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Identification of Coreplicating Chromosomal Sectors in *Bacillus subtilis* by Nitrosoguanidine-Induced Comutation

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The simultaneous replication of four regions of dichotomously replicating chromosomes of *Bacillus subtilis* has been detected by means of nitrosoguanidine-induced comutation. The map distance between successive rounds of replication has been measured as one-half a replicative arm in cells growing exponentially in rich medium.

Bacillus subtilis lacks a genetic exchange system capable of transferring long stretches of deoxyribonucleic acid (DNA), like conjugation in *Escherichia coli*. The systems available, transformation (23) and transduction (24), can transfer DNA molecules that correspond to 0.5 and 5% of the *B. subtilis* genome, respectively.

Three methods that do not rely upon co-transformability or co-transducibility introduced by Yoshikawa and Sueoka and by Borenstein and Ephrati-Elizur (marker frequency analysis [28], marker density transfer [29], and sequential excretion of DNA from germinating *thyAB* spores [3]) have been used in determining the relative order of replication of genetic markers. Initially, the data were interpreted in terms of a linear, unidirectional mode of replication.

Wake and his collaborators later provided autoradiographic evidence that the *B. subtilis* chromosome replicates bidirectionally in a symmetrical manner and that it has a circular structure (10, 25, 26). Lepésant et al. (15) and Hara and Yoshikawa (11) have produced genetic evidence for the bidirectionality of chromosome replication. Lepésant-Kejzlarova et al. (16, 17) have recently revised all linkage relations between the known markers (also with the aid of new, previously unmapped ones) and have produced a single, circular genetic linkage group (Fig. 1a). Harford (12) has extended marker density transfer analysis to a great number of genetic markers throughout the *B. subtilis* map and has produced a coreplication map composed of two replicative arms of equivalent length (Fig. 1b) perfectly compatible with the genetic map of Lepésant-Kejzlarova et al. (16).

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B. subtilis cells in exponential culture (28) (and even during spore germination [19]) in "rich" media replicate their chromosomes dichotomously; that is, they initiate a new replication round before the preceding one is completed. Thus, in dichotomously replicating chromosomes there are six growing points currently replicating four chromosome sectors (Fig. 3). The replicative map of Harford has been obtained with chromosomes that have a single replicative round going on and can predict neither the four sectors that are coreplicated nor the distance between successive rounds of replication in dichotomously replicating chromosomes.

In this paper, the property of the mutagen nitrosoguanidine of introducing mutations preferentially in the replication regions (9) has been evaluated in *B. subtilis* and used to enrich a chromosomal region of new genetic markers (by comutation within the same replication region) and to identify four chromosomal sectors coreplicating in chromosomes that undergo dichotomous replication.

MATERIALS AND METHODS

Bacterial strains. All *B. subtilis* strains used in this work are listed in Table 1. The mutants obtained and characterized during this work are listed in Table 7.

Culture media. Nutrient broth (Difco), penassay broth, (antibiotic medium no. 3, Difco), and minimal medium (Davis and Mingioli, 1950) supplemented with appropriate requirements were used for liquid cultures or solidified with 2% agar (Difco). For PBS-1 transduction, medium Y (27) and tryptose blood agar base (Difco) were used. For sporulation, AK medium no. 2 (BBL) was used.

Nomenclature of Pur⁻ phenotypes. Pur⁻ mutants able to grow on hypoxanthine, guanine, or adenine are indicated as Pur^{PUR}; the ones that grow on adenine but not on the other purines are indicated as Pur^{ADE}.

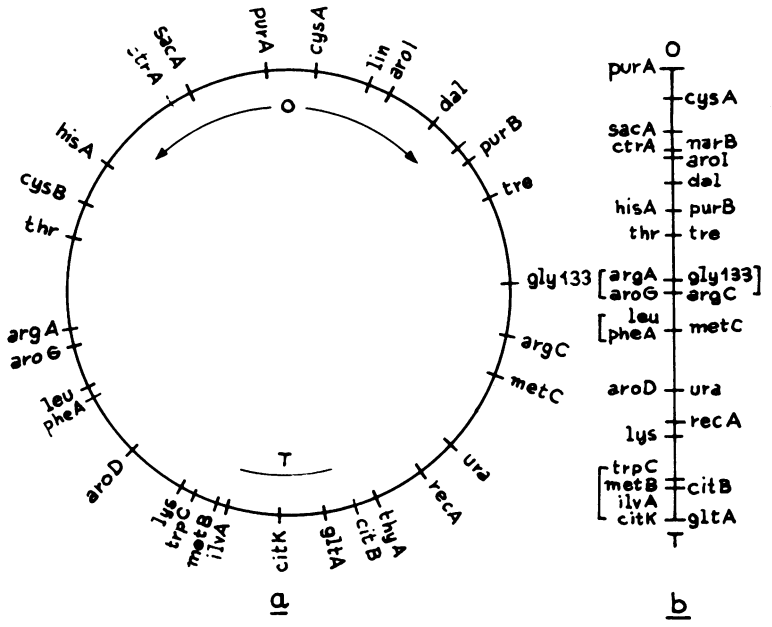

FIG. 1. *(a) PBS-1 transduction map of the B. subtilis chromosome, according to reference 16. O, Origin of replication; T, terminus. (b) Coreplication map. The order of replication was determined by the density transfer technique (12).*

TABLE 1. *List of strain*

Strain	Genotype	Origin
PB 566/1	*thyAB*	M. Polsinelli
PB 920	*purA16 leu-8 metB5*	Mu8u5u16, N. Sueoka
PB 3394	*purE26 metB5*	Mu5u26, N. Sueoka
PB 3356	*purB6 leu-8 metB5*	Mu8u5u6, N. Sueoka
PB 3361	*purB6 leu-8 metB5 trpC2 hisA1 lys-21 thr-5 ery-101*	Hll 39, P. Sheaffer
PB 1665	*purB6 leu-8 metB5 hisA1 lys-21 thr-5 sacA321*	J. A. Lepésant
PB 1683	*dal metC trpC2*	*ald*, E. Freeze
B 534	*pyrA26*	J. Copeland
QB 539	*cysB3 thr-5 trpC2*	J. A. Lepésant
SB 120	*aroD120 trpC2*	E. W. Nester
GSY 292	*gltA292 trpC2*	J. A. Hoch
WB 932	*aroG932*	E. W. Nester
WB 906	*aroI906*	J. A. Hoch
QB 693	*purB6 tre-12*	J. A. Lepésant

Mutagenesis with NTG. Exponential cells growing in nutrient broth medium were washed and suspended in maleate buffer (1), 7.5, at a concentration of 10^8 cells/ml and treated with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) per ml for 30 min. Survival was approximately 10^{-3}. The cells were then washed and resuspended in nutrient broth medium supplemented with adenine (20 μg/ml) and grown for 4 h at 30 C to allow expression of the mutations. Suitable dilutions were plated onto minimal medium plates supplemented with Casamino Acids (Difco) (0.02%) for selection of Pur^+ revertants and with hypoxanthine (20 μg/ml) for selection of Pur^{ADE+} revertants. Among the latter, Pur^{PUR} clones were then screened by replica plating on purineless plates. Pur^+ temperature-sensitive (ts) double mutants were screened by replica plating on the same medium at 46 C.

PBS-1 transduction mapping. Transduction was performed according to Hoch et al. (13). Donor and recipient strains were tested for motility on soft agar. After selection on appropriate media, recombinants were isolated on the same media before testing for unselected markers. Mapping data are expressed as percentage of recombination according to the convention: map distance = 100 − percent cotransfer (2).

RESULTS

Comutagenesis by NTG. NTG is known to induce multiple mutations preferentially at the growing point of the DNA replication fork (4). Guerola et al. (9) have demonstrated that there is a rather long stretch of DNA, the "replication region," that shows enhanced sensitivity to the mutagen.

We investigated the degree of such a "preference" for the replication regions in *B. subtilis* by evaluating the rate of NTG-induced reversion of three auxotrophic markers *(purA16, leu-8,* and *metB5)* in germinating spores of PB 920. The three markers are conveniently located near the origin, in the middle, and near the terminus of the *B. subtilis* replicative map (Fig. 1b) and can be induced to revert by NTG in exponential cultures at comparable frequen-

cies. Adding NTG at the onset of DNA replication, we found an induced reversion rate more than 100-fold higher for the origin-proximal marker *purA16* compared to the other two markers (see Table 2).

The peculiar properties of the mutagen make it possible to enrich a selected chromosomal region with new markers. The basic scheme of our project was to mutagenize strains PB 3356 (*purB6*) and PB 3394 (*purE26*) during exponential growth in nutrient broth medium, to select for Pur$^+$ revertants, and to search among these, by replica plating, for double mutants (e.g., Pur$^+$ ts mutants). Figure 2 describes the procedures used with the two parental strains, and Fig. 3 shows the possible sites at which comutations could occur in the case of dichotomous replication.

In the case of *purE26* (a strict adenine requirer that does not grow on hypoxanthine, Pur^{ADE} phenotype), we looked for two types of double mutants by selecting the revertants able to grow on hypoxanthine medium and then searching for both ts and Pur^{PUR} double mutants (on purineless medium). The only clone we found to show a purine requirement that could be satisfied by hypoxanthine (Pur^{PUR} phenotype), PB 2430, resulted in being due to mutation *pur-30*, which was shown to map very close to *purE26* and on its left (Fig. 4). This finding substantiates the hypothesis that NTG-induced comutagenesis can be used to place new mutations in proximity of known markers.

TABLE 2. *Frequency of reversion of purA16, leu-8, metB5 markers at the onset of DNA synthesis during spore germination (60 min)*

Markers	Spontaneous	NTG induced	Ratio (spontaneous/NTG-induced)
purA16	3.2×10^{-8}	3.4×10^{-4}	1.1×10^{4}
leu-8	7.8×10^{-9}	4.2×10^{-7}	5.4×10^{1}
metB5	5.2×10^{-9}	5.0×10^{-7}	9.6×10^{1}

Genetic analysis of ts markers linked to Pur$^+$ alleles. The map locations of the ts markers of 18 double mutants (15 from the *purB6* and 3 from the *purE26* parents; see Fig. 2) were then investigated using the Pur$^+$ ts strains as donor in back-crosses with their parents, selecting for Pur$^+$, and scoring ts clones; the donors in which the Pur$^+$ and ts markers are linked were expected to give a fraction of Pur$^+$ ts recombinants in the back-cross.

Only three double mutants, PB 2458 (Pur$^+$ *ts-58*), PB 2459, (Pur$^+$ *ts-59*) and PB 2472 (Pur$^+$ *ts-72*), showed a linkage between the Pur$^+$ and ts markers.

Table 3 shows the linkage relationships of the markers *ts-58*, *ts-59*, and *ts-72*; *ts-58* is closely linked to *purB6* and on its right (since it is further away from *dal*) and *ts-72* is linked to *purB6*, *pur-30*, and *tre* and is unlinked to *dal*.

ts-59 linked to the Pur$^+$ marker and *dal* and was unlinked to *purE26* and *pur-30*; these linkage relationships would be absurd if the Pur$^+$ marker were *purB6* and suggest an altogether different situation. Strain PB 2459 (*ts-59*) could be a suppressor strain with a Su marker linked to *dal* and the ts marker linked to the suppressor rather than to *purB6* itself.

The analysis of the three-factor cross illus-

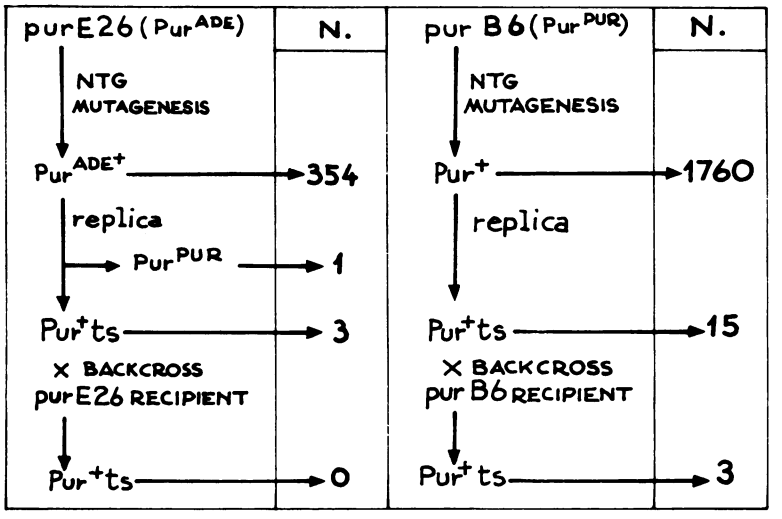

FIG. 2. *Scheme of the experimental protocol used for comutation experiments by means of NTG mutagenesis. Pur^{ADE} and Pur^{PUR} phenotypes are defined in Materials and Methods.*

trated in Table 4 substantiates this hypothesis, showing that the strain still contains the *purB6* allele; the relative order of the markers was established by constructing a *dal ts-59 su-59* (purB6) strain (PB 2535, Table 7) and using it as a recipient in the three-factor cross illustrated in Table 5. The results of the cross deserve some comment since two classes of possible recombinants share the same phenotype (i.e., *purB6*$^+$ and *purB6 su-59*) and two of the possible recombinant classes are empty. The results have to be looked at together with the genetic distances in Table 3 (*su-59* − *ts-59* = 45 and *dal* − *ts-59* = 88); thus two of the recombinant classes (*su*$^+$*dal*$^+$ and *purB*$^+$ *ts*$^+$) are excluded because the map distance exceeds the phage-carrying capacity. Therefore, the most probable order of the markers considered is *purB6, dal, ts-59, su-59*. As expected from the reported (16) linkage relationships of *dal* with *aroI, ts-59* maps very close to *aroI* (Table 3); *su-59* might be the same suppressor described and mapped near *lin* by Georgopoulos (8).

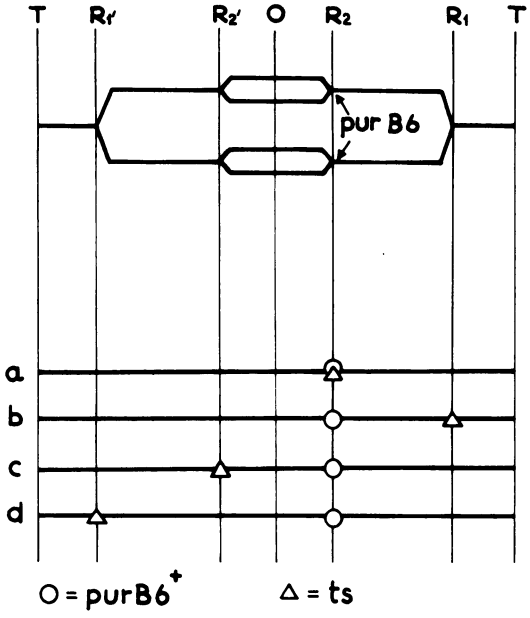

FIG. 3. *Model of the expected locations of comutations in dichotomously replicating chromosomes, following the experimental protocol depicted in Fig. 2.*

Four other Pur$^+$ revertants were due to suppression mutations rather than to back-mutations (see Table 7) since they showed a normal fraction of Pur$^-$ recombinants in crosses with *dal* recipients. Five Pur$^+$ ts derivates (Table 7) were asporogenic: the only spores recovered from AK slants were ts$^+$. In one case (PB 2450) we could recover ts bacilli and demonstrate that only ts$^+$ revertants could sporulate. No further studies on these mutants were made (and finally they were lost).

Identification of coreplicating regions on the basis of NTG-induced comutation. The remaining Pur$^+$ ts comutants are likely to be located in chromosomal regions that are coreplicated with *purB* (see Fig. 3).

Our such region can be expected to be the *hisA* region, shown by Harford to coreplicate with *purB* by marker density transfer (Fig. 1b); the other two regions cannot be located by marker density transfer (see above). The six ts markers unlinked to *purB6* (see Table 7) have been mapped by PBS-1 transduction (see Table 6); one of them maps in the *hisA* region, two in the *pyrA-recA* region, and three in the *lys-metB* region. The last two map locations are compatible with Harford's replicative map, since *lys* and *recA* regions do coreplicate (Fig. 1b).

The four chromosomal regions defined by NTG-induced comutation are indicated in Fig. 5 as being located about 0.5 replications apart. Such a pattern is expected if reinitiations occur when the preceding replicative forks are at the middle of the replicative arms (in the *aroG* and *argC* regions, respectively).

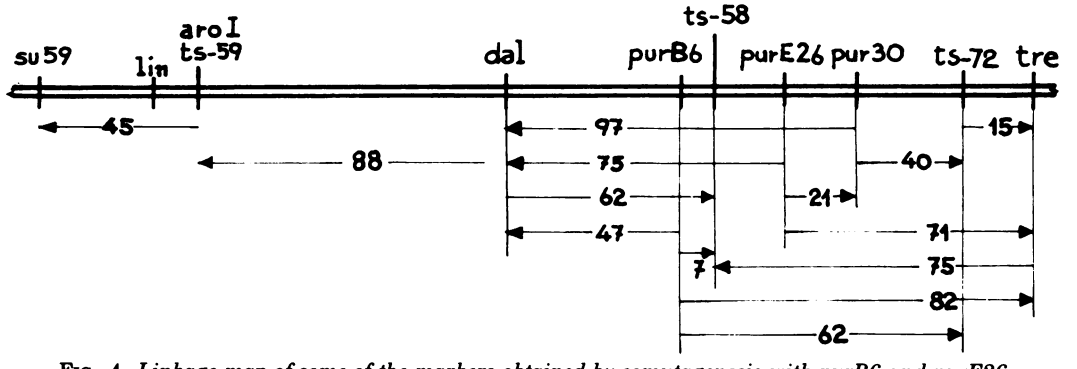

FIG. 4. *Linkage map of some of the markers obtained by comutagenesis with purB6 and purE26.*

TABLE 3. *Linkage of ts markers to known markers by PBS-1 transduction*

Donor	Selected markers				
	purB6	*pur-30*	*tre*	*dal*	*aroI*
ts-58	185/200[a]		25/100	38/100	0/100
ts-59	55/100	0/100	0/100	12/100	96/100
ts-72	38/100	60/100	85/100	0/100	

[a] ts fraction.

TABLE 4. *Demonstration of the persistence of the purB6 allele in strain PB 2459 by transduction*[a]

Unselected markers	No. examined
ts$^+$ Pur$^+$	120
ts$^-$ Pur$^+$	3
ts$^+$ Pur$^-$	64
ts$^-$ Pur$^-$	0

[a] Donor, PB 2459 (Pur$^+$ ts); recipient, PB 1683 (Pur$^+$ *dal*). Selected marker used was Dal$^+$.

DISCUSSION

The property of the mutagen nitrosoguanidine to introduce multiple mutations in the replicative regions has been used to identify a number of ts mutations arising concomitantly with Pur$^+$ reversions from *purB6* or *purE26* parents. Three of these ts mutations resulted in being linked with the Pur$^+$ marker, two of them being linked to *purB6* and one to *dal* and to a suppressor of *purB6* located between *aroI* and *lin*.

Marker density transfer experiments (12, 29) have enabled Harford (12) to determine which regions of the two replicative arms of the chromosome are replicated at the same time, indicating that the *purB6* and *hisA* markers are coreplicated (i.e., equidistant from the origin on the two different replicative arms).

During exponential growth in rich medium, *B. subtilis* cells replicate their DNA dichotomously, that is, with two simultaneous rounds of replication. NTG comutation permits the identification of the four chromosomal regions that are coreplicated (Fig. 3).

The ts mutations concomitant to Pur$^+$ reversions and unlinked to *purB6*, which are likely to identify chromosomal regions coreplicating the *purB6* in exponential cells, have been mapped near *hisA*, in the *pyrA-recA* region, and in the *lys-metB* region.

These data are compatible with Harford's coreplication map (Fig. 1b) for both pairs of map locations.

The four chromosomal regions where comutations have occurred in our experiments are shown in the dichotomous replication map (Fig. 5), which indicates that in exponential cells growing in rich medium reinitiations occur when the preceding round of replication is at the time center of the replicative arms (in the *aroG* and *argC* regions, respectively).

These conclusions coincide with the ones we have reached with *B. subtilis* spores germinating synchronously in rich medium (22); in that work we studied nuclear partitions by fluorescent microscopy. We defined the interval between the onset of DNA synthesis and the first nuclear partition (NP1) as 1 CPT (chromosomal percurrence time) and we found that NP2 and NP3 then followed at 0.5 CPT intervals, implying that new rounds of replication had also to be initiated at 0.5 CPT intervals, i.e., when the preceding rounds of replication were half completed.

Guerola et al. (9) have defined, in *E. coli*, the replication region as the chromosomal region that displays enhanced sensitivity to NTG mutagenesis. They evaluated the enhancement in sensitivity as a 220-fold increase and the length of the region as 2 min on the map of *E. coli* (approximately 8×10^4 base pairs, assuming a total length of 4×10^6 base pairs). Analogous measurements in *B. subtilis* give an enhancement value between 100 and 200 (see Table 2). A minimum estimated length of the sensitive region has been evaluated from the highest map distance found between comutations within the same replication region (i.e., 62

TABLE 5. *Three-factor cross showing the order: purB6, dal ts-59, su-59 (transduction)*[a]

Unselected markers	No. examined	Possible order
ts$^+$ Pur$^+$	124	*purB6, dal, ts-59, su-59*
ts$^-$ Pur$^+$	440	
ts$^+$ Pur$^-$	0	
ts$^-$ Pur$^-$	0	

[a] Recipient, PB2535 (*purB6*) *su-59 ts-59 dal metB5;* donor, PB 168, *trpC2*. Selected marker used was Dal$^+$.

TABLE 6. *Linkage of ts markers to known auxotrophic markers by PBS-1 transduction*

Donor	Selected markers					
	pyrA	*metC*	*lys-21*	*metB*	*hisA*	*thr-5*
ts-31	40/200[a]	0/180	0/100	0/200	0/200	0/100
ts-32	0/200	0/200	0/100	0/200	19/200	0/100
ts-36	60/200	0/200	0/100	0/200	0/200	0/100
ts-54	0/200	0/180	85/100	12/200	0/200	0/100
ts-57	0/200	0/200	80/100	18/200	0/200	0/100
ts-60	0/200	0/200	76/100	31/200	0/200	0/100

[a] ts fraction.

TABLE 7. *List of double mutants obtained by NTG-induced comutation and their characterization*

Strains	Genotype (phenotype)	Linkage
From PB 3394 + NTG:	*purE26*$^+$ *metB5 pur-30*	(= *pur 65*)
PB 2430	*purE26*$^+$ *metB5 ts-31*	*pyrA26*
PB 2431	*purE26*$^+$ *metB5 ts-32*	*hisA1*
PB 2432	*purE26*$^+$ *metB5 ts-36*	*pyrA26*
PB 2436		
From PB 3356 + NTG:	*purB6*$^+$ *leu-8 metB5 trpC2* (ts Spo$^-$)	
PB 2446	*purB6*$^+$ *leu-8 metB5 trpC2* (ts Spo$^-$)	
PB 2449	*purB6*$^+$ *leu-8 metB5 trpC2* (ts Spo$^-$)	
PB 2450	*purB6*$^+$ *leu-8 metB5 trpC2* (ts Spo$^-$)	
PB 2453	*purB6*$^+$ *leu-8 metB5 trpC2* (ts Spo$^-$)	
PB 2456		
PB 2448	*purB6 leu-8 metB5 trpC2* (ts Su)	
PB 2452	*purB6 leu-8 metB5 trpC2* (ts Su)	
PB 2455	*purB6 leu-8 metB5 trpC2* (ts Su)	
PB 2459	*purB6 leu-8 metB5 trpC2 ts-59 su-59*	*dal aroI*
PB 2458	*purB6*$^+$ *leu-8 metB5 trpC2 ts-58*	*purB6*
PB 2472	*purB6*$^+$ *leu-8 metB5 trpC2 ts-72*	*purB6*
PB 2535	*(purB6) su-59 ts-59 metB5 dal* = PB 1683 $\xrightarrow{tf}$ PB 2459[a]	
PB 2454	*purB6*$^+$ *leu-8 metB5 trpC2 ts-54*	*lys*
PB 2457	*purB6*$^+$ *leu-8 metB5 trpC2 ts-57*	*lys*
PB 2460	*purB6*$^+$ *leu-8 metB5 trpC2 ts-60*	*lys*

[a] The arrow indicates that the strain was constructed by DNA transformation (*tf*) at saturating DNA concentration.

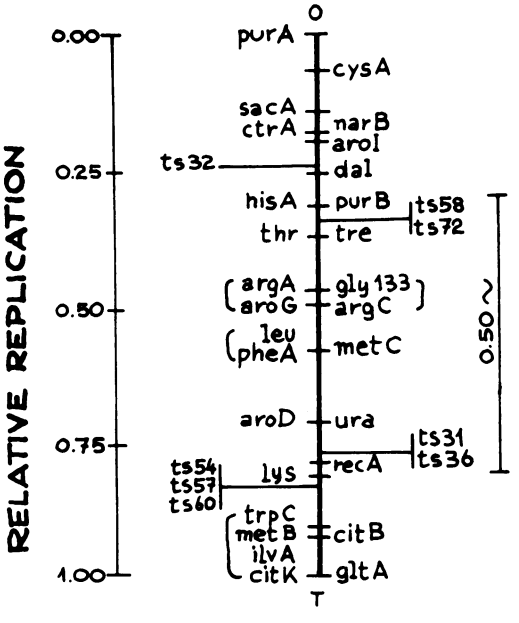

FIG. 5. *Position on the coreplication map (12) (Fig. 1b) of the replication regions defined by NTG comutation with purB during dichotomous replication.*

PBS-1 map units); assuming a total chromosome length of 2,100 PBS-1 map units and 6×10^6 base pairs, this distance corresponds to 2.1×10^4 base pairs, only one-fourth of the length calculated in *E. coli*.

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