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Characterization and Mapping of Temperature-Sensitive Division Initiation Mutations of *Bacillus subtilis*

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Two temperature-sensitive filamenting mutants of *Bacillus subtilis* (*ts1* and *ts12*) have been shown to be defective in the initiation of septation. Recombination index mapping showed that these mutations mapped in two different but closely linked genes. A third proposed initiation mutation, *tms-12*, probably maps in the same gene as *ts12*. Another proposed initiation mutation was not linked with these genes by transformation, indicating that there was a minimum of three genes involved in the initiation of division. PBS1 transduction mapping located these three genes close to the *pyr* cluster.

The process of cell division can be considered as occurring in three stages: initiation, cross-wall and membrane synthesis (septal growth), and cell separation (27). Several mutants temperature sensitive in one or more of these stages have been isolated for both *Escherichia coli* (9) and *Bacillus subtilis* (21). On transfer to the nonpermissive temperature many of these mutants stop dividing but otherwise continue to grow normally, forming long, multinucleate, apparently septationless filaments. Although such mutants have sometimes been described as division initiation mutants (17, 21, 23, 38), in no case has this been definitively established. Indeed, there is considerable difficulty in distinguishing between initiation mutants and those defective in septal growth (27).

For a filamenting mutant to be classified as an initiation mutant, two conditions must be met. The filaments should not contain any incompleted septa, nor any membrane or wall aberrations more consistent with a defect in the early stages of septum formation. Also, it should be possible to show that the disappearance of partial septa is due to their completion rather than to some form of septal resorption (27). The occurrence of such septal resorption has been described previously (23, 33, 35).

This paper presents work showing that two temperature-sensitive filamenting mutants of *B. subtilis*, *ts1* and *ts12*, isolated by Nukushina and Ikeda (24), behave as expected for mutants defective solely in division initiation. This is the first time that residual cell increase after a temperature shift has been related to the number of partial septa in cells at the time of the shift, thus overcoming former objections to defining division initiation mutants on the basis of residual division (27). We also present mapping data for these and two other proposed initiation mutants (21), describing the possible number and chromosomal location of division-initiation genes in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains. The strains of *B. subtilis* used and their sources are given in Table 1.

Growth conditions and light microscopy. Overnight stationary-phase cultures in a supplemented glucose-salts medium (5) at 34°C were diluted into fresh medium at 34°C to give an absorbancy at 590 nm of 0.05. After growth into mid-exponential phase (absorbancy at 590 nm, 0.4 to 0.6), cultures were diluted 1:3 into prewarmed medium at either 34 or 49°C. For phase-contrast and fluorescence microscopy, 0.1- or 0.5-ml samples were taken into equal volumes of 20% Formalin at appropriate times after the shift. Fluorescence microscopy using acridine orange staining was performed as described previously (19).

Total cell counts. The terms "bacillus," "filament," and "cell" are used in this paper according to the definitions of Breakefield and Landman (2). Thus, bacillus refers to a wall-bounded unit particle which may or may not contain septa, and cell refers to an individual compartment bounded by either cross-walls or bacillary ends or both.

To determine the residual cell increase samples were taken into Formalin as described above. The bacillus concentration was determined by using a Petroff-Hauser counting chamber in a Zeiss photomicroscope at a magnification of ×630. The number of visible septa was then counted in at least 100 bacilli with phase-contrast microscopy at a magnification of ×1,000. The cell concentration of each sample was calculated as follows: cells per milliliter = bacilli per milliliter × (1 + average septa per bacillus).

Electron microscopy. Filament morphology was examined by electron microscopy of samples prepared by the method of Whitehouse et al. (34) with some modifications. This method is designed to increase the number of bacilli longitudinally oriented in the plane of the section by centrifuging cells through molten agar onto a solid agar cushion. The major change was

TABLE 1. *Strains of B. subtilis*

Strains[a]	Genotype	Source
ts1	160 *trp-3 ts1*	J.-I. Nukushina
ts12	160 *trp-3 ts12*	J.-I. Nukushina
160	160 *trp-3*	J.-I. Nukushina
tms-12 (BC101)	168 *purA16 leuA8 metB5 tms-12*	O. Landman
tms-12$^+$	168 *purA16 leuA8 metB5 tms-12*$^+$	O. Landman
divD32 (VA322)	168 *trpC2 divD32*(Ts)	A. Hitchins
BR77	168 *thr5 trpC2*	A. Hitchins
Kit3 (QB934)	168 *tre12 metC3 glyB133 trpC2*	R. Dedonder
Kit4 (QB943)	168 *pyrD1 ilvA1 thyA1 thyB1 trpC2*	R. Dedonder
MB50	168 *pyrD1 cysC7*	P. Piggot
SB19	168 *trp*$^+$	E. Nester

[a] Previous designations are given within parentheses.

that samples were treated by the original procedure of Kellenberger et al. (15) until the agar resuspension step.

Samples (10 or 20 ml) were taken immediately before and 1 or 2 h after the temperature shift and prefixed for 5 min with a 0.1 volume of Kellenberger fixative. (The compositions of this and the other reagents used are given in reference 15.) Samples were centrifuged at 1,000 × *g* for 15 min, washed once with buffer, and fixed overnight in 1.8 ml of fixative plus 0.18 ml of tryptone medium. After fixation the suspensions were diluted with 8 ml of buffer, centrifuged, washed once with buffer, and finally suspended in 0.15 ml of 3% Noble agar (Difco Laboratories) at 65°C. The samples were then treated as described by Whitehouse et al. (34), except that no further osmium tetroxide fixation was needed, and uranyl acetate staining was for 2 h. The blocks were dehydrated in acetone for longer than usual, being left for about 2 h at each step, and were finally embedded in Spurr Low Viscosity Embedding Medium (Polysciences Inc., Warrington, Pa.).

Extraction of DNA and recombination index mapping. The preparation of spores on potato extract medium at 30°C was as described previously (4). Extraction of spore DNA with thioglycolic acid and urea was also as described previously (4), except that extracts were treated with 50 μg of RNase (prepared by the method of Marmur [18]) per ml before the addition of detergent and deproteinized with phenol rather than chloroform-isoamyl alcohol. When vegetative cell DNA was required, it was extracted from cultures in mid-exponential growth and deproteinized with phenol as described previously (30, 32). In these cases, care was taken to ensure that both the mutant and its parent strain were growing at the same rate so that the distribution of genetic markers within each strain was as similar as possible.

Preparation of competent cells and all transformations were done at 30°C, but otherwise as described previously (4). Selection of temperature-insensitive transformants was at 48°C on freshly made tryptose blood agar base (Difco) plates for *tms-12* cells and nutrient broth (Difco)–1.5% agar (Difco) plates (24) for *ts1* cells.

The recombination index (RI) was determined by the method of Karamata and Gross (12). Wherever possible spore DNA was used, as spores contain only completed chromosomes (4). When vegetative DNA was used, all ratios were normalized to parental spore DNA. The RI could then be corrected for the difference in marker distribution caused by different states of replication in mutant and parent donors. To do this correction, both vegetative and nonreplicating parental DNA must be used, as well as vegetative mutant DNA, and all ratios must be determined within the same experiment (14). The formula for the corrected RI was derived from the equation of chromosome replication in the theory of marker frequency analysis developed by Sueoka and Yoshikawa (28), and is described here for a general case.

$$\text{Corrected RI} = (ts^+/X^+)_M/(ts^+/X^+)_P^{[\log(A^+/B^+)_M/\log(A^+/B^+)_P]}$$

where *A* and *B* are any two genetic markers isogenic in both mutant and parent donors, and *X* is any genetic marker, including either *A* or *B*, also isogenic in the two strains. *M* and *P* denote normalized ratios from mutant and vegetative parent DNA, respectively. It is essential that all ratios substituted in this equation first be normalized to nonreplicating parental DNA. The equation assumes that the map location of all recipient markers (*ts*, *X*, *A*, and *B*) is the same in both mutant and parent donors, but does not require these positions to be known exactly. In the experiments presented here, *purA16* was used for the markers *X* and *A*, and *metB5* was used for *B*.

Phage preparation and transduction mapping. PBS1 transducing lysates were prepared as described by Coote (7); stock lysate was prepared on SB19. For transduction, recipient cells were grown in Penassay broth (Difco antibiotic medium no. 3) at 30°C for 18 h, by which time they were generally highly motile. A 1-ml amount of culture was mixed with 0.03 to 0.1 ml of lysate and incubated at 30°C with shaking for 20 min. The cells were centrifuged at 1,000 × *g* for 4 min and suspended in 1 ml of Spizizen salts (1), and 0.1-ml volumes were plated on appropriate selection plates. These normally contained 1.5% agar, but for selection of *cys*$^+$ recombinants of strain MB50 1.5% Noble agar was used.

To determine cotransfer of temperature-sensitive markers with the selected auxotrophic marker, recombinants were patched onto the same selective medium and incubated at 30°C. These were then replica plated onto duplicate plates of the same medium, one of which was incubated at 48°C and the other of which was incubated at 30°C. Results of transduction mapping have been expressed in two ways. Map distances are conventionally given as percentage of recombination (100 − percentage of cotransduction). In this paper we have also expressed map distances as the percentage of the length of the transducing fragment (100 × *t*), calculated from the formula of Kemper: $C = 1 - t + t\ln t$, where *C* is the cotransfer of markers (10, 16). The *t* value appears to be a more representative measurement of physical map distance (10).

RESULTS

Growth and morphology at 49°C. Nukushina and Ikeda have previously shown that *ts1* and *ts12* form filaments which can be seen by phase-contrast microscopy both after spore germination at 48°C and when vegetative cells are transferred to 48°C. They have also shown that total RNA and DNA synthesis at 48°C is normal (24). When exponentially growing cultures of *ts1* and *ts12* were transferred from 34 to 49°C, the cell mass, measured by absorbancy at 590 nm, continued to increase in a manner similar to that of the parent strain (data not shown). Phase-contrast microscopy showed the formation of filaments, whereas fluorescence microscopy showed many nuclear bodies distributed regularly along the full length of the filaments (data not shown), indicating that these mutants are not defective in DNA segregation.

Electron microscopy. Samples were taken for electron microscopy immediately before and 1 h after a temperature shift, by which time filaments were about three times the normal cell length at 34°C. Typical electron micrographs are shown in Fig. 1, 2, and 3. Few completed septa and no partial septa were seen in filaments of either *ts1* or *ts12*, whereas in the parent strain the ratio of partial to completed septa seen in longitudinal sections remained the same at 49 as at 34°C (Table 2). Furthermore, there were no obvious differences in the appearance of the cell membrane or cell wall between the mutants growing at 34 and 49°C or between the parent strain and either mutant at 49°C, even at high magnifications. There may have been an increase in wall turnover with temperature, indicated by the increased amount of debris associated with the external surface of the cells at 49°C, but this occurred in both parent and mutant strains. The same results were obtained by using filaments taken 2 h after transfer.

Residual cell division. To establish that partial septa were completing in *ts1* and *ts12* the residual cell increase was followed and related to the number of divisions in progress at the time of transfer. Table 2 gives the ratio of partial septa to completed septa as seen by electron microscopy in *ts1* and *ts12* at the time of transfer. The number of bacilli with no septum, either partial or complete, was less than 10%, and correcting for this made no significant difference to the calculations which followed.

When similar samples were examined by phase-contrast microscopy, as many as 28% of *ts12* and 58% of *ts1* bacilli showed no visible septa. For *ts1* (in which very few bacilli were seen with more than one septum) this corresponds very closely to the percentage of partial septa seen by electron microscopy (Table 2). A similar correspondence is not seen for *ts12* because this strain tends to grow in chains, but the proportion of bacilli with no visible septum in phase-contrast microscopy was still more than three times that seen by electron microscopy. These results indicate that, for *B. subtilis* at least, only completed or very nearly completed septa are visible under the conditions of phase-contrast microscopy used here.

Figure 4 shows the residual increase in both bacillus and cell numbers for *ts1* and *ts12* after transfer to 49°C. The difference between these curves in each case shows the change in the number of visible (and therefore completed) septa. The increase in cell number of *ts12* suggests that at least some partial septa were being completed, whereas the increase in bacillus number and the eventual disappearance of visible septa indicates that subsequent cell separation proceeded normally.

The initial rise in cell number of *ts1* indicates that in this mutant partial septa were at first completed, so becoming visible by phase-contrast microscopy, whereas the rise in bacillus number indicates that cell separation was occurring. The subsequent fall in cell number after 30 min at 49°C was unlikely to be due to septal resorption, since this would require resorption of septa after their completion. In fact, this fall, which was accompanied by a slight fall in bacillus number, could be accounted for by the considerable cell lysis seen in this mutant at the later times of this experiment. Although many lysed bacilli (identified as pale forms in the phase-contrast microscope) were seen at the high magnification used for counting visible septa, such lysed bacilli were not visible at the lower magnifications used with the Petroff-Hauser counting chamber and were therefore not included in the estimates of bacillus number. When the extent of lysis at 70 min was taken into account, the total cell number (from lysed and normal bacilli) was approximately the same as the cell number at 30 min, whereas total bacillus number had increased by a further 23% of the initial number. Therefore, the observed cell number of *ts1* at 30 min was used to estimate the observed residual cell increase of *ts1*. Because of the lysis, this residual cell increase would be, if anything, an underestimate of the real residual increase, and so would not affect the conclusions drawn.

Assuming that all visible septa present at the time of transfer were completed septa (see above), it was possible to calculate the number of partial septa present from the ratio of partial to completed septa seen in the electron micro-

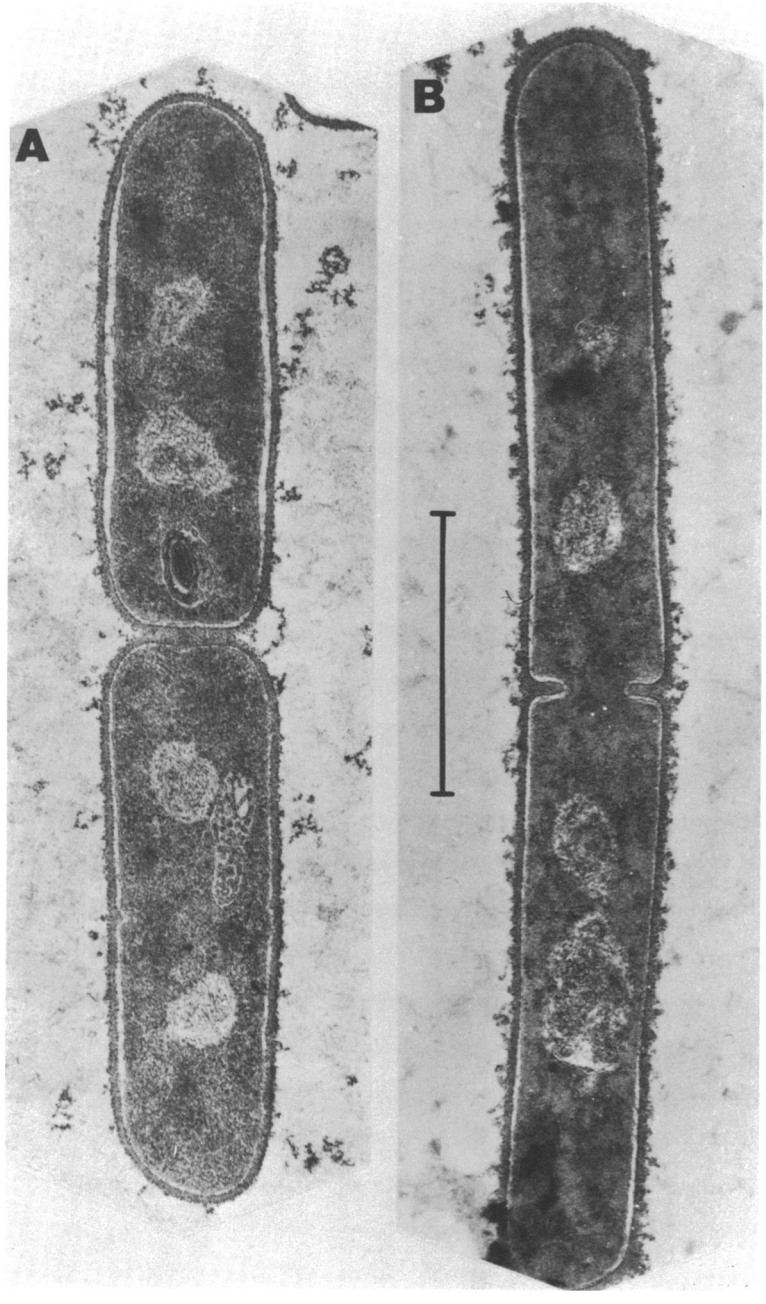

FIG. 1. *Electron micrographs of parent strain 160 at (A) 34°C at the time of transfer and (B) after 1 h at 49°C. At the time of transfer, an exponentially growing culture at 34°C was diluted 1:3 into fresh, prewarmed medium at 49°C. Bar, 1 μm.*

scope. The residual cell increase expected if all these partial septa were completed could then be calculated. From Table 3 it can be seen that the maximum cell increase observed for both *ts1* and *ts12* was very close to that predicted if these mutants were behaving as division initiation mutants.

RI mapping. RI mapping is a fine-structure mapping technique which can be used to estimate the distance between two mutations. Mu-

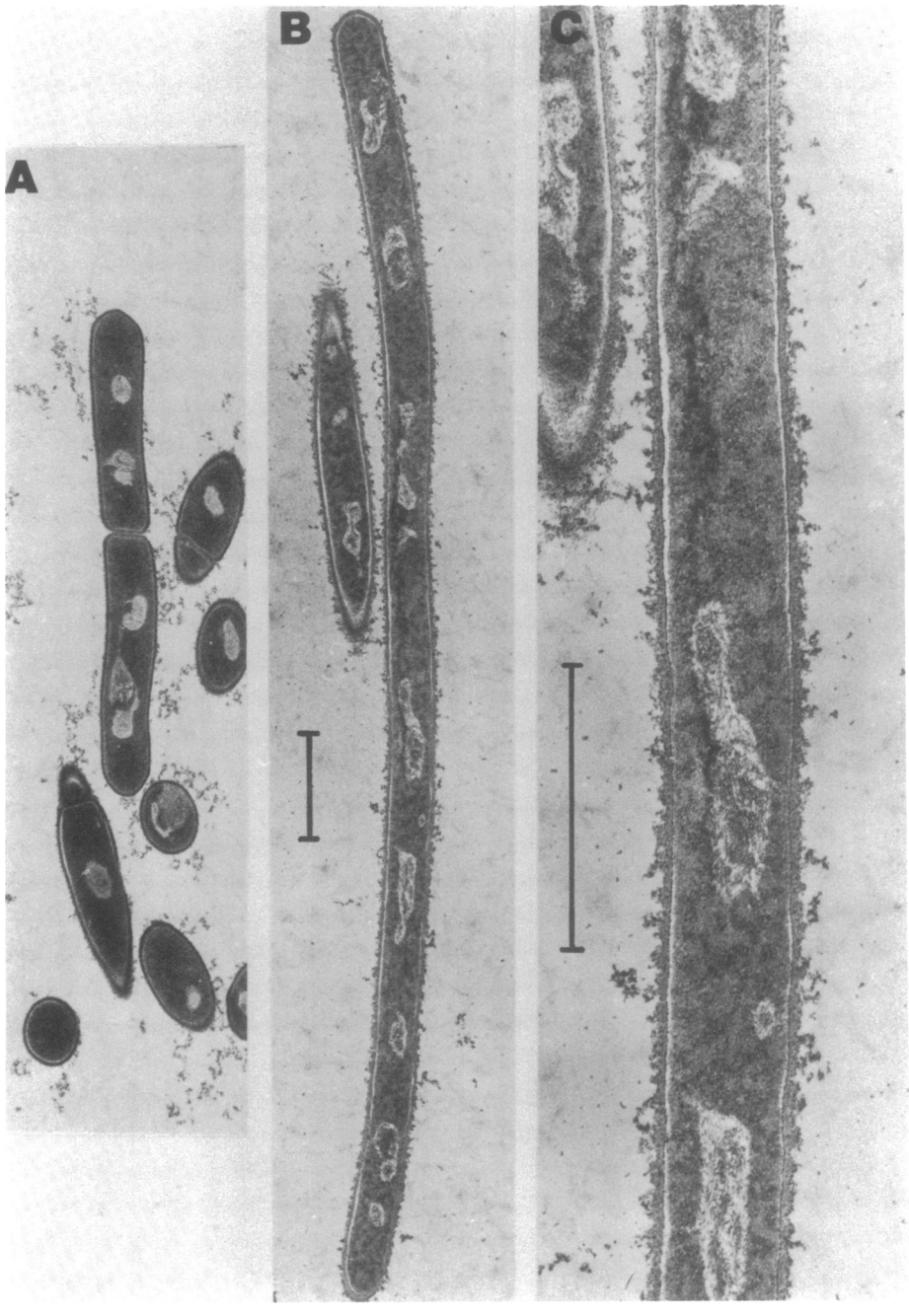

FIG. 2. *Electron micrographs of ts1 (A) at 34°C and (B) after 1 h at 49°C. (C) Higher magnification of central part of filament in B. Bars, 1 μm. A and B are at the same magnification.*

tations which are not linked will have a RI of 1, whereas an RI less than 1 indicates that two mutations are very close on the chromosome (12). The most detailed use of RI mapping has been to determine the order of over 50 mutations within the six genes of the pyrimidine cluster (14, 25). In this study, the highest RI obtained between two mutations within a single gene was

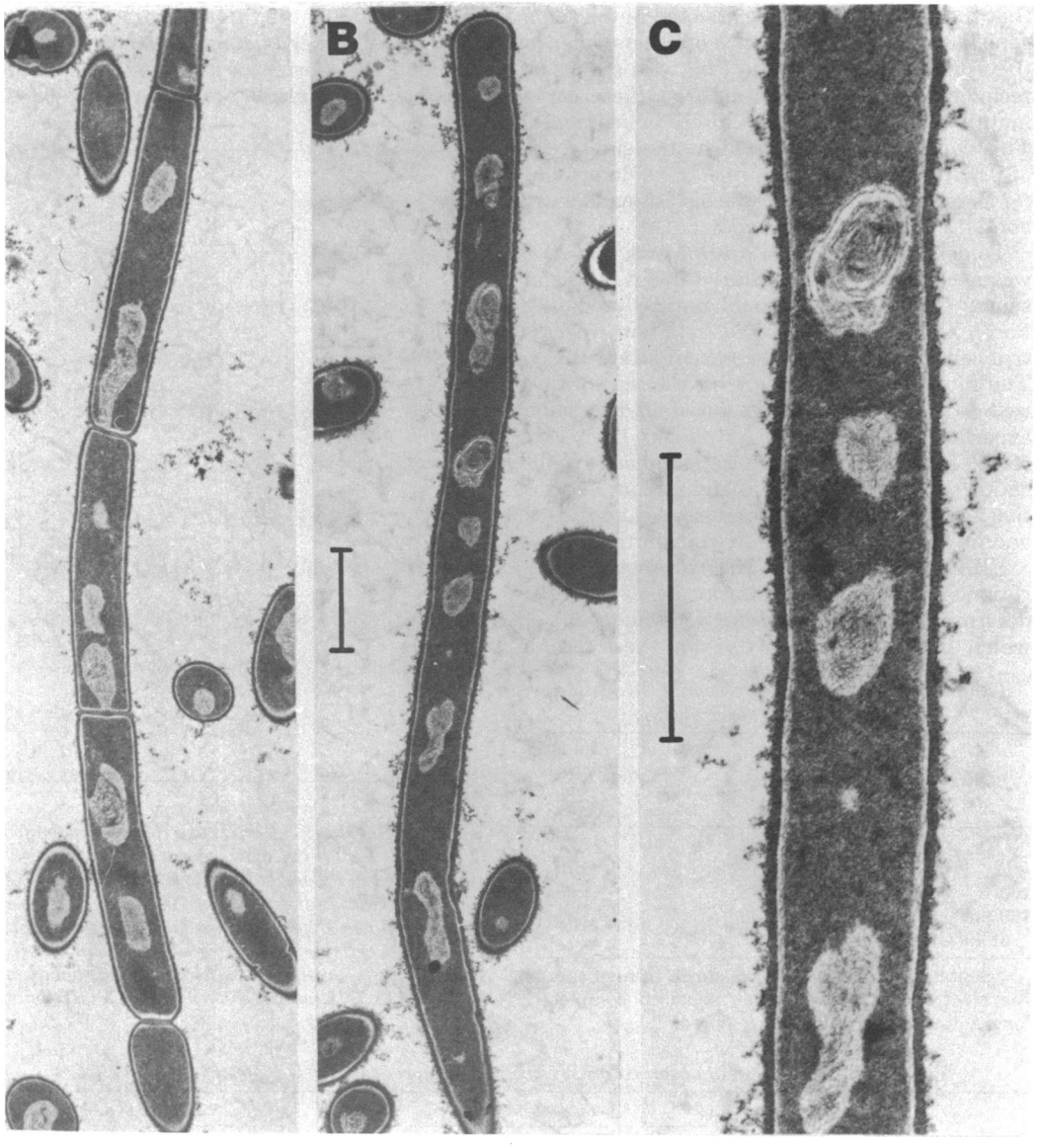

FIG. 3. *Electron micrographs of ts12 (A) at 34°C and (B) after 1 h at 49°C. (C) Higher magnification of central part of filament in B. Bars, 1 μm. A and B are at the same magnification.*

0.129 (25). An RI greater than 0.2 is generally considered to indicate that two mutations are in different genes (6, 11). Although an RI less than 0.1 is usually considered to indicate that two mutations are within the same gene (11), it is possible for mutations in adjacent genes to have very low RI values. For example, the RI of three mutations in the *pyrF* gene with a mutation in the adjacent *pyrD* gene varied from 0.03 to 0.08 (25). So, although RI mapping can suggest that two mutations are located in a single gene, this cannot be determined conclusively on the basis of RI alone.

Besides *ts1* and *ts12*, a number of other filamenting mutants of *B. subtilis* have been proposed as division initiation mutants (21). Of these we have investigated two: *tms-12* (2) and *divD32* (31). The results of mapping these mutations by the RI method are given in Table 4.

The RI of *ts12* with *tms-12* is 0.06, suggesting

that these mutants may be affected in the same gene, although in the absence of other evidence this is not certain. The RI of *ts1* with *tms-12* as recipient was 0.53, indicating that these carry mutations in different but closely linked genes. This conclusion is supported by the similar RI of 0.61 obtained by using *tms-12* as donor and *ts1* as recipient. These results also show that *ts1* and *ts12* are in different genes.

To determine the RI of *divD32* with *tms-12* it was necessary to use vegetative DNA, as clean spores of this mutant could not be prepared. Parental DNA was extracted from both vegetative cells and spores of the parent strain BR77. The ratios given in Table 4 have been normalized to BR77 spore DNA (used in the same experiment), and the RI given has been corrected for the difference in the number of replication positions in parental and mutant vegetative DNA. This RI of 0.95 indicates that *divD32* and *tms-12* are not linked by transformation.

PBS1 transduction. The chromosomal location of these division initiation genes has been determined by PBS1 transduction, the results of which are given in Table 5. From these results and the results of the three-factor cross given in Table 6, the order of markers shown in Fig. 5 was deduced. This order is consistent with previous map positions determined for *tms-12* (10;

TABLE 2. *Ratio of partial to completed septa at time of temperature shift*

Strain	No. of septa counted[a]	No. of partial septa	No. of completed septa	Ratio (partial/completed)
ts1	100	56	44	1.27
ts12	100	44	56	0.79
160	100	58	42	1.38
160 (after 1 h at 49°C)	65	38	27	1.41

[a] Septa were counted from a single thin section in the electron microscope. Only septa in completely longitudinal cell sections were scored.

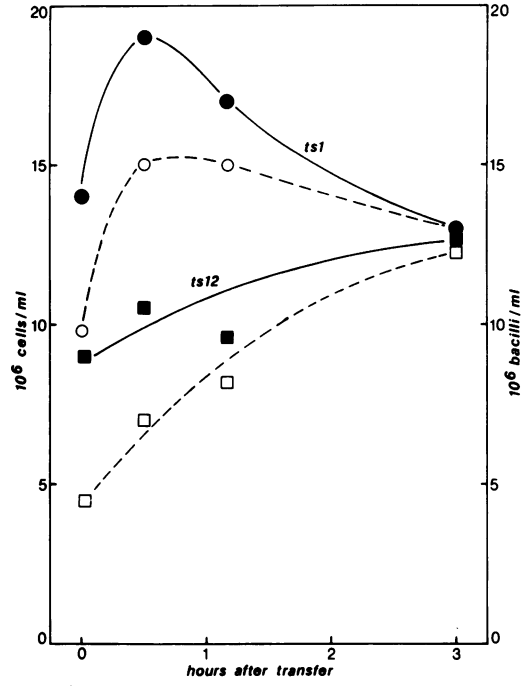

FIG. 4. *Residual cell increase after transfer from 34 to 49°C. At 0 h cultures were transferred to 49°C as described in the legend to Fig. 1, and samples were taken into an equal volume of 20% Formalin at appropriate times. The definition and measurement of bacilli and cells per milliliter are given in the text. Symbols: ●, ts1, cells per milliliter; ○, ts1, bacilli per milliliter; ■, ts12, cells per milliliter; □, ts12, bacilli per milliliter.*

TABLE 3. *Comparison of observed and residual cell increase after transfer from 34 to 49°C*

Strain	34°C (at time of transfer)					49°C		
	Bacilli per ml (10^6)[a]	Visible septa per bacillus[a]	Cells per ml (10^6)[b]	Visible septa per ml (10^6)[c]	Partial septa per ml (10^6)[d]	Maximum cells per ml (10^6)[a]	Observed cell increase (%)[e]	Predicted cell increase (%)[f]
ts1	9.78	0.42	13.89	4.11	5.23	18.80	35	38
ts12	4.56	1.00	9.12	4.56	3.58	12.63	38	39

[a] Definitions of bacillus and cell and measurement of bacilli and visible septa per milliliter are described in the text.

[b] Cells per milliliter = bacilli per milliliter × (1 + visible septa per bacillus).

[c] Visible septa per milliliter = bacilli per milliliter × visible septa per bacillus.

[d] Partial septa per milliliter = visible septa per milliliter × ratio of partial to completed septa (Table 2).

[e] Observed cell increase = $\frac{\text{maximum cells per milliliter} - \text{cells per milliliter at time of transfer}}{\text{cells per milliliter at time of transfer}} \times 100.$

[f] Predicted cell increase = $\frac{\text{calculated partial septa per milliliter at time of transfer}}{\text{cells per milliliter at time of transfer}} \times 100.$

the experimental data have not been published for this mutant) and for *divD32* (31). Although our mapping data place *divD32* somewhat closer to the *pyrD* locus than do previously published data (31), this probably reflects the variation normally seen in cotransduction frequencies from different laboratories. However, the relative map positions of *ts1*, *ts12*, and *divD32* should not be subject to such variation. Also, the high number of recombinants examined in the present work would make the map distances in Fig. 5 reliable.

Other transduction experiments (data not shown) indicate that the orientation of the *fur-pyr-cys* region given in Fig. 5, which has been a matter of some debate (10, 29, 36, 37), is correct. It is of interest to note that conversion of recombination values to *t* values gives good additivity between the closer markers, as expected if *t* is a meaningful measurement of physical distance (10). The additivity is not as good for more distant markers, such as *metC*, because of the considerable error in measuring cotransduction over such large distances.

TABLE 4. *Recombination index mapping of proposed division initiation mutations by using tms-12 as recipient*

Donor DNA	Ratios normalized to parental spore DNA		RI[a]
	ade$^+$/*met*$^+$	*ts*$^+$/*ade*$^+$	
ts12 (spore)	1.01	0.06 ± 0.01	0.06 ± 0.01[b]
ts1 (spore)	1.01	0.53 ± 0.09	0.53 ± 0.09[b, c]
divD32 (vegetative)	4.12	0.37	0.95[d]
BR77 (vegetative)	3.42	0.44	

[a] RI was determined as described in the text.
[b] Mean of four experiments ± average deviation.
[c] When *ts1* was used as recipient with *tms-12* and *tms-12*$^+$ spore DNA, the RI (*ts*$^+$/*trp*$^+$) was 0.61 ± 0.04 (mean of three experiments ± average deviation).
[d] Corrected RI calculated as in the text by using ratios normalized to BR77 spore DNA.

DISCUSSION

By using a combination of electron microscopy and phase-contrast microscopy to estimate the number of divisions in progress at the time of a temperature shift and by comparing the observed residual cell increase with that expected for this number of divisions, we have been able to establish that *ts1* and *ts12* are division initiation mutants. At present they are classified as GspIV mutants, that is, mutants unable to form a septum after spore germination (24, 36). However, since the results presented here were obtained by using filaments derived from vegetative cells, they should now be classified as DivI mutants as has already been proposed (21).

In the present work it was found that septa are not visible in the light (phase-contrast) microscope until virtually completed. This makes it imperative that electron microscopy be used

TABLE 6. *Three-factor transduction cross to map ts12*[a]

Recipient	Selected marker	Recombinants		Suggested order
		Class	No.	
MB50 (*pyrD1 cysC7*)	*cys*$^+$	*cys*$^+$ *pyr ts*$^+$	72	*cys-pyr-ts*
		cys$^+$ *pyr ts*	23	
		cys$^+$ *pyr*$^+$ *ts*$^+$	282	
		cys$^+$ *pyr*$^+$ *ts*	980	

[a] A PBS1 lysate of *ts12* was used to transduce MB50 to *cys*$^+$. Recombinants were patched onto the same selective medium and then replica-plated onto four plates, two containing and two without uracil. One of each type was then incubated at 48°C, and the other at 30°C.

TABLE 5. *PBS1 transduction mapping of initiation mutations*

Donor	Recipient	Selected marker	Total no. of recombinants examined	No. of recombinants which were also temperature sensitive	% of recombination	100 × *t* (%)
ts12	Kit3	*met*$^+$	521	30	94	68.0
	Kit4	*pyr*$^+$	1,395	1,053	25	6.6
	MB50	*cys*$^+$	1,455	1,079	26	7.1
ts1	Kit3	*met*$^+$	681	22	97	75.7
	Kit4	*pyr*$^+$	1,391	1,077	23	5.9
	MB50	*cys*$^+$	235	176	25	6.8
divD32	Kit3	*met*$^+$	228	2	99	87.0
	Kit4	*pyr*$^+$	1,517	1,348	11	2.3
	MB50	*cys*$^+$	103	85	17	4.2

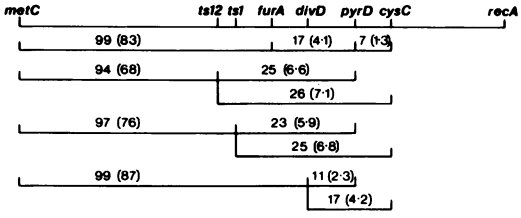

FIG. 5. *Map of the metC-recA region of B. subtilis. Distances between markers are given as percentage of recombination and within parentheses as percentage length of the transducing fragment (100 × t).*

to determine the absence of partial septa from filaments. It is also needed to establish that filaments contain no membrane or wall aberrations such as occur in *B. subtilis divC*, another filamenting strain (31).

Very few *E. coli* filamenting mutants have been examined by electron microscopy (3, 22, 33, 38). Although partial septa have not been observed, most of these mutants stop division immediately on transfer to the nonpermissive temperature, a behavior more consistent with mutations in septal growth rather than initiation (27). A possible exception is *E. coli ftsZ* (strain PAT84, formerly *ftsA*, [17]), for which both immediate stop behavior (3) and a small residual increase (33) have been reported. However, as the number of divisions in progress at the time of transfer was not known for either this mutant or for *ts52* (a mutant which shows a very high residual increase [38]), it is not certain that inhibition of division occurred at initiation. Since the disappearance of septa without any comparable rise in cell number has been reported in *E. coli* (23, 33, 35), such results must be interpreted with caution. Although the proposed initiation mutants of *B. subtilis* (21) have all been examined by electron microscopy and have residual cell increases consistent with this classification, the results presented here are the first to demonstrate that all partial septa present in a mutant at the time of a temperature-shift are subsequently completed.

From the RI mapping we have shown that there are at least two closely linked genes, represented by *ts1* and *ts12*, involved specifically in the initiation of division. There is a high probability that another division mutation, *tms-12*, maps in the same gene as *ts12*, whereas a fourth mutation, *divD32*, is not linked by transformation to the other three. Therefore, there are at least three division initiation genes in *B. subtilis*.

These three genes map in a single region of the chromosome (Fig. 5), about 70% of the distance from origin to terminus (10). There is also a minicell-forming mutation, *divIV-A1*, which maps in this region (26), but because of the difficulty in collating mapping data from different laboratories (10) its position relative to the mutations mapped in this work is uncertain. However, a comparison of the available data for markers in this region (8, 26, 31) with the results presented here suggests that *divIV-A1* maps very close to *divD32*. During exponential growth at the permissive temperature, *divD32* has cells much longer than normal, which may indicate that the mutation under these conditions influences the timing of initiation (31). This may be related to the high degree of division suppression seen in *divIV-A1* (20). The *rodB* marker, which has a glutamate-reversible effect on cell shape, has also been placed in this region on the current *B. subtilis* map (10), but this position is incorrect. This marker cotransduces with both *pheA12* and *leuA8* (13), mapping very close to the *attϕ105* and *divIV-B* markers on the opposite arm of the chromosome to the division genes mapped here (26).

It is possible that such an organization and location of these division genes may be of importance in the control of initiation of septation. Experiments to investigate the relationship between the cell cycle and the action of the *ts1* and *ts12* gene products are now being performed.

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LITERATURE CITED

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81**:741–746.
2. **Breakefield, X. O., and O. E. Landman.** 1973. Temperature-sensitive divisionless mutant of *Bacillus subtilis* defective in the initiation of septation. J. Bacteriol. **113**: 985–998.
3. **Burdett, I. D. J., and R. G. E. Murray.** 1974. Septum formation in *Escherichia coli*: characterization of septal structure and effects of antibiotics on cell division. J. Bacteriol. **119**:303–324.
4. **Callister, H., and R. G. Wake.** 1974. Completed chromosomes in thymine-requiring *Bacillus subtilis* spores. J. Bacteriol. **120**:579–582.
5. **Callister, H., and R. G. Wake.** 1977. Completion of the replication and division cycle in temperature-sensitive DNA initiation mutants of *Bacillus subtilis* 168 at the non-permissive temperature. J. Mol. Biol. **117**:71–84.
6. **Carlton, B. C.** 1966. Fine-structure mapping by transformation in the tryptophan region of *Bacillus subtilis*. J. Bacteriol. **91**:1795–1803.
7. **Coote, J. G.** 1972. Sporulation in *Bacillus subtilis*. Genetic analysis of oligosporogenous mutants. J. Gen. Microbiol. **71**:17–27.
8. **Dubnau, D., C. Goldthwaite, I. Smith, and J. Marmur.** 1967. Genetic mapping in *Bacillus subtilis*. J. Mol. Biol. **27**:163–185.
9. **Helmstetter, C. E., O. Pierucci, M. Weinberger, M.**

Holmes, and M.-S. Tang. 1979. Control of cell division in *Escherichia coli*, p. 517-579. *In* J. R. Sokatch, L. N. Ornston, and I. C. Gunsalus (ed.), The bacteria: a treatise on structure and function, vol. VII. Mechanism of adaptation. Academic Press, Inc., New York.

10. **Henner, D. J., and J. A. Hoch.** 1980. The *Bacillus subtilis* chromosome. Microbiol. Rev. **44**:57-82.
11. **Imada, S., L. E. Carroll, and N. Sueoka.** 1980. Genetic mapping of a group of temperature-sensitive *dna* initiation mutants in *Bacillus subtilis*. Genetics **94**:809-823.
12. **Karamata, D., and J. D. Gross.** 1970. Isolation and genetic analysis of temperature-sensitive mutants of *B. subtilis* defective in DNA synthesis. Mol. Gen. Genet. **180**:277-287.
13. **Karamata, D., M. McConnell, and H. J. Rogers.** 1972. Mapping of *rod* mutants of *Bacillus subtilis*. J. Bacteriol. **111**:73-79.
14. **Kelleher, R. J., Jr., and H. Gooder.** 1973. Genetics and biochemistry of pyrimidine biosynthesis in *Bacillus subtilis*: linkage between mutations resulting in a requirement for uracil. J. Bacteriol. **116**:577-581.
15. **Kellenberger, E., A. Ryter, and J. Séchaud.** 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4**:671-678.
16. **Kemper, J.** 1974. Gene order and cotransduction in the *leu-arg-fol-pyrA* region of the *Salmonella typhimurium* linkage map. J. Bacteriol. **117**:94-99.
17. **Lutkenhaus, J. F., H. Wolf-Watz, and W. D. Donachie.** 1980. Organization of genes in the *ftsA-envA* region of the *Escherichia coli* genetic map and identification of a new *fts* locus (*ftsZ*). J. Bacteriol. **142**:615-620.
18. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**: 208-218.
19. **McGinness, T., and R. G. Wake.** 1979. Completed *Bacillus subtilis* nucleoid as a doublet structure. J. Bacteriol. **140**:730-733.
20. **Mendelson, N. H.** 1975. Cell division suppression in the *Bacillus subtilis divIV-A1* minicell-producing mutant. J. Bacteriol. **121**:1166-1172.
21. **Mendelson, N. H.** 1977. Cell growth and division: a genetic viewpoint, p.5-24. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
22. **Nagai, K., and G. Tamura.** 1972. Mutant of *Escherichia coli* with thermosensitive protein in the process of cellular division. J. Bacteriol. **112**:959-966.
23. **Normark, S., L. Norlander, T. Grundström, G. D. Bloom, P. Boquet, and G. Frelat.** 1976. Septum formation-defective mutant of *Escherichia coli*. J. Bacteriol. **128**:401-412.
24. **Nukushina, J.-I., and Y. Ikeda.** 1969. Genetic analysis of the developmental processes during germination and outgrowth of *Bacillus subtilis* spores with temperature-sensitive mutants. Genetics **63**:63-74.
25. **Potvin, B. W., R. J. Kelleher, Jr., and H. Gooder.** 1975. Pyrimidine biosynthetic pathway of *Bacillus subtilis* J. Bacteriol. **123**:604-615.
26. **Reeve, J. N., N. H. Mendelson, S. I. Coyne, L. L. Hallock, and R. M. Cole.** 1973. Minicells of *Bacillus subtilis*. J. Bacteriol. **114**:860-873.
27. **Slater, M., and M. Schaechter.** 1974. Control of cell division in bacteria. Bacteriol. Rev. **38**:199-221.
28. **Sueoka, N., and H. Yoshikawa.** 1965. The chromosome of *Bacillus subtilis*. I. Theory of marker frequency analysis. Genetics **52**:747-757.
29. **Uehara, H., K. Yamane, and B. Maruo.** 1979. Thermosensitive, extracellular neutral proteases in *Bacillus subtilis*: isolation, characterization, and genetics. J. Bacteriol. **139**:583-590.
30. **Upcroft, P., H. J. Dyson, and R. G. Wake.** 1975. Characteristics of *Bacillus subtilis* W23 mutant temperature sensitive for initiation of chromosome replication. J. Bacteriol. **121**:121-127.
31. **van Alstyne, D., and M. I. Simon.** 1971. Division mutants of *Bacillus subtilis*: isolation and PSB1 transduction of division-specific markers. J. Bacteriol. **108**:1366-1379.
32. **Wake, R. G.** 1967. A study of the possible extent of synthesis of repair DNA during germination of *Bacillus subtilis* spores. J. Mol. Biol. **25**:217-234.
33. **Walker, J. R., A. Kovarik, J. S. Allen, and R. A. Gustafson.** 1975. Regulation of bacterial cell division: temperature-sensitive mutants of *Escherichia coli* that are defective in septum formation. J. Bacteriol. **123**: 693-703.
34. **Whitehouse, R. L. S., J.-C. Benichou, and A. Ryter.** 1977. Procedure for the longitudinal orientation of rod-shaped bacteria and the production of a high cell density of procaryotic and eucaryotic cells in thin sections for electron microscopy. Biol. Cellulaire **30**:155-158.
35. **Wolf-Watz, H., and S. Normark.** 1976. Evidence for a role of *N*-acetylmuramyl-L-alanine amidase in septum separation in *Escherichia coli*. J. Bacteriol. **128**:580-586.
36 **Young, F. E., and G. A. Wilson.** 1976. Revision of the linkage map of *Bacillus subtilis*, p. 686-703. *In* G. D. Fasman (ed.), Handbook of biochemistry and molecular biology, 3rd ed., section B, vol. II. CRC Press, Cleveland, Ohio.
37. **Young, M.** 1975. Genetic mapping of sporulation operons in *Bacillus subtilis* using a thermosensitive sporulation mutant. J. Bacteriol. **122**:1109-1116.
38. **Zusman, D. R., M. Inouye, and A. B. Pardee.** 1972. Cell division in *Escherichia coli*: evidence for regulation of septation by effector molecules. J. Mol. Biol. **69**:119-136.