Volume 16 Number 13 1988 Nucleic Acids Research

# Pulsed-field electrophoresis indicates larger-than-expected sizes for mycoplasma genomes

Louise E.Pyle, Lynn N.Corcoran[1]*, Benjamin G.Cocks, Andrew D.Bergemann, Jane C.Whitley and Lloyd R.Finch

Russell Grimwade School of Biochemistry, University of Melbourne and [1]Walter and Eliza Hall Institute, Parkville, Victoria 3052, Australia

Received February 12, 1988; Revised and Accepted June 1, 1988

## ABSTRACT

The sizes of large DNA fragments produced from genomes of members of the Mycoplasmataceae by digestion with restriction endonucleases having infrequent (1 to 3) cleavage sites within the genome were estimated from their mobility in contour-clamped homogeneous electric field (CHEF) agarose gel electrophoresis by comparison with yeast chromosomal DNA markers. The estimates of total genome size for 7 strains of 6 species ranged from approximately 900 kilo base pairs (kb) for Ureaplasma urealyticum 960$^T$ to 1330 kb for M. mycoides subsp. mycoides, GC-1176. The values derived from this new method are considerably higher than those of approximately 500 Mdaltons or 750 kb previously reported for genome sizes in members of the Mycoplasmataceae.

## INTRODUCTION

An attractive aspect to the study of mycoplasmas is their status as the smallest and simplest self-replicating cellular organisms with the smallest recorded genomes. The reported genome sizes for members of the Mollicutes fall into two clusters; one composed of Mycoplasma and Ureaplasma species with a genome size of about 500 MDaltons, or approximately 750 kilobase pairs (kb), and the other of Acholeplasma, Spiroplasma and Anaeroplasma species with a genome about twice the size (1, 2). Only a limited number of species have been examined and in most cases the sizes reported have been based on renaturation kinetics (3) supported in some cases by contour length measurements from electron microscopy of spread genomes. It has been pointed out (1, 4) that the published values should be reconsidered since DNA renaturation kinetics are affected by the (G + C) content, which is very low for mollicute DNA, and this factor was not taken into account in previous genome size measurements. Recently, estimates of genome size have been made for Escherichia coli JM101 and two strains of Acholeplasma laidlawii (5), using an innovative 2-dimensional restriction digest gel electrophoresis technique. The technique used measures the number and sizes of the additional restriction fragments produced when the electrophoretically separated

© IRL Press Limited, Oxford, England.

fragments from a digest by one enzyme are digested by a second enzyme and vice versa. Genome size is derived from this data on the assumption that the restriction sites are randomly distributed in the genome. These estimations gave a value of 3369 kb for E. coli JM101, within the previously reported range for K12 derivatives, and values of 1705 and 1483 kb for the A. laidlawii strains JA1 and K2, respectively. The latter values are within the previously reported range for Acholeplasmas as determined by DNA renaturation kinetics.

The development of techniques for the preparation and manipulation of large DNA molecules in agarose, and their separation by pulsed field agarose gel electrophoresis (PFGE) (6, 7, 8) suggests another approach to the determination of size for relatively small genomes. By choice of suitable restriction endonucleases (9), the genome can be cut to give one or a small number of fragments which might then be separated and sized by comparison with markers in PFGE. We report on the estimation of genome size for a small number of mycoplasmas (members of the Mycoplasmatales) by this approach.

## MATERIALS AND METHODS

### Sources of Genomic DNA

Mycoplasma mycoides subsp. mycoides strains Y and GC-1176 were grown in PPLO broth and harvested and washed as described previously (10). Ureaplasma urealyticum 960 (ATCC 27618) was grown, harvested and washed as reported before (11). The cultures of M. mycoides were treated with chloramphenicol (80 mg/l) for 1 1/2 h before harvest (12). The DNA of Mycoplasma hyopneumoniae strain J was kindly supplied by Dr. L. Lloyd of the CSIRO Animal Health Laboratory, Parkville, Vic. 3052 prepared in agarose blocks as described below. Similar preparations from Mycoplasma gallisepticum PG 31, Mycoplasma iowae 695, and Mycoplasma synoviae WVU 1853, were kindly supplied by Dr. K. Whithear, Department of Veterinary Para-Clinical Science, University of Melbourne, Parkville, Vic., 3052. DNA of a recombinant vaccinia virus, V-KAHRP, from WR strain with an insert of 1 kb in the HindIII J fragment, was kindly supplied in agarose by Dr. G. Langford of the Walter and Eliza Hall Institute, Parkville, Vic.

Preparation and digestion of DNA in agarose blocks Cells (approximately 4 x $10^9$/ml) were incorporated into low melting agarose blocks (FMC Sea Plaque) and their DNA released in situ as described by Schwartz and Cantor (6) with the modifications of Kemp et al. (13). Digestion with restriction endonucleases and testing for effectiveness of digestion was performed as previously described (12).

Agarose gel electrophoresis with yeast chromosomal DNA markers

Electrophoresis was performed using a CHEF apparatus, as described by Chu et al. (8). Agarose gels (1%) were run for 24 h in 44.5 mM Tris-borate, 44.5 mM boric acid, 1 mM EDTA (cooled to ∿12°C) in a voltage gradient of ∿ 5.7 v/cm. Pulse times varied between 20 s and 200 s, depending on the size range of molecules to be resolved. Some electrophoresis was also performed by the field inversion gel electrophoresis (FIGE) technique using a FIGET Pulse Programmer (Acronym Pty. Ltd., Boronia, Vic.) as described previously (12) Intact DNA molecules from chromosomes of *Saccharomyces cerevisiae* strain YP 80 (YNN 295) were used as molecular weight markers (14), together with those of another strain, YP148. YP 148 has one of its larger chromosomes fragmented to yield an additional small chromosome containing a DNA molecule of 97 kb.

The DNA molecules from *S. cerevisiae* strains provide suitable markers ranging from 97 kb up to over 2,000 kb (14, 15). YP 80 has the same pattern of chromosomal DNA molecules as YNN 295 on which data is given by Vollrath and Davies (14). Sizes for the 10 smaller chromosomal DNA molecules, up to 829 kb, were obtained through a chain of personal communication from these authors via Dr. D. Kemp. However, their sizes can be derived by comparison of their mobilities with those of λ concatamers in Fig. 1B of ref. 14. Consideration of the data in Figs. 1A, 1C and 2A of ref. 14 and extrapolations of our own data in Fig. 1 upward from the 829 kb band suggest values of 924 and 971 kb for the next two chromosomes for which Mortimer and Schild (15) give values of 991 and 1047 kb. Fig. 2 in ref. 15 shows the next two chromosomes as approximately 1150 and 1260 kb and the two largest chromosomes at about 1600 and 2200 kb. The data in ref. 14 are consistent for the former two molecules showing them running together in Fig. 2A and indicated as 1.2 Mb (1200 kb). However, for the latter two, ref. 14 quotes Carle and Olson (personal communication) to give a value of 2.5 Mb for the largest and notes that the two largest chromosomes differ by about 1 Mb in size. This would make the second largest chromosome about 1.5 Mb which is in approximate agreement with the value obtained by counting λ concatamer bands between it and the 1.2 Mb band in Fig. 2A of ref. 14.

## RESULTS

The technique of contour-clamped homogeneous electric field (CHEF) agarose gel electrophoresis is particularly suitable for the determination of molecular size of DNA fragments (8, 14) since the fragment separations on the gels show two regions of good resolution where the mobility approximates a

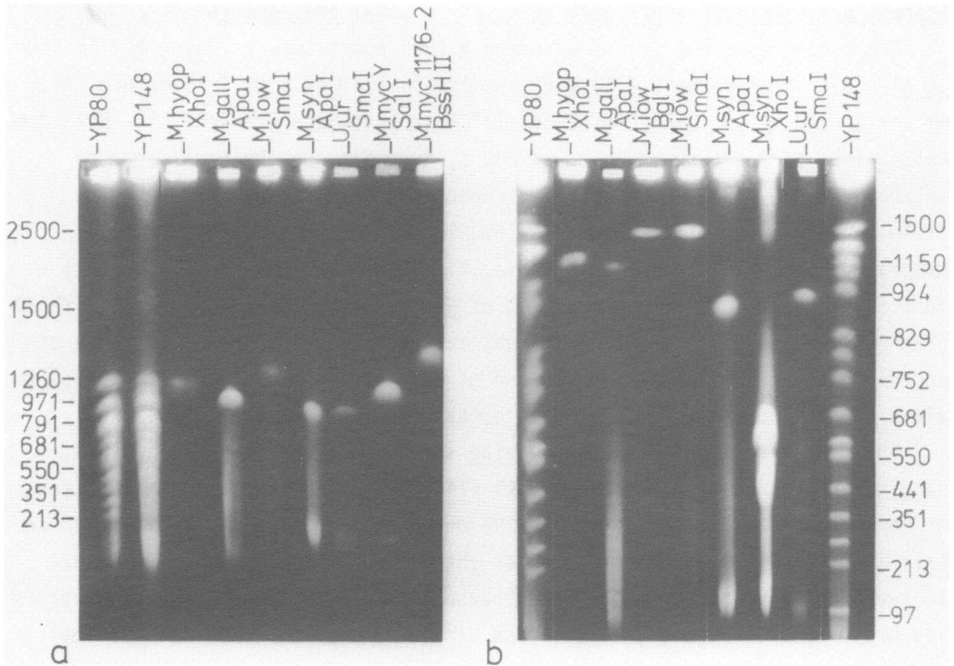


Fig. 1 Fragments separated from restriction digests of DNA from various mycoplasmas by CHEF agarose gel electrophoresis. The gels were run at pulse-times of 200 (a) and 100s (b) for 20 h, then stained with ethidium bromide and photographed on a UV transilluminator. The sizes (kb) of yeast DNA markers on the same gels are shown to the sides of the photographs. The lanes are labelled above to indicate the source of DNA and restriction endonuclease used for the individual samples.

linear function of molecular weight. The ranges of molecular sizes resolved can be altered by variation of the conditions of pulse-time and/or voltage (14).

Estimation of the size of mycoplasma genomes cannot be performed by direct comparison of their electrophoretic mobility with those of the yeast DNA markers because the genomes are circular and circular DNA molecules do not move readily in PFGE (8). It is therefore necessary to convert the genomic DNA to linear fragments by restriction endonuclease digestion before electrophoresis. For simplicity in the estimation of total genome size, it is desirable to use digests with only a small number of restriction fragments, preferably one. Initial studies to define suitable endonucleases for digestion of the DNA of the various mycoplasma strains were performed using the field inversion gel electrophoresis (FIGE) technique (16) with a FIGET pulse programmer as we have described elsewhere (12).

**Table 1** Apparent sizes of some mycoplasma genomes by comparison with yeast chromosomal DNA markers in pulsed-field electrophoresis

| Organism | Digest | Estimated Fragment size (kb) | | Apparent genome size (kb) |
|---|---|---|---|---|
| | | A | B | |
| M.hyopneumoniae, J strain | XhoI | 1140 | 1080 | 1140 |
| M.gallisepticum, PG 31 | ApaI | 1050 | 1050 | 1050 |
| M.iowae, | BglI | - | 1240 | - |
| | SmaI | 1280 | 1240 | 1280 |
| M.synoviae, WVU 1853 | ApaI | 900 | 880 | 900 |
| | XhoI | - | 580 + 400 | - |
| U.urealyticum 960 | SmaI | 900 | 890 | 900 |
| M.mycoides subsp. mycoides, Y strain | SalI | 1100 + 100 + 40 | | 1240 |
| M.mycoides subsp. mycoides, GC 1176-2 | BssHII | 1330 | | 1330 |

A and B. Estimates from gels electrophoresed at pulse-times of 200 and 100 s, respectively.

Various digests giving convenient numbers of restriction fragments were then run in CHEF agarose gel electrophoresis in comparison with yeast DNA markers at pulse times of 200, 100, 80, 40 and 20s. Lanes from the gels run at 200 and 100 pulse times are shown in Fig. 1, together with indication of the position and sizes of the yeast markers. Size estimates from the mobility of the mycoplasma DNA fragments on these gels are shown in Table 1. Measurements to compare mobilities of fragments and markers were made to the fronts of bands. The tabulated values for apparent genome size were taken from the estimates of fragment size on the gel run at a 200s pulse-time, because all the fragments and the relevant markers moved well into the gel under these conditions.

To obtain further estimates of genome size, fragments in several different restriction endonuclease digests of DNA from M.mycoides subsp. mycoides Y were separated on CHEF gels run at 80, 40 and 20s pulse times (Fig. 2). The λ ladder (λn) used in Fig. 2b too highly polymerised to show bands on this gel. The estimated sizes of the fragments are given in Table 2. The average for the total of the fragment sizes in the various digests approximates to 1230 kb as an apparent genome size, supporting the value observed from SalI digestion in Table 1. Except for the fragments from KpnI digestion, the values listed in Table 2 are those estimated from the gels on which the fragment concerned

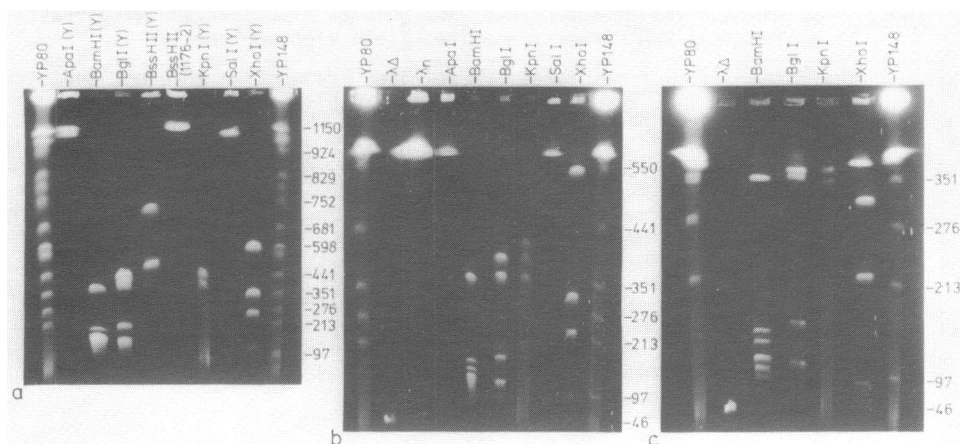


Fig. 2 Fragments separated from restriction digests of DNA from M. mycoides subsp. mycoides Y by CHEF agarose gel electrophoresis. The gels were run at pulse times of 80 (a), 40 (b) and 20 s (c) for 20 h. Other details as in Fig. 1.

ran closest to the middle of the gel. The sizes shown for the KpnI fragments are averages of the values estimated at 40s and 80s pulse times (see Table 3). The mobilities of all fragments of mycoplasma DNA relative to the yeast markers tended to be greater at the shorter pulse times. This had the effect

**Table 2 Apparent sizes of restriction fragments from DNA of M.mycoides subsp. mycoides Y by comparison with yeast chromosomal DNA markers in pulsed-field electrophoresis**

| Restriction Endonuclease | Estimated Fragment Sizes (kb) | Total (kb) |
|---|---|---|
| ApaI | 1130, 96 | 1226 |
| BamHI | 363, 151, 140, 117, 113, 106, 103, 95, 71 | 1259 |
| BglI | 405, 369, 160, 111, 109, 75 | 1229 |
| BssHI | 740, 490 | 1230 |
| KpnI | 443[a], 420[a], 373[a] | 1236[a] |
| SalI | 1100[b], 88, 30 | 1218 |
| XhoI | 575, 333, 232, 90, | 1230 |

a Average of values for 80 and 40 s pulse times.
b 200 s pulse-time (Table 1)

**Table 3** **Variation of apparent sizes of restriction fragments from DNA of M. mycoides subsp. mycoides Y with pulse-time in pulsed field electrophoresis by comparison with yeast chromosomal DNA markers**

| Restriction fragment | Pulse-time 20 s | 40 s | 80 s |
|---|---|---|---|
| | | Size (kb) | |
| Bm A | 350 | 363 | 370 |
| Bg A | NM | 405 | 430 |
| Bg B | 358 | 369 | 376 |
| Bg C | 160 | 174 | 200 |
| Kp A | NM | 425 | 460 |
| Kp B | NM | 400 | 440 |
| Kp C | 348 | 367 | 380 |
| Xo C | 90 | 100 | 120 |

N.M. Not measurable

that estimates of their size were smaller at shorter pulse-times, (e.g. a sample of bands in Table 3).

The variation in size estimates for mycoplasma DNA molecules by comparison with yeast chromosomal DNA markers introduced some range of uncertainty into the values and seemed likely to be due to differences in base composition. To obtain an alternative estimate which would be free of this source of uncertainty, a BamHI digest of DNA from M. mycoides subsp. mycoides Y, with a (G + C) content of 26-27% (17), was compared with a λ ladder (14) and intact and BglI digested vaccinia DNA in a separation by FIGE (Fig. 3). With FIGE separations the size of the molecules showing least mobility varies with the pulsing conditions and this size increases with pulse time. Under the conditions used in Fig. 3 the minimum mobility is shown at sizes of approximately 500 kb. The material showing between the designated λ concatamers derives from larger concatamers than those having minimum mobility. The vaccinia DNA has a size of 188 kb, (Dr. G. Langford personal communication, 18, 19) and a base composition of 32% (G + C) (20). BglI digestion gives fragments of 112, 43, 21 and 12 kb. A restriction fragment, HindIII D, sized at 16.2 kb, has been shown by sequencing to contain 16,059 base pairs (19), indicating that the sizes estimated for vaccinia DNA by electrophoretic comparison of its restriction fragments with λ DNA and by sucrose gradient centrifugation (18,19) are close to absolute values. The

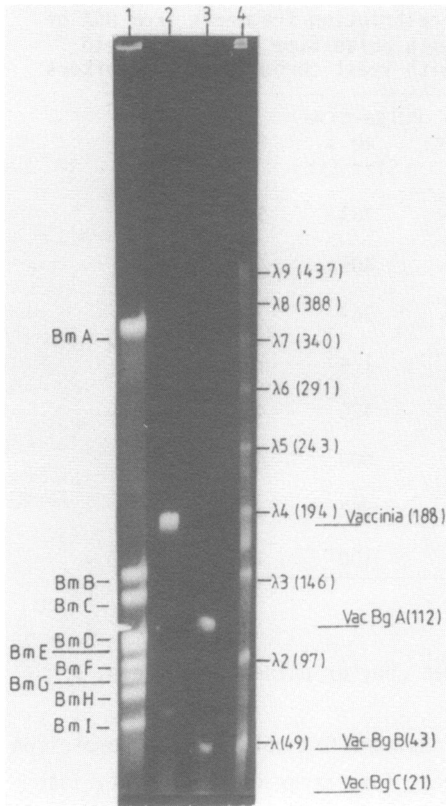


Fig. 3 Fragments separated from BamHI digests of DNA from M. mycoides subsp. mycoides Y by FIGE. Electrophoresis was carried out, as described previously in ref. 12, at 5.6 volt/cm with 8 h at a forward pulse time of 4.3 s and 32 h at 4.3 to 25.7 s increasing linearly with time. Lane 1, BamHI digest of M. mycoides DNA; Lane 2, Vaccinia DNA; Lane 3, BglI digest of vaccinia DNA; and Lane 4, λ and λ concatamers. The individual fragment bands from the M. mycoides DNA are designated on the left margin of the figure. The bands for the markers and their sizes are designated on the right.

results in Fig. 3 do not indicate any major deviation between the effects of pulsed field electrophoresis on the (A + T) vaccinia DNA and on λ DNA under the conditions used. Thus major deviations in the effect on M. mycoides DNA should not be expected.

The sizes of the BamHI digestion fragments from strain Y estimated by comparison of mobilities with those of the vaccinia DNA and λ concatamers are

**Table 4 Apparent sizes of BamHI restriction fragments of M. mycoides subsp. mycoides DNA by comparison with λ concatamers or vaccinia DNA as markers in FIGE pulse-field electrophoresis**

| Marker | Estimated fragment sizes (kb) | Total (kb) |
|---|---|---|
| λ ladder | 363,150,131,111,107,96,88,78,63 | 1187 |
| Vaccinia DNA | (363)[a],151,131,108,103,90,85,77,58 | 1166 |

a Estimate by comparison with λ concatamers

shown in Table 4. The sums of these fragment sizes confirm the size of the M. mycoides genome at approximately 1200 kb as observed by the electrophoretic separations in comparison with the yeast markers.

## DISCUSSION

The apparent sizes of the mycoplasma genomes shown in Tables 1, 2 and 4 are considerably larger than any of those reported previously for a range of mycoplasma species as tabulated in (2). The values in kb for strains of some of the same species as shown in Table 1 were: M.gallisepticum, 740, 820; U.urealyticum, 670, 710; and M.mycoides subsp. mycoides, 760, 860. While these values are considerably smaller than those shown in Table 1, there is a similarity of trend in that U.urealyticum shows the smallest values and M.mycoides subsp. mycoides the largest. Although the strains used in the current work are not identical with those used for the previous work, this difference is unlikely to be the reason for the large differences in the values observed. The differences presumably derive from the different methods used. Most of the previous values have been determined by DNA renaturation rate and some by electron microscopy. The lack of correction for (G + C) content in the renaturation studies does not account for the difference between the estimates, since its application would increase the discrepancies. The molecular complexity (equated with genome size) of DNA as determined by renaturation kinetics is inverse to the rate constant for renaturation. As reported by Wetmuir and Davidson (21), the rate constant for several DNAs ranging between 34 and 64% (G + C) is approximately proportional to (G + C) content, so that without correction the technique would give an overestimate, not an underestimate, of genome size for the DNAs of low (G + C) content. However, there are other potential sources of inacuracy, such as association between non-homologous sequences, in procedures for the analysis of DNA sequences by renaturation kinetics, and Britten et al (23), have stressed strongly the need for appropriate corrections to be made in the evaluation of reassociation rates from the Wetmuir and Davidson (21) technique. The estimation of mycoplasma genome sizes was an early application of this technique and it may be that the values which were reported suffered from a lack of the appropriate corrections. The published data are insufficient to allow reassessment of the values presented.

The small number of values for mycoplasma genome sizes measured by electron microscopy are close to those determined by renaturation kinetics. It is not apparent whether some source of error might effect these estimates. One early estimate for the molecular weight of M.gallisepticum DNA, by Riggs,

quoted in a review (22) was 10 x $10^8$ daltons. As pointed out by the reviewers, this estimate, made by autoradiography, electronmicroscopy and chemical analysis, was per growing cell and so must be multiplied by 0.69 to obtain the value for the completed genome of a newly formed cell. The resultant value approximates closely to the 1050 kb reported here for the M.gallisepticum genome.

To obtain size estimates for the fragments of the different mycoplasma DNAs under comparable conditions, the values reported here were based on the mobilities observed when the pulsing conditions caused the fragment bands to run near to the middle of the gel. Although differences in pulsing conditions cause some variation in the estimates of the size of the mycoplasma DNA fragments by comparison with the yeast chromosomal DNA markers, these values should nonetheless give reliable comparative measures of the sizes of the various mycoplasma genomes studied. The substantiation of the value for the size of the genome of M. mycoides subsp. mycoides Y as approximately 1200 by the sizing of its BamHI digestion products in comparison with λ concatamers and the high (A + T) DNA of vaccinia therefore gives confirmation to the approximate values reported here for the other mycoplasma genomes. It is therefore concluded that the values obtained by this method give valid estimates for the sizes of those mycoplasma genomes studied and that these are therefore considerably larger than the estimates previously reported for mycoplasma species from renaturation kinetics or electron microscopy.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian Research Grants Scheme and during tenure of Australian Postgraduate Awards by A.D.B. and J.C.W.

*Present address: The Whitehead Institute, Nine Cambridge Center, Cambridge, MA 02142, USA

REFERENCES

1. Razin, S. (1985). Microbiol. Revs. 49: 419-455.
2. Razin, S., Barile, M.F., Harasawa, R., Amikam, D. and Glaser, G. (1983). Yale J. Biol. Med. 56: 357-366.
3. Bak, A.L., Black, F.T., Christiansen, C. and Freundt, E.A. (1969) Nature (London) 224: 1209-1210.
4. Bove, J.M. (1984) Isr. J. Med. Sci. 20: 817-825.
5. Poddar, S.K. and Maniloff, J. (1986) Gene 49: 93-102.
6. Schwartz, D.C. and Cantor, C.R. (1984) Cell 37: 67-75.
7. Carle, G.F. and Olson, M. (1984) Nucleic Acids Res. 12: 5467-5664.
8. Chu, G., Vollrath, D. and Davis, R.W. (1986) Science 234: 1582-1585.

9. McClelland, M., Jones, R., Patel, Y. and Nelson, M. (1987) Nucleic Acids Res. 15: 5985-6005.
10. Mitchell, A., Sin, I.L. and Finch, L.R. (1978) J. Bacteriol. 134: 706-712.
11. Cocks, B.G. and Finch, L.R. (1987) Int. J. Syst. Bacteriol. 37: 451-453.
12. Pyle, L. and Finch, L.R. (1987) Nucleic Acids Res. 16: 2263-2268.
13. Kemp, D.J., Corcoran, L.M., Coppel, R.L., Stahl, H.D., Bianco, A.E., Brown, G.V. and Anders, R.F. (1985) Nature 315: 347-350.
14. Vollrath, D. and Davis, R.W. (1987) Nucleic Acids Res. 15: 7865-7876.
15. Mortimer, R.K. and Schild, D. (1985) Microbiol. Revs. 49: 181-212.
16. Carle, G.F., Frank, M. and Olson, M.V. (1986) Science 232: 65-68.
17. Fasman, G.D. Editor (1975) Nucleic Acids, Vol. I. p 562.
18. De Filipes, F.M. (1982) J. Virol. 43: 136-149.
19. Niles, E.G., Condit, R.C., Caro, P., Davidson, K., Matusik, L. and Seto, J. (1986) Virology 153: 96-112.
20. Fasman, G.D. Editor (1975) Nucleic Acids Vol. II, p 143.
21. Wetmuir, J.G. and Davidson, N. (1968) J. Mol. Biol. 31: 349-370.
22. Maniloff, J. and Morowitz, H.J. (1972) Bacteriol Revs. 36: 263-290.
23. Britten, R.J., Graham, D.E. and Neufield, B.R. (1974) Methods Enzymol 29: 363-418.