Characterization of the Replication Terminus of the *Bacillus subtilis* Chromosome

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DNA in the terminal region of the chromosome of *Bacillus subtilis* was labelled by a procedure in which cells in sporulation inducing conditions were pulse-labelled with [³H]thymidine and then treated with *p*-hydroxyphenylazouracil, an inhibitor of DNA synthesis. The labelled DNA in isolated spores yielded a small number of restriction fragments. About 14 *Eco*RI fragments with a total length of 80 kb were labelled in a 2.5 min pulse. A fragment of 4.0 kb had the highest specific radioactivity in terminally labelled DNA from several strains. One of these strains lacked the 120 kb prophage of SP β that is normally integrated close to the terminus. Loss of the 120 kb prophage did not affect the point of termination which must therefore be regarded as a specific 'stop' sequence. Labelled terminus DNA has been used to identify lambda (Charon 4A) clones containing sequences derived from the terminal region. The total length of the restriction fragments present was 150 kb and adds another 90 kb to the 150 kb region mapped previously. Only one group of these sequences was present in a *B. subtilis* strain (CU1695) carrying a deletion spanning from SP β to the right of the terminus and *gltA*. This suggests that the terminator sequence found in the wild-type can be deleted but presumably this strain has an alternative mechanism of termination.

INTRODUCTION

The genetic evidence now available from *Escherichia coli* and *Bacillus subtilis* suggests that replication of the circular chromosome proceeds bidirectionally from the origin to a site approximately opposite to the origin. There is evidence from *E. coli* and *B. subtilis* that this is not merely the point at which replication forks meet but is a sequence which arrests replication forks (Kuempel *et al.*, 1977; Louarn *et al.*, 1977; O'Sullivan & Anagnostopoulos, 1982; Weiss *et al.*, 1982). The biochemical basis of this phenomenon is unknown. Termination sequences have been identified with considerable accuracy in certain plasmids (Germino & Bastia, 1981). In both organisms, the terminus region is deficient in genetic markers, and contains no markers which define essential functions. The region surrounding the terminus may have important interactions with the cell surface which determine sites of cell division, although the membrane binding site found at *ilvA-ilvD* is a substantial distance from the terminus (Sargent & Bennett, 1982). The region may also contain sequences that are substrates for DNA-topoisomerases that dissipate topological constraints generated during replication (Davern, 1979). These are speculations that can be substantiated only after a physical investigation of the region and the isolation of the relevant parts.

The structure of the terminal region of *E. coli* and *B. subtilis* can now be studied at the level of restriction fragments. A 400 kb restriction map of the terminal region of *E. coli* has been constructed using DNA of a temperature-sensitive mutant, pulse-labelled under its restrictive conditions (Bouché, 1982; Bouché *et al.*, 1982). On this map, the point of termination lies within a 50 kb region. Using a method devised for *B. subtilis* that is not applicable to *E. coli*, the point of termination can be determined more precisely. Cells of *B. subtilis* in a sporulation-inducing

medium were provided with [^3H]thymidine for a short period followed by an inhibitor of DNA synthesis (such as *p*-hydroxyphenylazouracil) which prevents sporulation in cells that have not completed their chromosomes. Labelled spores were thus obtained only from those cells whose chromosomes were completed during the pulse period, and were therefore labelled only in the terminus (Sargent 1980*a, c*; Adams & Wake, 1980). The labelled DNA yields a small number of fragments on digestion with restriction enzymes (Sargent & Monteiro, 1982; Weiss *et al.* 1981), and is indeed replicated last during subsequent cycles of replication (Adams & Wake, 1980). Weiss & Wake (1983) have now compiled a 150 kb restriction map of the *B. subtilis* terminus using DNA labelled by this procedure. They have shown that one arm of the chromosome reaches the terminus about 5 min before the other; an observation which indicates that the terminus acts as a specific 'stop' sequence. In this report we present a study of terminus-labelled material in several strains of *B. subtilis*, and of cloned terminal fragments which confirm and extend the conclusions of Weiss & Wake (1983).

METHODS

Bacteria. The bacteria used for these studies were *B. subtilis* 168 *trpC2* ($\text{SP}\beta^+$) (referred to as $\text{SB}\beta^+$), 168 *trpC2 thyA thyB* (Callister & Wake, 1974) (referred to as Thy^-), CU1050 $\text{SP}\beta^-$ *sup-3 metB5 thr-5 leuA8 purA16* (Warner *et al.*, 1977) (referred to as $\text{SP}\beta^-$), CU1695 *trpC2* $\Delta(\text{SP}\beta^- \text{ kauA citK gltB gltA})$ (Zahler, 1982).

Induction of sporulation and labelling of the terminus region. Conditions for induction of sporulation and for terminus labelling were described previously (Sargent, 1980*a*).

The pattern of restriction fragments labelled was determined by fluorography (Lasky, 1980) using DNA isolated from spores (Sargent, 1980*c*).

Identification of Charon 4A clones containing DNA from the terminus region. One thousand individual plaques from the Charon 4A bank of Ferrari *et al.* (1981) were arranged in a three-dimensional matrix with axes *x*, *y*, and *z*, numbered 0–9. For each point on the *x* axis, all the phage in the other two dimensions were pooled (i.e. 100 clones). The procedure was repeated for the *y* and *z* axes, giving 30 pools. After amplification (deWet *et al.*, 1980), the phage DNA was prepared and used for hybridization and transformation experiments.

Cloned DNA to be probed with either ^3H -terminus or ^{32}P -nick-translated DNA was denatured and bound to nitrocellulose filters which were then soaked in prehybridization medium (Sargent *et al.*, 1983). In typical hybridization experiments, ^3H -labelled DNA preparations contained approximately 10^5 c.p.m. spore DNA labelled for 10 min (specific activity approximately 10^3 – 10^4 c.p.m. g^{-1}) and were incubated with filters for 5 d. Approximately 5×10^5 c.p.m. ^{32}P -nick-translated probes (Jeffreys & Flavell, 1977) were incubated with filters for 24 h. ^{32}P and ^3H probes were denatured by boiling in neutral buffer or 0.5 M-sodium hydroxide, respectively (Sargent 1980*b*; Sargent *et al.*, 1983). Filters were washed (Sargent *et al.*, 1983), and the radioactivity bound to the filters was determined by liquid scintillation counting. Positive pools were identified as having substantially greater radioactivity than blanks. The co-ordinates of positive clones present in the three-dimensional matrix were given by the pool numbers in the *x*, *y* or *z* series which gave substantial signals. Thus if positive responses were obtained in 3*x*, 7*y* and 2*z*, this would indicate that clone number 372 was positive. Stocks of each phage were prepared starting from single plaques. Hybridization properties were then confirmed using purified phage DNA.

Some clones described were isolated by plaque hybridization (Benton & Davis, 1977).

Phage handling, DNA isolation and restriction analysis. Single plaques were used to prepare 10 ml primary lysates of the Charon 4A host, DP50 *supF*. These were used to prepare secondary lysates with titres of 10^{11} – 10^{12} ml^{-1} (deWet *et al.*, 1980). Phage were purified with CsCl , and their DNA was isolated after phenol treatment (Sargent *et al.*, 1983). Restriction fragments to be used as probes were isolated from gels by electrophoresis onto DEAE-cellulose (Dretzen *et al.*, 1981). The elution buffer used was 0.2 M-Tris/HCl, pH 9.0, 1.5 M-sodium chloride, 1% (v/v) sarkosyl, 0.05 M-EDTA.

Genetic methods. The presence of genetic markers in recombinant phage was determined using DNA mediated transformation (Sargent & Bennett, 1982). The following strains were used: MS146 *purA16 hisA1 metB5 gltA292 ilvA1 trpC2 pheA12* (Sargent & Bennett, 1982); QB9432 *trpC2 ilvA1 thyA1 thyB1 gln-9* (Reysset, 1981); CU281 *citB trpC2* (supplied by S. A. Zahler); CU1323 *gltA2 citK1 kauA1 metB5* (Rosenthal *et al.*, 1979); UTB600 *gltB1 leuA8 metB10* (Deshpande & Kane, 1980).

Determination of the time of replication of the $\phi 715$ and $\text{SP}\beta$ region. Samples (10 ml) of a sporulating culture of 168 *trpC2* were pulse-labelled at various time intervals (Fig. 5) with [^3H]thymidine [$50 \mu\text{Ci}$ (1.85 MBq) per sample]. Labelling was terminated in all cultures with *p*-hydroxyphenylazouracil ($50 \mu\text{g ml}^{-1}$) 2 h after T_0 (Sargent, 1980*a*). Spores were isolated 16 h later and purified. The radioactivity per 10^8 spores was determined using a Coulter counter to count the spores, and the DNA was then extracted and purified (Sargent, 1980*b*). DNA hybridization and subsequent washing steps were carried out as described by Sargent *et al.* (1983). Exactly $1 \mu\text{g}$ denatured DNA in 0.5 ml of hybridization buffer was incubated with each filter. The filters were loaded with $4 \mu\text{g}$ denatured DNA of $\phi 715$ and $\text{SP}\beta$.

RESULTS

[³H]Thymidine-labelled DNA synthesized during the terminus labelling protocol described above yielded a small number of restriction fragments. The patterns observed by fluorography (Fig. 1) were highly reproducible and there were considerable similarities between different strains (Fig. 1, Table 1 and 2). Thus in a 2.5 min pulse, at least 11 clearly defined restriction fragments were visible in fluorographs of *EcoRI* digests of DNA from the three strains. The strains examined included the parental *B. subtilis* strain (168 *trpC2*), a *Thy*⁻ derivative thought to contain non-isogenic regions in the terminus (Callister & Wake, 1974) and a strain (CU1050) (Warner *et al.*, 1977) that had lost the 120 kb prophage that is present in the terminus region of most 168 strains.

The last fragment to replicate in the chromosome can be labelled at any time during the pulse period and thus will have the highest specific activity. The proximity of other fragments to the terminus is given by their specific radioactivity relative to the hottest fragment. To calculate the

Fig. 1. Fluorographs of terminus DNA labelled for 2.5 min. Lanes A, B and C, 168 *trpC2* digested with *Bam*HI, *Sal*I and *Eco*RI, respectively; D, E and F, 168 *trpC2* *Thy*⁻ digested with *Bam*HI, *Sal*I and *Eco*RI, respectively; G and H, CU1050 digested with *Bam*HI and *Sal*I, respectively; I, 168 *trpC2* *Thy*⁻ digested with *Eco*RI; J, CU1050 digested with *Eco*RI; Numbers are sizes in kb: left-hand side, tracks A-H; right-hand side, tracks I and J.

Table 1. *Relative specific radioactivities of EcoRI fragments labelled in 2.5 min*

	Size (kb)	Specific radioactivity (relative units)		
		168 <i>trpC2</i>	168 <i>trpC2</i> Thy ⁻	CU1050
A	4.0	100	100	100
B	2.85	76	64	0
C	6.0	67	67	53
D	14.5	45	36	35
E	9.3	36	57	30
F	2.32	33	51	14
G	2.5	32	50	56
H	10.3	28	43	11
I	7.6	27	28	0
J	7.1	27	36	12
K	1.95	20	51	28
L	8.3	19	35	0
M	2.15	14	28	66
N	1.65	12	22	86
Total length (kb)		80.5	80.5	61.8

Table 2. *Relative specific radioactivities of BamHI and SalI fragments labelled in 2.5 min*

		Specific radioactivity (relative units)		
	Size (kb)	168 <i>trpC2</i>	168 <i>trpC2</i> Thy ⁻	CU1050
<i>Bam</i> HI				
A	14.3	—	—	100
B	18.3	100	100	—
C	25.0	76	67	73
D	5.65	66	63	73
E	15.2	62	+	—
F	10.8	48	55	—
G	40.0	23	45	—
H	1.65	+	+	+
I	12.3	+	+	—
<i>Sal</i> I				
A	15.8	100	100	100
B	23	+	+	86
C	25	89	89	49
D	11.3	75	90	—
E	14.5	+	+	—
F	10	+	+	—
G	5.58	+	+	—
H	2.25	+	+	—

— Indicates absence of band; + indicates presence of a band not resolved sufficiently clearly to measure density of image.

specific radioactivity, the area under each peak seen on the fluorograph was obtained from the densitometer trace (Fig. 2) and divided by the size of the fragment. The values for *Eco*RI fragments labelled in 2.5 min are shown in Table 1 relative to the value for the 4 kb fragment which is clearly the most heavily labelled in the three strains tested. Most of the 14 bands seen in the wild-type were reproducibly visible in the other strains. There were, however, some differences. Thus a 2.85 kb fragment (B), which was among the three most highly labelled fragments in the SP β ⁺ strains was absent from the SP β ⁻ strain. Fragments I and L, which were amongst the minor bands in 168 *trpC2* were absent from the SP β ⁻ strain. Fragments G, M and N appeared to be more prominent in the SP β ⁻ strain than in the SP β ⁺ strains. However, there was clearly a fundamental similarity between the three strains examined.

Using enzymes that cut *B. subtilis* DNA less frequently, the similarity between the labelling

Fig. 2. Densitometer traces from fluorographs of terminus-labelled DNA digested with *Eco*RI. (a) 168 *trpC2*; (b) 168 *trpC2* Thy^- ; (c) CU1050.

patterns was less apparent. The *Bam*HI fragment with the highest specific activity was 14.3 kb (A) in CU1050 and 18.3 kb (B) in the other two strains. *Bam*HI fragments C and D were present in all three strains but the minor fragments F and G were not seen in CU1050. As the *Eco*RI fragments from the three strains were similar, fragment A was probably homologous to fragment B and probably contained a deletion or rearrangement.

All three strains gave 16 kb (A) and 25 kb (C) *Sal*I fragments when labelled for 2.5 min. In addition to these CU1050 gave a 23 kb band (B) and the other two strains gave an 11.3 kb band (D). The sum of the lengths of the restriction fragments labelled in 2.5 min was about 80 kb in the $\text{SP}\beta^+$ strains and about 62 kb in CU1050 ($\text{SB}\beta^-$). DNA replication probably occurs at a slower rate in the $\text{SP}\beta^-$ strain.

Identification of clones

Direct methods of screening clone banks (Benton & Davis, 1977) were impractical with a probe of such low specific activity as ^3H -terminus-labelled DNA. However, using the three-dimensional matrix described in Methods, a substantial number of clones were rapidly identified. These were characterized by restriction analysis. The presence of fragments in common between different phages was noted and from this information, linear maps were constructed. The terminal *Eco*RI fragments from each group were used, after nick-translation, to identify further clones either by screening the three-dimensional bank or by plaque hybridization. The *Eco*RI, *Bam*HI and *Sal*I restriction maps of the phage isolated by this procedure are illustrated in Fig. 3. A variety of tests were used to establish that the fragments obtained in each clone were contiguous in the chromosome (unpublished observations). Several clones were found in which non-contiguous fragments had presumably been ligated together during construction of the bank; these have not been included.

Fig. 3. Restriction maps of Charon 4A clones containing sequences from the terminus region. A-F represent composite restriction maps of groups of clones that contain sequences that overlap. *EcoRI* fragments (sizes in kb) are shown above the line and *Bam*HI (Ba) and *Sal*I (S) fragments are shown below the line. Clones containing parts of these sequences are represented by arrows below the maps.

To confirm that the DNA present in the clones was from the terminus region, it was immobilized on nitrocellulose and hybridized quantitatively with two kinds of probe. These were ^3H -terminus-labelled DNA (labelled for 10 min) and ^{32}P -labelled DNA of a *B. subtilis* strain (CU1695) which contains a large deletion extending from *SP β* to the right of *gltA*. The amount of hybridization was corrected for any possible variation in the amount of cloned DNA on the filters by using a parallel set of filters which were incubated with ^{32}P -labelled phage λ . This hybridizes with a substantial part of the vector DNA and therefore estimates the amount of bound DNA. The extent of hybridization per unit of cloned DNA was expressed per kb of insert (determined from the *EcoRI* restriction pattern).

All the groups of clones shown in Fig. 3 hybridized with terminus-labelled DNA (Table 3). The highest rate of hybrid formation per μg of cloned DNA, per kb, was with $\phi 553$ (group C) with lower rates in descending order A, D, E, B and F (56, 35, 28, 11 and 5%, respectively). Within any one group there was a comparable specific radioactivity in all the members of a group.

Hybridization with CU1695 DNA clearly differentiates between groups A-E and group F. The amount of hybrid formed in groups A, B, D and E was about 2% of the level seen with group F.

A slightly higher level of hybridization was found with $\phi 553$. This is probably explained by the presence of another region (6.6 kb) in CU1695 which has weak homology with $\phi 553$ (M. J. Monteiro, unpublished observations). This indicates that the majority of the clones obtained contain DNA from the region defined by the CU1695 deletion. None of the clones contain *SP β* fragments (unpublished observations).

Table 3. Hybridization of Charon 4A clones with ^3H -terminus labelled DNA and DNA of CU1695

Group	Phage	Size of insert (kb)	^3H -Terminus (c.p.m. per kb)*	Average	Percentage of maximum	^{32}P -Labelled CU1695 DNA†
A	326	12.0	61.8	66	56%	5
A	1006	9.8	71.7			8
B	866	17.8	15.8			6
B	703	11.8	11.8	13	11%	4
B	704	15.4	12.9			4
C	553	13.9	117.0	117	100%	27
D	570	15.4	41.4	41	35%	3
E	964	14.5	29.5	33	28%	2
E	715	16.5	36.7			3
F	537	14.3	7.4			197
F	688	10.4	9.4	6.5	5%	218
F	539	17.8	5.5			194
F	328	17.2	5.1			224
F	559	12.7	11.8			342
F	659	15.7	2.9			134
F	524	13.9	5.8			151
F	563	10.0	6.0			141
F	906	16.5	4.6			155

* T/LK ; where T = ^3H -terminus-labelled DNA hybridized with phage DNA; L = ^3H λ DNA hybridized with phage DNA; K = size of insert in kb.

† C/LK ; where C = ^{32}P -labelled CU1695 DNA hybridized with phage DNA; L and K as above.

Table 4. Sizes of *Bam*HI and *Sal*I fragments of 168 *trpC2* DNA homologous with clones in groups A–E

Clone group	Sizes (kb) of chromosomal fragments of 168 <i>trpC2</i> DNA produced by enzymes:		
	<i>Bam</i> HI	<i>Sal</i> I	<i>Bam</i> HI– <i>Sal</i> I
A	35.0	13.3, 17	13.3
B	13.0, 16.0	35.0	ND
C	40.0	22.5	15.0,* 20.0
D	23.0	35–40, 2.8	18.2,* 3.2, 2.8
E	7.6, 23.5	23.5	14.0

ND, Not done.

* Substantially more heavily labelled than other bands.

Blot hybridization of clones with *Bam*HI and *Sal*I fragments of 168 *trpC2* DNA

The relationship between the cloned sequences and the *Bam*HI and *Sal*I restriction fragments seen in fluorographs was investigated by blot hybridization (Southern, 1979). The sizes of the *Bam*HI, *Sal*I and *Bam*HI–*Sal*I doubly digested fragments hybridizing with cloned DNA are given in Table 4. Where two or more clones constitute the group, the data were compiled from experiments with each probe. The data obtained have been used to determine the location of clones relative to the Weiss & Wake (1983) map (see Discussion).

Hybridization of ϕ 715 with terminus-labelled DNA

Comparison of the restriction maps of ϕ 715 (group E) and the map of Weiss & Wake (1983) indicated that the sequences in this clone originate from close to the terminus (see Discussion). An experiment was devised in which the replication of this region could be compared with that of SP β , which lies on the left-hand arm (Fig. 4).

DNA was prepared from spores obtained after pulse-labelling sporulating cells for various lengths of time. This was hybridized with either ϕ 715 or SP β DNA bound to nitrocellulose

Fig. 5. Time of replication of $\phi 715$ and $SP\beta$ DNA. DNA (1 μ g) from spores pulse-labelled for the times shown was incubated with nitrocellulose filters containing 4 μ g denatured $SP\beta$ (●) or $\phi 715$ (○) DNA as described in Methods.

filters. The effect of pulse length on the radioactivity bound is illustrated in Fig. 5. The amount of hybrid formed using either phage starts to increase sharply after about 5 min. If group E was to the left of the terminus (i.e. on the late replicating arm), the region would be heavily labelled in very short pulses. As it is not labelled for 5 min, but is tightly linked to the most terminal fragment, it must be replicated from the right. The simultaneous labelling of the $SP\beta$ prophage and the $\phi 715$ region indicates that termination of the right-hand arm occurs as the $SP\beta$ prophage is replicated. The total radioactivity bound to $SP\beta$ is approximately 10 times that bound to $\phi 715$ but $SP\beta$ is 7.5 times greater in size than the insert in $\phi 715$.

Association of genetic markers with cloned DNA

The genetic map of the terminus region contains the markers *kauA*, *citK*, *gltB*, *gltA*, *citB*, and *glnA* which may be considered sufficiently close to the terminus to merit investigation (Henner & Hoch, 1980). Suitable recipients were transformed with approximately 1 μ g purified DNA of the isolated clones. Clones containing *kauA*, *citK*, *citB* and *glnA* were not identified. However $\phi 926$ and $\phi 715$ were positive for *gltB1* and *gltA2*, while $\phi 715$ was positive for *gltA292*. Studies with isolated fragments indicated that the 9.0 kb *EcoRI* fragment in group E corrected *gltB* and *gltA2* and the 7.5 kb fragment corrected *gltA292*, and thus confirmed and extended the observations of Fisher & Kane (1981).

DISCUSSION

The sensitivity of the terminus labelling technique provides an opportunity to determine with considerable precision whether the chromosomal terminus is merely the point at which replication forks meet or whether a specific sequence arrests replication forks. Weiss & Wake (1983) have used this technique to show that there is a fixed terminus and that there is a ≤ 5 min difference in the time of termination of the right- and left-hand forks. According to the former model, the presence of a deletion in one arm of the chromosome would change the position of the terminus, or according to the latter would have no effect. The test used here was to compare the wild-type *B. subtilis* which is lysogenic for $SP\beta$ with strains which lack the 120 kb prophage. When the terminal region of the *B. subtilis* chromosome was labelled for 2.5 min, only a small number of *EcoRI* restriction fragments with a total length of 80 kb were labelled and the patterns of the three strains examined were qualitatively very similar. Fragments of 4 and 6 kb had the highest specific activity in the three strains examined and furthermore, 11 out of 14 bands seen in the $SP\beta^+$ strains were present in the $SP\beta^-$ strain. As the total length of DNA labelled in 2.5 min was smaller than the $SP\beta$ prophage, a substantial change in pattern in $SP\beta^-$

strains would be expected if the chromosomal terminus was merely the point at which chromosomal forks meet. The close similarity of $SP\beta^+$ and $SP\beta^-$ strains suggests that there must be a specific stop sequence.

There were, however, differences in the restriction patterns of $SP\beta^+$ and $SP\beta^-$ strains. The absence of one major 2.8 kb *EcoRI* band from the $SP\beta^-$ strain probably indicates that in addition to the deletion resulting from the loss of $SP\beta$ there is another small rearrangement or deletion in the terminal region. Differences between the much larger *SalI* and *BamHI* restriction fragments were also observed but these differences were probably consequences of the deletion as the *EcoRI* patterns were so similar. Several minor *EcoRI* bands seen in the wild-type were missing from the $SP\beta^-$ strain labelled for 2.5 min, but as the rate of DNA replication was slightly reduced compared with the wild-type these were probably not labelled. No convincing differences between 168 *trpC2* and the Thy^- strain have been demonstrated.

The relative specific radioactivities of the *BamHI* and *SalI* fragments observed in 168 *trpC2* were consistent with the order of replication suggested in the map of Weiss & Wake (1983). Our most radioactive *SalI* fragment, A, is 15.8 kb and is probably identical to the 16.2 kb *SalI* fragment that Weiss & Wake (1983) consider to be the terminal fragment; our fragment D (11.3 kb) corresponds to their 11.0 kb fragment which is next to the 16.2 kb fragment. Our fragment C (25 kb) probably results from superimposition of the 26.9 and 24.1(a) kb fragments. The order of *BamHI* fragments on the left-hand arm of the terminus shown by Weiss & Wake (1983) is 24.8(a) (terminus), 5.6, 1.4, 18.3 and 36.9 kb. Equivalents of all of these (C, D, H, B and G, respectively) were seen in our fluorographs of 2.5 min labelled DNA, although the order of labelling did not correspond exactly. However, this probably reflects the limitation of the method of determining specific activity.

The restriction fragments labelled in the wild-type in 2.5 min, which are absent from CU1050 are the 11.3 kb *SalI* and 18.3 kb *BamHI* fragments. This indicates that the rearrangement or deletion lies in the region marked X in Fig. 3.

Independently of Weiss & Wake (1983), we have been constructing a map of the terminus using fragments of DNA cloned in Charon 4A. The probe used to screen for these clones was prepared from spores of CU1050, in order to eliminate $SP\beta$ clones from our survey. Pulses of 10 min were employed to ensure that the fast replicating arm would be labelled more strongly than with shorter pulse periods.

Five of the six groups of clones come from a very restricted region of the chromosome, defined by the deletion in CU1695 that extends from $SP\beta$ to the right of *gltA* (Zahler *et al.*, 1982). The position of groups C, D and E were identified satisfactorily in the Weiss-Wake map by means of the *BamHI* and *SalI* sites (Fig. 3). Blot hybridization experiments confirmed that the chromosomal fragments generated by *BamHI*, *SalI* or *BamHI-SalI* double digestions were of the size indicated by the map of Weiss & Wake (1983) (Table 4 and Fig. 3). This information indicated that groups D and E were almost exactly contiguous, but unfortunately we were unable to obtain a clone from the λ bank which overlapped the two groups. However, using an independent approach we have cloned, in a plasmid, a chromosomal fragment that does overlap the two groups (M. G. Sargent, unpublished observation).

Group C contained only one *SalI* site but could be assigned to the position shown in Fig. 3 on the basis of the *BamHI* (40 kb), *SalI* (23 kb) and *BamHI-SalI* (20 and 15 kb) fragments to which it hybridized. The 15 kb *BamHI-SalI* fragment observed was considerably more heavily labelled than the 20 kb fragment and therefore indicates the orientation of the clone.

Sequences like groups A and B are quite definitely not present in the Weiss-Wake map and not present in CU1695. Group A has a substantial specific activity of hybridization with terminus-labelled DNA and probably originates from a region just to the left of the Weiss-Wake region. Group B we more tentatively assign to the same region.

The total length of cloned DNA from the region defined by the CU1695 deletion is now more than 150 kb and substantially greater than the distance suggested by analysis of the available transduction data (Weiss & Wake, 1983).

The group F contained a 48 kb length of DNA that hybridized with CU1695 and was weakly labelled by the 10 min terminus probe. It probably is located to the right of the Weiss-Wake

region on the early terminating arm of the chromosome. Direct evidence for this has been obtained by genetic mapping experiments. Non-replicative plasmids which contain the terminal *EcoRI* fragment of group F have been inserted into the chromosome by homology and the introduced chloramphenicol resistance mapped by transformation. The 3.95 kb fragment of $\phi 537$ was linked to *gltA* while the 2.1 kb fragment of $\phi 906$ was not. This indicates that there is only a small gap between group F and the *gltA* region (M. G. Sargent, unpublished observations).

The genetic evidence for the location of the terminus of replication rests on now classical density transfer studies which placed the terminus close to the markers *citK* and *gltA* (Harford, 1975). More recently, it has been suggested that the terminus lies to the right of *gltA* (O'Sullivan & Anagnostopoulos, 1983). Using merodiploid strains in which the length of the left-hand side was increased by a large duplication, these authors showed that replication of *gltA* was delayed and that this marker was still the last to replicate. If the terminus had been between *gltA* and *SP β* for example, *gltA* would have been replicated by the right-hand fork at the normal scheduled time.

The *gltA292* mutation was corrected by the 7.5 kb *EcoRI* fragment of $\phi 715$, and *gltA2* and *gltB1* corrected by the 9 kb fragment of $\phi 715$. All of these are presumably on the 13.7 kb *BamHI*–*SalI* fragment (T_1 in Fig. 4), thus supporting the tentative suggestion of Weiss & Wake (1983). However, almost all of this fragment lies to the right of the terminus indicated by their map, and therefore contradicts the conclusion of O'Sullivan & Anagnostopoulos (1982) which suggests that *gltA* is to the left of the terminus. *EcoRI* fragments of close to 7.5 and 9.0 kb are present in 2.5 min terminus-labelled DNA, but these are unlikely to be the fragments in $\phi 715$, as the kinetics of labelling indicate that the region does not become radioactive unless pulse-labelled for 5 min or more. Even in a 10 min labelling period, the specific activity determined by DNA–DNA hybridization (Table 3) of this region was only one-third of that of $\phi 553$. This observation suggests that a more accurate position for the terminus is slightly to the left of T_1 (Fig. 4), suggested by Weiss & Wake (1983), at T_2 . The data of O'Sullivan & Anagnostopoulos (1982) may be explained by suggesting that the region around *gltA* is inverted so that *gltA* is on the late terminating arm of the chromosome.

The possibility of an attachment of the terminal region of the chromosome to the cell membrane has been widely discussed. We have shown previously that the *ilvA*–*ilvD* region is enormously enriched in a membrane fraction while the terminal region marker *gltA* is not (Sargent & Bennett, 1982). Attempts to demonstrate enrichments of terminally labelled DNA in the membrane fraction indicate that only a small portion of the terminal region can be membrane-bound and this part is probably a substantial distance from the terminus (Sargent & Bennett, 1982). None of the cloned fragments described above were membrane-bound and furthermore membrane-bound DNA in CU1695 had an identical composition to membrane-bound DNA in the wild-type (M. G. Sargent, unpublished observations). Although there may be membrane-bound regions present in the uncloned regions, the major membrane binding site in the late replicating part of the chromosome appears to be at *ilvA*–*ilvD*, which is at least 240 kb from *gltA*.

There is no obvious explanation of the apparently paradoxical findings of the fixed nature of the terminus on the one hand and its dispensibility in CU1695 although, presumably, it indicates that alternative mechanisms of termination must be present in the bacterial cell. Unfortunately, we are unable to determine the sequence used in CU1695 as this strain is unable to sporulate.

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