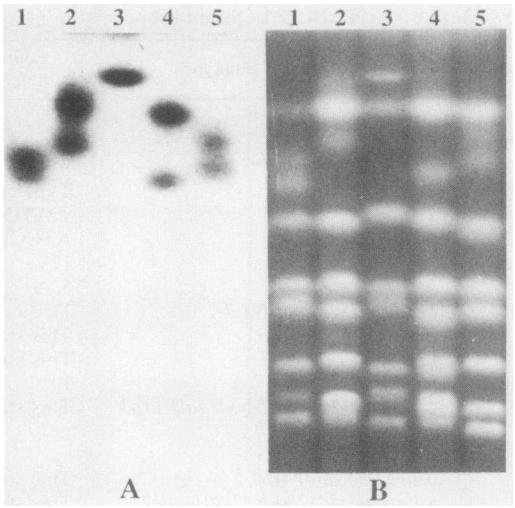

FIG. 2. Chromosome R has different mobilities in different strains. (A) Blots, probed with a labeled rDNA clone, of electrophoretic karyotypes of B4497 (lane 1), B311 (lane 2), 1012A (lane 3), 3153a (lane 4), and FC18 (lane 5). (B) Gel of panel A stained with ethidium bromide.

strains examined they hybridize only to the putative chromosome R. Lasker et al. showed that rDNA hybridized only to the largest band in 1012A (7) (Fig. 1A). We therefore asked whether *ADE1* and *RDN1* (the rDNA locus) were linked. In *C. albicans*, which lacks a sexual cycle, genetic linkage can be demonstrated by spheroplast fusion followed by heat shock, which leads to chromosome loss. If two markers are always or nearly always lost together after heat shock, one can infer that they are on the same chromosome. Although a majority of *C. albicans* isolates contain a particular restriction pattern in the rDNA repeat (the *RDN1-1* allele, or type I), a minority contain a polymorphism (*RDN1-2*, or type II) (8, 14). When the two types are crossed, inheritance of the parental rDNA types can be followed by Southern blots of the heat-shocked progeny. By examining the cosegregation of any trait with the rDNA pattern, linkage can be established. We accordingly crossed 1012M, a Met^- derivative of 1012A (type II) with hOG24 (type I), a well-characterized *ade1* strain. The resulting fusants were heat shocked to cause them to lose chromosomes, and red (Ade^-) progeny were picked; 12 of 12 had type I rDNA, indicating that the two loci are linked in 1012M (Table 2), since the alleles from this strain (*ADE1* and *RDN1-2*) are invariably lost together. Thus, chromosome R most likely contains an active *ADE1* locus as well as the *RDN1* locus in 1012M, whereas hybridization results show that there is a second *ADE1* locus that migrates with chromosome 2. Similar linkage results for *RDN1* and *ADE1* were found in a cross of hOG24 with strain 983, but in strain 983 *ADE1* hybridizes to a single band.

TABLE 2. Linkage of genes on chromosomes 1 and R

Cross	Segregant selected	Cosegregation
hOG24 (*ade1 pro suf RDN1-1*) × 1012M (*met RDN1-2*)	Ade^-	*ade1-RDN1-1* (12/12)
hOG24 × 983 (*arg pro RDN1-2*)	Ade^-	*ade1-RDN1-1* (11/11)
hOG24 × 1161 (*gal1 ser57 RDN1-2*)	Gal^-	*gal1-ser57* (10/14) *gal1-RDN1-2* (2/14)[a]

[a] Ten of the 14 segregants were heterozygous for *RDN1*.

Do all *C. albicans* strains contain chromosome R? The demonstration of chromosome R in 1012A does not prove that all *C. albicans* strains have eight chromosomes. However, refined pulsed-field electrophoresis techniques have succeeded in separating chromosome R from chromosome 1 in several strains, including FC18, the strain on which the previous electrophoretic karyotype was based. Figure 2B shows the karyotypes of five strains, including 1012A and FC18, and Fig. 2A shows the radioautograms of the transfers probed with rDNA. It is evident that chromosome R does not always migrate in the same place relative to chromosome 1. In FC18 and B4497 (the type strain of *C. albicans*), both homologs migrate faster than those of chromosome 1; in B-311 and 3153A, one homolog overlaps with chromosome 1 and the other migrates more rapidly. We therefore propose to call the rDNA-containing chromosome chromosome R, since any numbering system based on relative chromosome position would be incorrect for some strains.

Which of the other "chromosome 1" genes are on chromosome R? The strain with the most easily separable chromosome R, 1012A, has at least one nonstandard linkage, the presence of *ADE1*-hybridizing sequences in the band with chromosome 2; hybridization data from this strain must therefore be confirmed in other strains to ascertain their generality. Table 3 shows the hybridization data from several experiments. *ADE1*, *CDC10*, *SOR9*, and *RDN1* are all found on chromosome R, whereas the rest of the genes previously assigned to chromosome 1 are found on what seems from physical evidence to be a single linkage group.

To define chromosome 1 genetically as well as physically, we carried out a series of linkage studies with strain 1006 (3). J. Gorman (2) has shown that selection for resistance to 2-deoxygalactose will yield *gal1* strains; such a strain was constructed from strain 1006, which also contains the *ser57* marker, to yield strain 1161. This strain was then fused to hOG24, and heat-shock experiments to determine linkage

TABLE 3. Gene assignments by hybridization for chromosomes 1 and R

Chromosome 1	Chromosome R
ACT1	*ADE1*[a]
CDC3	*CDC10*[a]
GAL1	*RDN1*
LYS2	*SOR9*[a]
TRP1	*MGL1*
TUB2	

[a] *ADE1*, *SOR9*, and *CDC10* also blot to the band containing chromosome 2 in 1012A.

were carried out as before. The *ser57* and *gal1* genes are linked and *gal1* is not linked to rDNA (Table 2). We can therefore define chromosome 1 genetically as the location for the *GAL1* and *SER57* markers and chromosome R as the location for the *ADE1* locus.

Why does strain 1012A have such a large chromosome R? In contrast to most other strains observed, chromosome R in 1012A is much larger than chromosome 1 and, based on the rDNA hybridization data, does not appear to separate easily into homologs. To determine whether the rDNA is evenly apportioned in this strain, we digested the DNA with *Hin*dIII, an enzyme that does not cut within the rDNA repeat. Thus, the sizes of the fragments generated containing the rDNA give maximum estimates for the sizes of the repeats. In FC18, the sizes of the two fragments generated are about 650 and 900 kb (Fig. 3, lane 1), whereas digestion of 1012A yields one large fragment of about 2,200 kb. The simplest explanation for these results would be unequal crossing over, so that one homolog of chromosome R contains approximately 180 rDNA repeats and the other contains one or a few. If the *SOR9*, *ADE1*, and *CDC10* genes were centromere proximal to the rDNA, the finding that they hybridize to a band running at the same place as chromosome 2 as well as to the large chromosome R band could be explained, since the smaller crossover product might run there. The failure to see an rDNA band at the position of chromosome 2 in the 1012A lane in Fig. 2 is due to the very short exposure time needed for the highly repeated genes on the large homolog. Some highly overexposed radioautographs of blots probed with the rDNA gene do show a faint band at the position of chromosome 2 (data not shown).

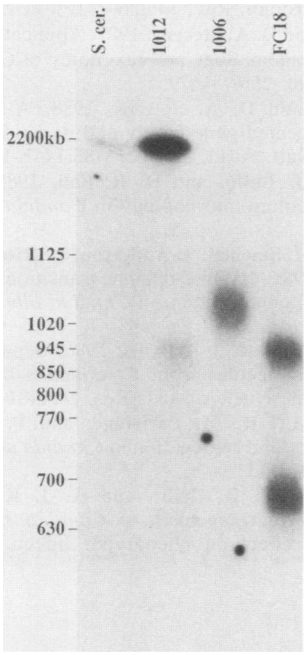

FIG. 3. Sizes of the rDNA repeats in FC18, 1006, and 1012A. Genomic DNA was digested with *Hin*dIII, and the digest was separated by pulsed-field electrophoresis (24 h with a 60-s switch time, 16 h with a 90-s switch time; 200 V). The separation was then blotted and probed with a labeled rDNA clone. The size markers were *S. cerevisiae* chromosomes.

DISCUSSION

The number of linkage groups in the chromosomes in *C. albicans* has been very difficult to determine. Classically, the chromosome number is equated with the number of centromeres, as determined by the mapping of centromere-linked genes. In the absence of meiosis, this counting becomes very difficult. Poulter et al. (10) began the first assignment of genes to separate linkage groups; within limits, this approach can provide an accurate counting of chromosomes, but a very large number of markers is needed before all linkages can be determined. Chromosome loss by the heat shock method of Hilton et al. (4) can be used in lieu of meiosis to demonstrate linkage across centromeres, since genes on both arms are lost together and the chromosome number is thus determined by counting the number of separate linkage groups. E. Rikkerink (12) attempted this and arrived at a chromosome number of seven. Obviously, this sort of counting requires that the available genetic markers include at least one on every chromosome arm and that all possible two-way crosses be made.

Pulsed-field electrophoresis can provide a rapid way to count chromosomes, but it is also complicated by the problem of resolving all of the chromosomes; this problem is compounded when the chromosomes are all in the 1- to 5-mb range, as is the case with *C. albicans*, and are thus technically difficult to separate. Additional difficulties arise due to the dimorphic nature of this organism. Predominantly yeast cells are easy to work with, but cultures containing abundant pseudohyphae do not yield clear karyotypes. Pseudohyphae can form because of switching or of environmental factors such as growth temperature or medium. We have found that agar cultures show less pseudohypha formation than do broth cultures. This culture method also works well with strains that contain mostly budding yeasts and has the added advantage of being easier to use. Estimates of chromosome number based on the electrophoretic karyotype of *C. albicans* have ranged from six to eight (7, 9, 18). We feel confident of our present count of eight, arrived at by using genetic techniques combined with pulsed-field electrophoresis and blotting to identify separated homologs. Although it is still formally possible that chromosome 1 contains two unresolved sets of homologs, our inability to separate any new bands and the genetic evidence for two linkage groups, chromosomes 1 and R, render this possibility unlikely. The possibility that any of the other bands of the electrophoretic karyotype conceals two sets of homologs is also low. In comparing different strains under a variety of conditions, we have never seen one of these bands separate into two that were not demonstrably homologs. These are strains in which apparent translocation has changed the normal linkage rela-

tionships, but these can be rationalized on the basis of an eight-chromosome karyotype.

We have chosen to preserve the chromosome numbering scheme of Magee et al. (9), since the largest number of genes has been assigned to chromosomes with this system. Since that scheme involves numbering from the largest to the smallest chromosome, it presents a problem with regard to the new large chromosome. We propose to resolve this issue by calling the rDNA-containing chromosome chromosome R. This both avoids the problem of changing the old numbers and recognizes the fact that, in contrast to the other chromosomes, there is no consistent place in the electrophoretic order for this band. It is sometimes found as the slowest band, sometimes it is coincident with chromosome 1, and sometimes it runs (usually as split homologs) between chromosomes 1 and 2. It is important to note that this behavior would render problematic any scheme that numbers all eight chromosomes according to their migration rates in any given strain. In contrast to chromosome R, the rest of the karyotype is quite consistent among the strains we have studied in this paper and elsewhere (9); although homologs resolve in some cases, we have not yet seen a strain in which chromosome 4 has a higher mobility than chromosome 5, for example. The frequent resolution of homologs of chromosome R is no doubt due to the uneven distribution of rDNA repeats on the two homologs due to mitotic recombination. These unequally sized homologs must, however, be reasonably stable or the bands would not be visible. Still, minor variations in the size of the homologs may account for the fuzzy nature of the bands in FC18, for example. Strain 1012A may provide an example of a very large difference in homolog size. The homolog of chromosome R, which is easily resolvable from chromosome 1, seems likely to be the result of a crossover that has left all but one or a few rDNA repeats on one homolog. The smaller homolog may be migrating at the same rate as chromosome 2, thus accounting for the apparent hybridization of *ADE1* to that chromosome. Such an outcome would require that *ADE1*, *SOR9*, and *CDC10* be centromere proximal to the *RDN1* gene. We have no other evidence of the gene order on chromosome R at this time, but further work on determining linkage and better chromosome resolution should determine whether this explanation is correct.

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ADDENDUM IN PROOF

During the processing of this paper, we were made aware of a study by Rustchenko-Bulgac et al. (E. P. Rustchenko-Bulgac, F. Sherman, and J. B. Hicks, J. Bacteriol. **172**:1276–1283, 1990) which also presented evidence for eight chromosomes.

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