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Completed Chromosomes in Thymine-Requiring *Bacillus subtilis* Spores

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Origin:terminus genetic marker ratios (both *purA*: *metB* and *purA*:*ilvA*) were measured in extracts of spores of *Bacillus subtilis* strains W23 *thy his* and 168 *thy*. For strain W23 *thy his*, normalized to W23 spore deoxyribonucleic acid, both ratios were equal to unity and were consistent with the presence of only completed chromosomes in the spores. The same ratios in extracts of spores of 168 *thy*, normalized to strain 168 or the prototroph SB19, were abnormal, i.e., 2.26 ± 0.10 and 0.71 ± 0.06 for *purA*:*metB* and *purA*:*ilvA*, respectively. These values were unaffected by the extent of extraction of the spore deoxyribonucleic acid, the richness of the medium on which they are formed, and the thymine phenotype. The high ratio for *purA*:*metB* is in agreement with the results of earlier workers but, because of the low *purA*:*ilvA* ratio, cannot be explained simply by the presence of partially replicated chromosomes in spores of strain 168 *thy*. Furthermore, *purA*:*leuA* in such extracts is 1.01 ± 0.06, consistent with the presence of only completed chromosomes. It is concluded that the abnormal origin:terminus marker ratios are only apparent and result from non-isogenicity between strains 168 *thy* and 168 in the *metB thyB ilvA* chromosome region introduced during construction of 168 *thy* by transformation of strain 168 with W23 *thy* deoxyribonucleic acid. It is concluded further that the chromosomes of strain 168 *thy* spores are in a completed form.

The thymine-requiring mutants of *Bacillus subtilis* strains W23 and 168 isolated by Farmer and Rothman (3) have been used widely in studies on deoxyribonucleic acid (DNA) replication. Spores of the thymine-independent strains contain completed chromosomes (6); i.e., replication forks are absent. Therefore considerable use has been made of germinating spores of both the thymine-independent and thymine-requiring strains to attain synchrony in initiation of chromosome replication.

Recently, Ephrati-Elizur and Borenstein (2) measured, by transformation, the ratio of origin to terminus (*purA*:*metB*) genetic markers in spores of *B. subtilis* 168 *thy* and found a value close to 2, not one as would be expected for completed chromosomes. The ratio is consistent with the presence of partially replicated chromosomes in the spore. Measurements of the relative frequency of other markers in germinated spore extracts suggested that, within the spore population, the forks were distributed along the chromosome as in the case of an exponentially growing culture.

In this paper we conclude, from measurements of the relative frequency of various markers, that chromosomes in spores of the thymine-requiring W23 strain are in a completed form. In addition, we show that the high *purA*:*metB* ratio obtained for spores of the thymine-requiring 168 strain does not reflect the presence of replication forks. It can be accounted for by an alteration in the region of the chromosome near the *thyB* gene, which is linked to *metB*, and introduced when the thymine auxotroph is constructed by transformation of strain 168 with W23 *thy* DNA. We conclude that thymine-requiring 168 spores contain only completed chromosomes.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* strains W23 and W23 *thy his* were obtained from N. Sueoka. Strains of 168 and 168 *thy* were provided by J. L. Farmer. SB 19 (prototroph derived from 168 [*trpC*]) and additional samples of 168 and 168 *thy* were obtained from the Genetics Department, Stanford University, Stanford, Calif. (It should be noted that all the *thy* strains originated from Farmer and Rothman). J. Marmur provided BD 72 (168 *purA leuA metB ilvA*), the recipient strain for transformation. All gene designations are according to Young and Wilson (14).

Spore preparations. Spores of all strains were prepared on plates of potato extract in Spizizen medium (5) supplemented with required amino acids (50 μg/ml), of thymine (20 μg/ml), or both. They were

collected after 4 to 6 days of growth at 37 C and cleaned by lysozyme and detergent treatment followed by exhaustive washing in water (6). Spores of strain 168 *thy* were also prepared on solid Schaeffer medium (10) and collected after growth at 37 C for 28 h.

Extraction of DNA. DNA was extracted after the spores were sensitized to lysozyme by treatment with thioglycolic acid and urea according to the method of Sakakibara et al. (8). Lysozyme (500 μg/ml) was added to the washed, treated spores (5×10^9/ml) in 0.1 M NaCl-0.01 M ethylenediaminetetraacetate (pH 8) and incubated at 37 C for 1 h. Sodium lauryl sulphate (1% wt/vol final concentration) was added, and the suspension was heated at 60 C for 10 min. After the solution was cooled to room temperature, sodium perchlorate (final concentration 1 M) was added, and the mixture was shaken with an equal volume of $CHCl_3$-isoamyl alcohol (24:1) for 30 min. After centrifugation, the clear aqueous layer was collected and dialyzed exhaustively against 0.15 M NaCl-0.015 M sodium citrate (pH 7) at 4 C.

The extract contained both DNA and ribonucleic acid (RNA). The extent of DNA extraction was not measured directly. However, from the absorbancy of the extract at 260 nm, in addition to the known spore DNA content (4) and DNA:RNA ratio (12), the extent of the various DNA extractions was calculated to be in the range of 3 to 20%.

Transformation. Competent BD 72 cells were prepared and frozen as described by Strauss and Marone (9). The concentration of extract mixed with the recipient cells was such as to give a linear response with respect to the number of transformants obtained. After incubation of cells plus extract for 40 min at 37 C, deoxyribonuclease (20 μg/ml) was added. After 10 min, 0.10-ml samples were spread in triplicate on appropriate plates. Controls consisted of DNA extracts treated with deoxyribonuclease before incubation with recipient cells. In the majority of cases, 200 to 700 transformants of each class were counted for each extract in any experiment.

RESULTS

Completed chromosomes in spores of B. subtilis W23 thy his. By transformation, the ratios *purA:metB* and *purA:ilvA* in extracts of W23 *thy his* spores, normalized to values of 1.00 for W23 extracts, were found to be 0.90 ± 0.10 and 1.05 ± 0.01, respectively (means of two separate measurements in each case). Spores of these two strains are thus not significantly different with respect to the ratios of these particular pairs of markers. *purA* is very close to the origin of replication, and both *metB* and *ilvA* are near the terminus (7; see Fig. 1). Thus, one can conclude that, as in the case of strain W23, spores of W23 *thy his* contain only completed chromosomes.

Configuration of chromosomes in spores of B. subtilis 168 thy. The *purA:metB* ratios in extracts of various preparations of 168 *thy* spores were measured (Tables 1 and 2). In all cases they were the same, just greater than 2, when normalized to the ratio in extracts of spores of either strain 168 or the prototroph SB 19. Over all measurements, the ratio is remarkably constant, 2.26 ± 0.10 (mean of 10 measurements ± average deviation). Neither differences in the richness of the medium for preparation of spores—potato extract in Spizizen medium versus Schaeffer medium—nor in the extent of DNA extraction from the spores—a minimum of 3% [168 *thy* (3)] versus a maximum of 20% [168 *thy* (2)]—has any effect on this ratio. The strains of 168 and 168 *thy* used had been kept among our stocks and subcultured over several years (Table 1). Equivalent strains used for the

TABLE 1. *Marker ratios in extracts of spores of various B. subtilis 168 thymine-requiring and independent strains*[a]

Ratio measured	Marker ratio of spore preparation:				
	168 (1)	SB 19	168 *thy* (1)	168 *thy* (2)	168 *thy*$^{+}$[b]
purA:metB		*1.00*		2.26	
		1.00	2.10	2.10	
	1.00	1.10	2.62	2.22	2.35
	1.00		2.16		2.15
purA:ilvA		*1.00*	0.69	0.69	
		1.00	0.68	0.72	
	1.00	1.10	0.66	0.78	0.59
	1.00		0.54		0.50
purA:leuA		*1.00*		0.98	
		1.00	1.10	0.95	

[a] Each line across shows the results of a separate lot of measurements. The value *1.00* indicates that the other ratios on the same line have been normalized to it in that particular experiment.

[b] 168 *thy*$^{+}$ is a spontaneous revertant of 168 *thy* to thymine independence.

TABLE 2. *Marker ratios in extracts of spores of various B. subtilis 168 strains prepared on different media*[a]

Ratio measured	Marker ratio of spore preparation:		
	168 (2)	168 *thy* (3)	168 *thy* (4)
purA:metB	*1.00*	2.29	2.35
	1.00	2.25	2.30
purA:ilvA	*1.00*	0.68	0.76
	1.00	0.76	0.83

[a] Strain 168 (2) and 168 *thy* (3) were grown on solid potato extract in Spizizen medium; 168 *thy* (4) was grown on solid Schaeffer medium. Each line across shows the results of a separate lot of measurements.

spore preparations from strains 168 (2) and 168 *thy* (3) were obtained recently from J. L. Farmer (Table 2). There is no difference in the two lots of data. Finally, reversion of strain 168 *thy* to *thy*$^+$ has no effect on the *purA*:*metB* ratio in spore extracts (Table 1); so one cannot account for the high value simply as being a result of the thymine phenotype.

The explanation offered by Ephrati-Eluzur and Borenstein (2) for the high *purA*:*metB* ratio—that the chromosomes of 168 *thy* spores are partially replicated—is difficult to reconcile with the constant amount of DNA found in spores of all strains of *B. subtilis*, including 168 and 168 *thy* (1, 4, 8). We, therefore, tested more rigorously the possibility of there being partially replicated chromosomes in spores of strain 168 *thy*.

Figure 1 shows the replication order, established by O'Sullivan and Sueoka (7) by density transfer experiments, of some particular markers in *B. subtilis*. *ilvA* replicates after *metB*; thus, if the value of 2.26 for *purA*:*metB* reflects simply the presence of replication forks between the origin and terminus, the *purA*:*ilvA* ratio should be similarly high. The data in Tables 1 and 2 show that, when normalized to the ratio in 168 or SB 19 spores, the value for 168 *thy* is actually lower than 1.00, i.e., 0.71 ± 0.06 (11 measurements). Thus, one cannot explain the apparently high *purA*:*metB* ratio in terms of partially replicated chromosomes.

What is the cause of the normalized ratio of *purA* to both *metB* and *ilvA* being significantly different from 1.00? Table 1 shows also the ratio of *purA* to a mid-replication order marker, *leuA*, to be 1.01 ± 0.06 (three determinations). This would be consistent with the presence of completed chromosomes in spores of strain 168 *thy* and raises the possibility that the *metB* and *ilvA* genes in strain 168 *thy* are different from those in 168 and SB 19.

The procedure of Farmer and Rothman (3) for construction of the *thy* auxotroph of strain 168 involved transformation by DNA from strain W23 *thy*, the latter being a spontaneous thymine-requiring mutant of W23. The Thy phenotype has since been shown to result from mutations in two genes, *thyA* and *thyB* (11). Mutation in only one of these two results in the Thy$^+$ phenotype. Thus, strain 168 *thy* is actually 168 *thyA thyB*. It is probable that, during integration of *thyA* and *thyB* from strain W23 *thy* DNA into the 168 chromosome, changes in genes close to and on both sides of these loci were also introduced. By coincidence, *thyB* has been shown by transformation to be linked to both *metB* and *ilvA* (11); it is actually located between them. Double transformants for both *metB* and *ilvA* can be obtained readily (11, 13), and it is therefore likely that a significant portion, or perhaps even all, of the *metB thyB ilvA* region originates from strain W23 *thy*.

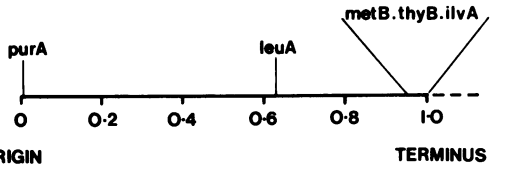

FIG. 1. *Replication order map of Bacillus subtilis, based largely on the analyses of O'Sullivan and Sueoka (7). thyB has been shown by transformation to be linked to both metB and ilvA, and is located between them (11).*

Such a situation, that in strain 168 *thy* a significant portion of the chromosome in the *metB thyB ilvA* region is derived from W23, could account for all the results on genetic marker ratios obtained here. In particular, it would explain fully the apparently abnormal *purA*:*metB* and *purA*:*ilvA* ratios in conjunction with a normal *purA*:*leuA* ratio. Confirmation that the *metB* and *ilvA* genes in strain 168 *thy* are more similar to those in W23 than in 168 has been obtained by comparing the *metB*:*ilvA* ratios in the various strains. The ratio in spore extracts of strain 168 *thy*, normalized to 168, is very low, i.e., 0.31 (calculated from the data in Tables 1 and 2). In an additional experiment, using triplicate samples throughout, the same ratio, normalized to a W23 spore extract, was found to be much closer to unity, actually 0.79.

In a separate experiment, we showed directly that the numbers of *metB*$^+$ and *ilvA*$^+$ transformants obtained when 168 *thy* was the donor were both independent of added thymine. Thus, the unusual ratios of *purA* to both *metB* and *ilvA* in strain 168 *thy* cannot be due simply to some effect mediated by expression of the *thyB* gene, which would be integrated to a significant extent with both *metB*$^+$ and *ilvA*$^+$ in such transformants.

DISCUSSION

The data presented here are consistent with the presence of only completed chromosomes in spores of *B. subtilis* strains W23 *thy his* and 168 *thy*. Normal origin:terminus marker ratios, indicative of such completion, were obtained when extracts of W23 *thy his* spores were compared with W23. The abnormal ratios obtained when the comparison was between spores of 168 *thy* and 168 cannot be interpreted as being due to the presence of partially replicated chromosomes. This is most obvious from the

inconsistency in the normalized ratios of *purA*:*metB* and *purA*:*ilvA*. Because *metB* and *ilvA* are linked and very near the terminus, the ratios, to be indicative of partially replicated chromosomes, should have both been >1.00 and of approximately the same value, but they were, respectively, 2.26 and 0.71. Furthermore, the ratio of *purA* to a mid-replication marker, *leuA* (Fig. 1), was consistent with the presence of completed chromosomes in 168 *thy* spores.

The apparently abnormal ratios of *purA* to both *metB* and *ilvA* in 168 *thy* spores were shown not to be dependent upon the extent of DNA extraction, the richness of the medium on which the spores formed, or the thymine phenotype. The variance of the ratios from unity can be explained after taking into account the method of construction of the 168 *thy* strain, transformation of 168 with W23 *thy* DNA. Because of the necessity for mutations in two genes, *thyA* and *thyB*, for thymine dependence and the linkage of *thyB* to both *metB* and *ilvA*, part or all of the *metB thyB ilvA* region of the chromosome in strain 168 *thy* would have originated from W23. Comparison of *metB*:*ilvA* ratios in the various strains confirmed this. It is concluded that non-isogenicity between strain 168 *thy* and 168 within the *metB thyB ilvA* region of their chromosomes is the cause of the apparently abnormal origin:terminus marker ratios observed here and elsewhere (2). Furthermore, the difference in the method of introduction of the thymine requirement to give W23 *thy his* (derived from W23 *thy*, which is a *spontaneous* mutant of W23) explains why, on the other hand, the origin:terminus marker ratios in spores of this strain, normalized to W23, are normal, i.e., equal to unity.

The reason for the high *purA*:*leuA* and *purA*:*hisA* ratios in spores of strain 168 *thy* found by previous workers (2), and which have been used as supporting evidence for the presence of partially replicated chromosomes, is not immediately obvious. But the fact that these particular ratios were measured only in extracts of germinated, not ungerminated, spores should be kept in mind. It could be that some replication of the *purA* marker occurred under the germination conditions used.

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