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A physical map of the genome of *Mycoplasma mycoides* subspecies *mycoides* Y with some functional loci

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ABSTRACT

A physical map is presented for the 1200 kb genome of Mycoplasma mycoides subsp. mycoides Y, locating 32 cleavage sites for 8 restriction endonucleases. The large restriction fragments involved were separated and sized by pulsed-field agarose gel electrophoresis. Their locations on the map were determined by probing Southern blots of digests with individual fragments isolated from other digests and by correlating the products of double and triple digestions. Loci for 2 ribosomal RNA operons and 2 tRNA operons have been determined by probing with cloned genes and the broad regions of the replication origin and terminus have also been outlined by in vivo labelling studies.

INTRODUCTION

Physical genomic maps will be very useful to the development of genetic analysis for those procaryotes in which gene transfer systems are not readily available. The recent publications of physical maps for the genome of Escherichia coli (1,2) show the potential of the techniques now available. In the first of these publications, pulse field electrophoresis was used for the separation of large restriction fragments. The latter technique, with selective use of restriction endonucleases in relation to the base composition of the DNA involved (3) and attention to its state of replication (4), give much promise for determination of maps for procaryotic genomes. The mycoplasmas are attractive subjects for such studies since they appear to have the simplest of cellular genomes and have so far been inaccessible to genetic study. We describe here the construction of a physical map for Mycoplasma mycoides subsp. mycoides Y solely by physical techniques and give approximate loci for some physiological functions.

METHODS

Preparation and separation of restriction fragments from single digestions.

M. mycoides subsp. mycoides strain Y was grown in PPLO broth and harvested and washed. The cells were then incorporated into low melting agarose for

preparation of DNA and digestion with restriction endonucleases as previously described (4). Restriction fragments were separated by field inversion gel electrophoresis (FIGE) (5) controlled by a FIGET pulse programmer (Acronym Pty Ltd, Boronia Vic.) as previously described (4) except that 1% agarose (Ultra-Pure DNA Grade Agarose, Biorad) gels were used in later experiments. Pulsing conditions were varied according to the fragment size range over which maximum separation was required.

Digestion by two or more restriction endonucleases.

Most multiple digestions of the DNA were done in the agarose blocks before FIGE separation of the fragments. Where the appropriate buffers for the different endonucleases were of similar composition the digestions were effected by simultaneous incubation with two (or more) enzymes. Where the buffers differed significantly the digestions were performed sequentially with washing of the blocks and appropriate change of the buffer between incubations. In some cases, second digestions were performed on individual fragments from the first digest after they had been separated by FIGE. In these separations samples from the first digestion were run in adjacent lanes in FIGE then the lanes were cut apart and one stained with ethidium bromide (5 ug/ml) to visualise the fragment bands under UV light. The positions of fragment bands in this lane were then used to guide the excision of equivalent bands from the other, unstained lane. The excised blocks of agarose were washed three times for two hours per wash with TE (10 mM Tris/HCl, 1 mM EDTA pH 8.0) and then incubated individually with a second restriction endonuclease for 16 hours under the conditions specified by the enzyme supplier. After the second digestion the sections of agarose were either placed in 1% Sarkosyl, 0.5 M EDTA, 10 mM Tris pH 9.5 for storage or washed with TE and subjected to FIGE for characterisation of the products.

Southern transfer of DNA to nylon membranes.

Agarose gels from FIGE separations were stained with ethidium bromide and photographed to locate bands containing restriction fragments, then treated for 2 x 10 min in 0.25 M HCl before immediate commencement of transfer to Gene Screen Plus (NEN) nylon membranes. With 1.0% agarose gels the alkaline Southern blotting technique (7) was used with 0.4 M NaOH as the eluant. With 1.5% agarose gels transfer with 1M ammonium acetate, 0.2M NaOH, followed by baking of the filter at 80°C for two hours (8) gave better results.

Preparation of DNA probes and hybridisation to membrane bound DNA.

DNA probes were prepared by the oligolabelling method (9) using random

hexamers (Pharmacia) as primers, [^{32}P]dATP (Bresa), unlabelled deoxynucleoside triphosphates and Klenow enzyme (Boehringer Mannheim). When restriction fragments were used for the preparation of probes, their electrophoretically-separated bands were excised from the gel following location by ethidium bromide staining. The DNA from the individual bands was then extracted and purified using Geneclean (BIO 101 Inc.) according to instructions supplied. This procedure recovered 70-80% of the DNA of large restriction fragments. Hybridisation of the probe to membrane bound DNA was effected in 50% formamide at 37°C for 16 hours with the membrane sandwiched between two sheets of filter paper (Schleicher and Schuell 595) saturated with probe solution as described by Reed (10).

Identification of functional genetic loci on the physical map.

The plasmid pMC5, with a 4.8 kb insert coding for part of the ribosomal RNA operon (rrn) of Mycoplasma capricolum (11), was kindly made available by Professor S. Razin. Two plasmids, pMM22 and pMM25 (12), containing inserts coding for tRNA genes of Mycoplasma mycoides sp. capri, were kindly supplied by Professor T. Samuelsson. These plasmids were used as probes as described above to identify the loci of homologous DNA within restriction fragments of the genome of M.mycoides subsp. mycoides strain Y. The probe from pMM25 was prepared by first excising a 2.2 kb HindIII insert fragment from the host plasmid by restriction endonuclease digestion followed by gel electrophoresis. This insert fragment contained an ApaI site and both of the HindIII-ApaI subfragments obtained from it were also used as probes. The restriction fragments were extracted and purified from the gel as described above.

Localisation of the origin and terminus of replication.

A culture of M.mycoides subsp. mycoides strain Y was grown in PPLO broth (13) without the addition of calf thymus DNA. At mid-logarithmic phase, chloramphenicol (Cm) was added to the culture at a final concentration of 80 µg/ml. Immediately, and at 20, 40, 60, 70, 80 and 90 min after Cm addition, 0.75 ml was taken from the culture and added to 3.0 µCi [^{3}H]dTMP (42 Ci/mmol., Amersham). After incubating at 37°C for 5 mins to give a pulse of [^{3}H]dTMP labelling, unlabelled dTMP was added to a final concentration of 80 µM and the incubation continued for 60 min. 0.5 ml of cells from each time sample was harvested and washed, for incorporation into agarose blocks for restriction endonuclease digestion and fragment separation as described in ref 4. The remaining 0.25ml was used for estimation (14) of the total incorporation of ^{3}H-label into DNA.

Table 1. Data on restriction fragments from the DNA of Mycoides subsp. mycoides, Y

Enzyme	Fragment	Size (kb) Est.	Size (kb) Map	Response to probes	Products of digestion[a]
ApaI	ApA	1130	1120	BmE,XoB,XoC SmaB,XoD(w), pMC5	Xo[D40,A,B,C180]
	ApB	80	80	ApB, SmB XoC, XoD	Bm[F69,(C11)],Bg[(E3),B77], Sl[C,A48],Sm[B65,(C13)],Xo[C43,D39]
	Total	1210	1200		
BamHI	BmA	363	374	BmA,BgA,*BgB, XoB, pMC5(s)	Bg[A270,C106],Bs[B298,A67], Kp[B123,C245],Xo[A178,B190]
	BmB	151	154	BgA,BgB	Bg[A77,B77],Bs[B113,A40],
	BmC	131	134	ApB,BgB,XoD, pMC5(s)	Ap[(B11),A119],Xo[D55,A78]
	BmD	110	110	BmD,XoB,XoC	Bg[F31,D78],Kp[(C36),A74], Xo[B52,C57]
	BmE	106	106	BmE, XoC	Bg[E73,F32],Sl[A56,B45]
	BmF	93	93	ApB,BgB,XoC XoD	Ap[(A24),B69],Bg[E25,B68], Sl[B35,C,A32],Sm[B89,(C4)], Xo[C67,D26]
	BmG	90	90	BgB,pMC5(w)	Kp[(A14),B76]
	BmH	78	78	XoB,pMC5(s)	Bg[C54,D25]
	BmI	60	60	(BgA)	
	Total	1182	1200		
BglI	BgA	405	405	BmA,BgA, BgB(w),XoB	Bm[B77,I,A270],Kp[B255,C140], Xo[A340,B77]
	BgB	369	369	BgA(w),BgB *XoB(w),XoC XoD,pMC5	Ap[B77,A300],Bm[F68,C,G,B77], Bs[A340,B35],Kp[A230,B144], Sl[C35,A330],Xo[C45,D,A245]
	BgC	160	160	BmA,*BgB,XoB, pMC5	Bm[A106,H54],Bs[B35,A131]
	BgD	104	104	BmD, XoB (XoC)	Bm[H25,D78],Kp[C58,A46],Xo[B75,C26]
	BgE	101	101	BmE, XoC	Ap[A96,(B3)],Bm[E73,F25], Sl[A20,B,(C3)]
	BgF	61	61	BmD(w),BmE(w) XoC	Bm[E32,D31]
	Total	1200	1200		
BssHII	BsA	740	722	BmE,BgA(w) BgB,XoC pMC5(s)	Bm[A67,H,D,E,F,C,G,B40], Bg[C131,D,F,E,B340], Xo[B209,C,D,A201]
	BsB	490	478	BgA,BgB(w)	Bm[B113,I,A298],Bg[B35,A,C35], Xo[A375,B113]
	Total	1230	1200		

KpnI or Asp718	KpA	443	431	BmE,pMC5	Ap[A208,B,A133],Bm[D74,E,F,C,(G14)], Bg[D46,F,E,B230]
	KpB	420	408	BmA(w)	Bm[G76,B,I,A123],Bg[B144,A255]
	KpC	373	361	BmA(s),pMC5	Bm[A245,H,(D36)]
	Total	1236	1200		
SalI	SlA	1100	1085	BmA,BmD,BmE, BgB,XoC, pMC5(s)	Ap[(A1050),B48],Bm[F32,C,G,B, I,A,H,D,E56],Bg[B350,A,C,D,F,E20], Xo[(C9),D,A,B,C105]
	SlB	77	77	BmE, XoC	Bm[E45,F35]
	SlC	38	38	BgB,XoC	Bg[(E3),B36]
	Total	1215	1200		
SmaI	SmA	691	684	pMC5(s)	Bm[A,I,B,G],Bg[B160,A,C106], Xo[A500,B190]
	SmB	395	389	SmB,pMC5(w)	Bm[H,D,E,F89],Bg[C58,D,F,E,B64], Xo[B131,C,D22]
	SmC	135	137	pMC5(s)	Ap[(B16),A120],Bm[(F4),C], Xo[D57,A78]
	Total	1221	1200		
XhoI	XoA	575	572	BmA,BgA,BgB pMC5	Bm[C78,G,B,I,A178],Bg[B245,A340], Bs[A201,B375], Sm[C78,A500]
	XoB	333	322	*BmA,BmD,BgA, BgB,pMC5	Bm[A190,H,D52],Bg[A77,C,D75], Bs[B113,A209],Sm[A190,B131]
	XoC	232	228	BmD,BmE,BgB XoC	Ap[A180,B43],Bm[D57,E,F67] Bg[D26,F,E,B45],S1[A105,B,C,(A9)],
	XoD	77	77	BgB,XoD	Ap[B39,A40],Bm[F26,C55]Sm[C57,B22]
	Total	1217	1200		

(a) The product fragments are shown in clockwise order around the genome as mapped in Figure 1. Thus as an example, the representation Xo[D40,A,B,C180] in the first line indicates that the ApA fragment was cleaved by XhoI to give 40 kb from the clockwise end of XoD, plus XoA and XoB, plus 180 kb from the anti-clockwise end of XoC. Fragment representations enclosed in round brackets are predicted but were not observed for sizing.

* The apparently anomalous response may be explained by noting that both the target fragment and the probe respond to pMC5 as a probe and so contain rrn operon sequences which may hybridise although they derive from different loci.

RESULTS

Size of Fragments

Sizes of fragments from the digestion of M. mycoides subsp. mycoides DNA with several restriction endonucleases have been estimated previously (6) by comparison with yeast chromosomal DNA molecules as markers. The size

estimates for fragments greater than 150 kb (Table 1 column 3) are quoted from this reference, except for the SmaI fragments. For the fragments smaller than 150 kb, sizing was made on FIGE by comparison with a sample (6) of vaccinia DNA (188 kb) or its BglI digestion fragments (112, 43, 21 and 12 kb) as exemplified for the Bam H1 fragments in ref 6. The SmaI fragments and those from double digestions were sized by comparison with other fragments of M. mycoides DNA. Sizing could vary by a few kb between gels.

Ordering of fragments from individual digests.

The primary method to determine the order on the genome of fragments from particular digests was to test for hybridisation and therefore overlap with individual fragments separated from other digests. Membranes containing sets of separated fragments from digestions with several endonucleases were probed with a DNA fragment isolated from another gel. The results are shown under "Response to probes" in Table 1. Also shown in this column are the responses to pMC5 which would hybridise to ribosomal RNA operons (rrn). Fragment bands varied in the relative intensity of their responses to individual probes and these are indicated in some cases with (s) for strong and (w) for weak responses. Where a probe fragment is designated in brackets, a response may have occurred but was too faint for certainty. Where the probes are asterisked, an apparently anomalous response may be explained by noting that both the target fragment and the probe fragment respond to pMC5 and so contain rrn operons which can hybridise together but come from different loci.

Identification of products in double digests.

Information from probing could be sufficient to establish the order on the genome for the restriction fragments from individual digests. Taken together with the values for fragment size this could allow definition of sets of restriction maps for single enzymes. To correlate these individual maps into a single map requires data on the distances between restriction sites for the different enzymes. These can be obtained by sizing the fragments from double digestions and identifying the products arising from the fragments in the initial single digests. In making this identification consideration was given to the bands digested, the sizes of the resultant products and consistency with the data on order and overlap. Where necessary, identification was confirmed by probing of the double digests with individual single digest fragments or with pMC5, by performing the second digestion on fragment bands excised from gels, or by performing triple digestions. Table 1 shows the products of second digestions of individual fragments in clockwise order as plotted in Figure 1. The only evidence of digestion of BamHI

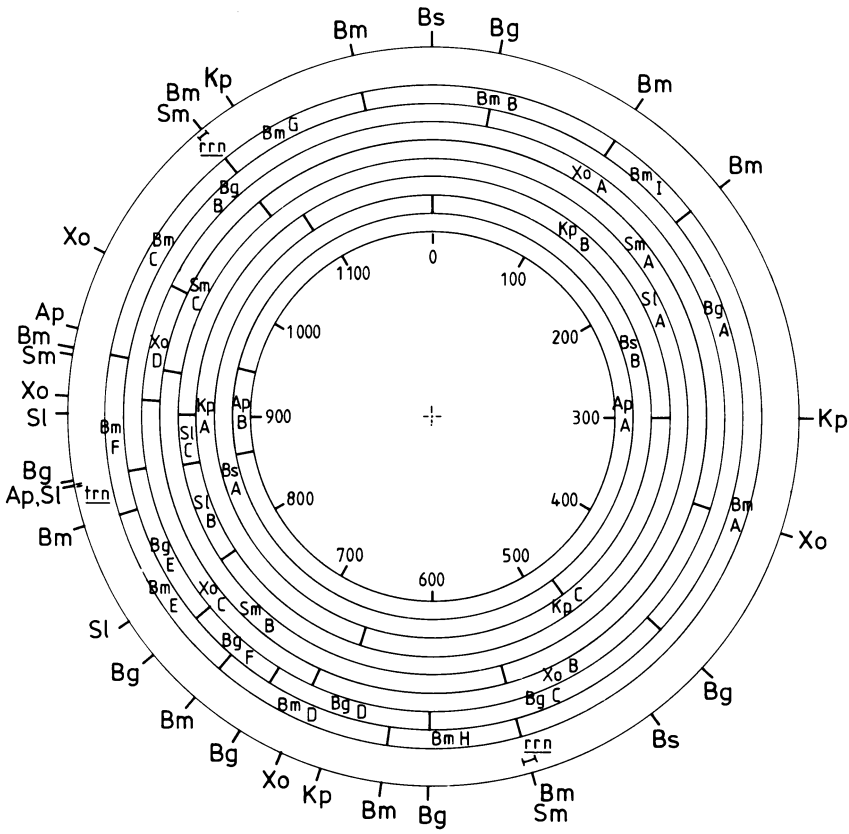

Figure 1: Restriction endonuclease map of the genome of M. mycoides subsp. mycoides, Y. The map was constructed as described in the text from the data on the restriction fragments presented in Table 1 to give an estimated total genome size of 1200 kb. The abbreviations Ap, Bm, Bg, Bs, Kp, Sl, Sm and Xo are used for the restriction endonucleases ApaI, BamHI, BglI, BssHII, KpnI, SalI, SmaI and XhoI, respectively to mark their cleavage sites around the outer circle or to identify the segments corresponding to particular restriction fragments within the inner rings. The segments marked rrn within the outermost ring indicate the loci identified by probing with pMC5, whereas as that marked trn was identified with pMM25. The inner circle shows distances clockwise around the genome in kb starting from the BssHII site in BmB.

fragments by SmaI was a small (4 kb) decrease in size of BmF, suggesting that two of the three SmaI sites coincided very closely with BamHI sites.

Construction of map.

The first step undertaken in the construction of the restriction map shown in Fig. 1 was to order the single digestion products. For example, with the

XhoI digestion products, linkage of XoA and XoB was indicated by hybridisation with BmA and with BgA; of XoB and XoC with BmD; of XoC and XoD with ApB. Assumption of a circular genome would require linkage of XoD and XoA. Reaction of BgB with XoC, XoD and XoA was consistent with this ordering of the XhoI fragments and the respective sizes of the fragments, but the fact that BgB also reacted with XoB presented an anomaly. This anomaly was subsequently resolved by the location of two ribosomal RNA operons (shown as rrn in Fig. 1) by probing with pMC5 and the consistent observation that fragments containing one rrn locus hybridised to fragments containing the other. Thus the apparently anomalous reaction indicated hybridization of the rrn locus within BgB with the other rrn locus in XoB and not overlap of the two fragments.

With the provisional ordering of the fragments from individual digests integration of these individual maps required an assumption of a standard genome size since the totals of the fragment sizes for the various digests differed (Table 1). The size assumed was 1200 kb corresponding to the total of the sizes for the BglI fragments. BglI sites were then plotted relative to the BssHII site in BgB which was arbitarily placed at 0 kb in Fig. I. Sites for the other restriction endonucleases were integrated with the BglI sites to achieve coherence with the data from probing and double or triple digestions. The map in Fig. 1 accomodates all the data within a few percent. Column 4 of Table 1 lists the sizes of the single digest restriction fragments as plotted on the map after correlating all the data on double digestions.

Interpretation of the data on response to probes allowed ordering of the restriction fragments from the various digests except that there was no appropriate data for the SmaI fragments or to define the position of BmI. Ordering of the SmaI restriction fragments came from the data obtained with pMC5 as a probe on the SmaI and BamHI digestion fragments and their double digestion products with XhoI (see below). These results indicated that SmA linked to SmB near the linkage of BmA and BmH and that SmC linked to BmA near the linkage of BmC to BmG.

The combination of data from probing and double digestion indirectly defined a unique position for BmI. More direct information came from partial digestions. A number of these were obtained and gave results fully interpreted by the map in Fig. 1. BamHI partial digests showed bands corresponding to fragment sizes of approximately 430 and 450, consistent with the linkage of BmA to BmI and BmH, respectively.

One set of results in Table 1 not explained by the map in Fig. 1 was that BgB and BsA respond weakly to BgA and that BgA responds weakly to BgB. This

may be due to the large size of both the probes and the target fragments and the presence within them of some repeated sequences.

Identification of functional loci

Results for the probing of membrane bound restriction fragments of M. mycoides DNA with pMC5 (Table 1) are consistent with strain Y having two rrn loci as reported for most mycoplasmas studied (15). Results for the probing of fragments from double digests (which are not tabulated) deserve some comment. One of these results of particular significance came from the probing of fragments from a double digestion with XhoI and SmaI. The strong response evident with SmA was found to derive from two sites - a weak reaction with the 500 kb fragment from XoA and a strong reaction with the 190 kb fragment from XoB. The weak response of SmB was associated with a 131 kb fragment from XoB whereas the strong response of SmC was associated with a 78 kb fragment (from XoA). BamHI digests also showed four fragments responding to pMC5: two hybridizing strongly (BmA and BmC) and two hybridizing weakly (BmG and BmH). The double digest with XhoI showed the weakly responding BmG and BmH unchanged, but new strongly reacting bands of 190 and 78 kb, identical in size to the two strongly responding fragments from the SmaI/XhoI double digest. These observations were consistent with a close coincidence of BamHI and SmaI sites within both of the regions hybridising with pMC5. They therefore allow the fairly precise definition of the two rrn loci show in Fig. 1.

Probing with pMM22 gave responses with BmB, BgA, KpB and XoA, whereas probing with the insert from pMM25 gave strong responses with BmF, BgE, KpA, both SlB and SlC, and XoC. The response to probing with pMM22 which contains a $tRNA^{Arg}$ gene (12), demonstrates homology within the overlap of BmB and BgA in the region 35 to 115 kb on the map in Fig. 1, suggesting a putative locus for the $tRNA^{Arg}$ gene within this region.

The responses to probing with the insert from from pMM25 indicated a tRNA locus spanning the SalI site at 864 kb on the map in Fig. 1 and within the overlap between BmF and BgE. This SalI site has a closely associated ApaI site. An ApaI site also occurs within the plasmid insert arising from the sequence coding for the first six nucleotides of the $tRNA^{Ala}$ gene which also includes a SalI (AccI) site 45 bases downstream (12) suggesting that these ApaI and SalI sites on the map in Fig. 1 may be within a locus for a $tRNA^{Ala}$ gene of M. mycoides subsp. mycoides Y. The insert of pMM25 from M. mycoides subsp. capri includes sequences coding for nine tRNAs specific for arginine, proline, alanine, methionine, isoleucine, serine, formylmethionine, aspartic

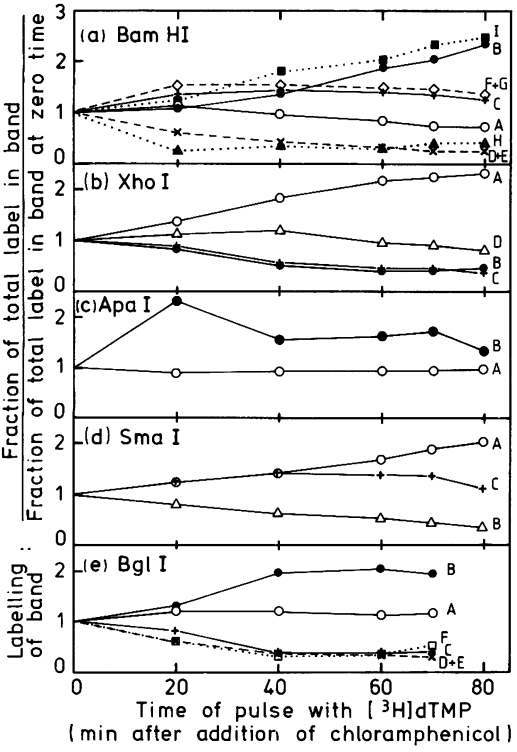

Figure 2: Effect of duration of chloramphenicol treatment before pulse-labelling with [^{3}H]dTMP on the distribution of label into various restriction fragments of the genome of M. mycoides subsp. mycoides Y. Cultures were pulse-labelled with [^{3}H]dTMP at various times after the addition of Cm and assayed for the distribution of label as described under Materials and Methods.

acid and phenylalanine in that order (12). The same sequential grouping of tRNA genes occurs in Bacillus subtilis (15) and probably Spiroplasma (16) so that it is very likely to occur also in M. mycoides subsp. mycoides Y. Thus the data is presumed to define the loci of all these nine tRNA genes. ApaI digestion of the 2.2 HindIII fragment from the insert of pMM25 gave two fragments of 1170 and 1071 kb. The latter was cut to about 1025 kb by AccI indicating that it is the subfragment containing the genes for the tRNAs for alanine, methionine, isoleucine, serine, formylmethionine, aspartic acid and phenylalanine whereas the larger subfragment would contain the genes for $tRNA^{Arg}$ and $tRNA^{Pro}$. In probing ApaI digests of M. mycoides DNA, the smaller subfragment reacted only with ApA, and the larger only with ApB indicating that the tRNA operon is transcribed anticlockwise with respect to the genomic map shown in Fig. 1.

Localisation of the origin and terminus of replication.

The time course for the effect of prior Cm treatment on the incorporation of label into the DNA of cells pulsed with [^{3}H]dTMP indicated that DNA

synthesis decreased over the 90 minute period as observed previously (4). When the distribution of label into different restriction endonuclease fragments was examined the time-course for the relative labelling varied with the fragments within individual digests. To show the different relative effects for the different fragments, the counts in each band were expressed as a percentage of the total of counts in all bands from that digest for each sample in the time course. The % value thus obtained for each fragment at each time point was then divided by the value for that fragment obtained for the zero time sample. Variation in the relative incorporation of ^{3}H into a fragment is then seen as change from the value of 1.0 at zero time. The results shown in Fig. 2 indicate regular patterns in the change, with some fragments showing increases and others showing decreases or little change. Detailed examination of the data in Fig. 2 shows the fragments BmB and BmI, XoA, SmA and BgB as the ones which most clearly increase in relative incorporation of pulse-label during the period of the experiment. Inspection of the map in Fig. 1 shows that these bands overlap a region centred around the BssHII site at 0 kb in BgB. This region, exhibiting DNA synthesis longest following Cm treatment, is presumed to contain the terminus of replication. Approximately opposite on the map are the bands BmH, BmD and BmE, XoB and XoC, SmB, and BgC, BgD, BgF and BgE which all decrease strongly in relative labelling. This region, centered near 700 kb on the map in Fig. 1 thus rapidly loses sites of DNA synthesis after Cm treatment and is therefore presumed to contain the origin of replication. Between these two regions on either side are fragments which are intermediate in response: BmC, XoD, SmC and BgA. This is consistent with a replication proceeding bidirectionally from the origin to the terminus.

Fragments not included in the above interpretation are BmF and BmG in Fig. 2 (a) and the ApaI fragments in Fig. 2 (c). BmF and BmG were not adequately separated on the gel and so were counted together. The results for the mixture are very close to those for BmC which they flank. The overall result is therefore likely to be an average of responses in which BmF is intermediate between BmE and BmC, and BmG is intermediate between BmC and BmB. The large ApA fragment contains more than 90% of the genome so that the absence of much relative change in its labelling is to be expected. ApB however shows very strong increase in its relative labelling at 20 min after Cm addition, consistent with early loss of replication sites close to the origin region, but not within the intermediate region which includes ApB. By 40 min after Cm treatment its relative labelling has dropped and continues to decrease,

consistent with replication sites having moved beyond it at the later times.

In steady state exponential growth, copy number should be highest for DNA sequences at the origin with a gradient of decreasing representation away from it to the terminus (18, 19). To seek confirmation of the origin region by estimation of the relative copy number for different restriction fragments in the replicating genome during steady state growth, cells in exponential phase were pulse labelled with [^{3}H]TMP, then treated with Cm and processed for assay of the distribution of label into BamHI restriction fragments. Other factors being equal the chance of incorporation of label into particular fragments should be proportional to the product of their length multiplied by their copy number. Incorporation of ^{3}H per unit length of DNA should then be proportional to copy number. When the observed incorporation of label was expressed per unit length of fragment, using the map fragment sizes shown in Table 1, the relative values were: A, 1.0; B, 1.3; C, 1.1; D, 1.66; E, 1.76; F, 1.59; H, 1.55; G, 1.29; and I, 1.11. These results may be subject to some error through differences in recovery of fragments, or even in their base composition. However, they suggest that copy number is highest within the region covered by BmH, BmD, BmE and BmF, and so support the conclusion that the origin lies in this region.

DISCUSSION

Examination of the distribution of restriction sites shown on the map of the *M. mycoides* genome in Fig. 1 suggests that they are not randomly distributed. Thus the SmA fragment on one side of the two *rrn* operons contains 10 sites in 684 kb (68 kb/site) compared with SmB + SmC on the other side containing 18 sites in 530 kb (30 kb/site). The sites involved here are ones of high (G + C) content chosen for infrequent occurrence within the genome.

Muto (20) has discussed the (G + C) content of different components of the *M. capricolum* genome in relation to their evolution under a biassed mutation pressure replacing GC pairs by AT pairs to give the overall low (G + C) content characteristic of mollicutes. He concluded that the (G + C) level of functionally less important parts of the *M. capricolum* genome would have become lower than that of more important parts. Spacer regions, being functionally least important, could evolve fastest, whereas rRNA and tRNA genes would be most invariable and retain a high (G + C) content because their sequences are directly concerned with biological functions. The nucleotide sequences coding for proteins, though functionally important, can be more

variable than those of stable RNA genes because of the possibility of synonymous codon changes and conservative amino acid changes.

In this context retention of (G + C) rich restriction sites may be indicative of the functional importance of the DNA in which they are situated. Occurrence of the BamHI and SmaI sites within the rrn operons and of the ApaI and SalI sites within $tRNA^{Ala}$ may be examples of such retention through functional importance. If retention of such sites is indicative of functional importance it is of interest that they are more frequent to that side the rrn operons where the origin is situated and gene duplication would occur earliest to increase copy number during DNA replication.

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