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Division Blocks in Temperature-Sensitive Mutants of *Streptococcus faecium* (*S. faecalis* ATCC 9790)

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Two hundred nine temperature-sensitive growth or division (or both) mutants of *Streptococcus faecium* ATCC 9790 were isolated. These strains were examined for timing of the division block in the cell division cycle. About 42% of the isolates were blocked at terminal stages of cell division. A second large group appeared to be blocked at various stages of septation. Only five of the temperature-sensitive isolates were blocked at a stage before the completion of chromosome replication. Thirty temperature-sensitive isolates lysed after one or more doublings at the nonpermissive temperature.

Bacterial cell division mutants have been classified according to a variety of genetic, biochemical, and morphological criteria (9, 14, 16, 18, 21). However, systematic studies of the relationship between division blocks in mutants and the timing of cell division cycle events have not been done. A useful method to estimate timing of the division block assumes that in a population of randomly dividing exponential-phase cells, only cells that are past a division block are capable of division (4, 7). Therefore, by examining the number of residual divisions occurring in a culture after induction of a block, the timing of the block in the cell division cycle can be estimated. For example, with inhibitors of DNA synthesis, the method has been used to derive the timing between completion of chromosome replication and cell division (4, 7, 10, 17). Other inhibitors have been used to establish cell division requirements (3, 10, 17).

In this study we have screened 209 temperature-sensitive division or growth (or both) mutants of *Streptococcus faecium* ATCC 9790 (*S. faecalis*) for residual divisions after a shift from the permissive to the nonpermissive temperature. In parallel, the wild type and randomly selected mutants were exposed to various antibiotics known to inhibit cell division in *S. faecium* (7, 10). Analysis of the data permits estimates of the timing of the various temperature-sensitive blocks within the cell division cycle. Studies of cell division in streptococci are of interest, since the cells divide in a single plane and form new poles semiconservatively (reviewed in reference 7). The formation of division poles is uncomplicated by the process of cylindrical rod elongation. In this system, pole formation is responsible for both surface enlargement and septation.

MATERIALS AND METHODS

Mutant selection. The mutants screened were of two kinds; 132 were selected after nitrosoguanidine mutagenesis (NT1 and NT2) as described previously (6), and the remaining 77 mutants were spontaneous (SP). For the selection procedure, cells were grown in a complex yeast extract-tryptone medium (SB; 11) at 30°C. At an absorbance at 650 nm of 0.1 the cultures were transferred to a 42°C water bath. Penicillin G (PEN) was added after 2 h at 42°C (25 μg/ml for NT1 and SP, and 125 μg/ml for NT2), and the cultures were incubated further at 42°C until clearing of visible turbidity. The residue was then pelleted by centrifugation, washed once with SB, plated on SB agar, and incubated at 30°C. Colonies were picked and replica plated at 30 and 42°C, and those that failed to produce colonies at 42°C were selected, recloned, retested for temperature sensitivity, and stored on SB agar slants.

Estimations of residual divisions. Screening for residual divisions (r) was performed on cells grown overnight on brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) containing 3% NaCl. An 0.1-ml sample of the overnight culture was transferred to 20 ml of fresh broth and incubated at 30°C to an absorbancy at 650 nm of approximately 0.1. At that time 10 ml of the culture was transferred to 42°C, and both cultures were sampled at 0, 2, and 4 h. Cell counts (N) were obtained with a Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.), and the A_{650} was determined with a Beckman DU spectrophotometer (Beckman Instruments, Inc., Irvine, Calif.). The r value was determined at 4 h as $r = (\ln N/N_0)/\ln 2$, and the number doubling time (t_D) was estimated at 2 h as $t_D = \ln 2/(\ln N/N_0) \times 120$. The minutes of residual divisions (t_R) are the product of $r \times t_D$. We used r or t_R

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in preference to other measures of cell cycle age because doubling times were not always estimated accurately by this procedure. In a separate detailed analysis, the mean t_D of 12 randomly selected mutant cultures grown at 30°C was 37.4 ± 10 min, whereas the t_D of the wild type was 39.5 ± 11.8 min.

Residual divisions were also determined after the addition of chloramphenicol (CAP; 10 μg/ml), mitomycin C (MIT; 0.5 μg/ml), or PEN (0.2 μg/ml) to cultures (*r* values indicated as *r*CAP, *r*MIT, and *r*PEN, respectively). The time of chromosome replication (*C* time) was estimated from the fractional increase in DNA content (7, 10) after the addition of CAP (10 μg/ml) to cultures. The brain heart infusion cultures contained 3% NaCl, [^{3}H]thymidine (5 mCi/ml), and carrier unlabeled thymidine (30 μg/ml) and were precipitated in 10% trichloroacetic acid and counted as described previously (19).

Microscopic examination was performed using 10× concentrated cells fixed in 8.3% Formalin. The cells were air dried and then stained with 0.01% crystal violet for 10 min. The frequency of septated cells (*s*) was determined, and $\bar{p}$ was derived as $\bar{p} = [\ln(1 + s)]/\ln 2$. It can be shown that $\bar{p} = t_S/t_D$, where t_S is the time from completion of the septum to division (7). $t_D \times \bar{p}$ represents the time between septum completion and division. The fraction of the cell cycle required to complete a septum was calculated as SPT = *r*MIT − $\bar{p}$. These relationships are shown schematically in Fig. 1. In each of these cases the minutes between onset of the specific, antibiotic-induced block and division would be the product of the respective *r* value, of SPT, or of $\bar{p}$ and t_D. In Fig. 1 an *r* value of 1 represents one interdivision time (t_D). The cell division cycle from initiation of chromosome replication to cell division (9) is usually longer than t_D. The blocks imposed on cells affect the cell division cycle in progress and may therefore permit divisions over a period of time greater than t_D (e.g., *r* may be greater than 1).

RESULTS

No substantial difference was found in the *r* or t_R values of wild-type cultures exposed to either MIT or CAP after pregrowth at 30°C and incubation after the addition of the antibiotic at either 30 or 42°C (Fig. 2). These results indicate that the *r* and t_R values are essentially the same when incubated after antibiotic addition at either temperature and represent the residual divisions of cells pregrown at a constant exponential rate of growth at 30°C. The t_D values of 30 and 42°C cultures (which were not measured here) differ by a factor of about 2 during balanced exponential-phase growth.

Previously, we discussed (7) the functional identity of t_R determined after MIT addition and the *D* time, as defined by Cooper and Helmstetter (5). The t_R time after CAP addition was defined as a later portion of the *D* interval (*D*2 phase), and is the time when continued protein synthesis is no longer required for division (10). From our observations at 30°C, the time between completion of a visible division septum and division is usually somewhat longer than *D*2 (e.g., $\bar{p}$ is longer than *r*CAP; Fig. 1).

For the wild type the mean *r* value obtained after MIT was 1.24, whereas the mean *r*MIT value for six randomly selected mutants was 1.36 (Table 1). Assuming a mean t_D of 39.5 and 37.4 min for the wild type and mutants at 30°C, respectively, the corresponding t_R values are 49.0 and 50.8 min, respectively, reasonably consistent with a 40-min value obtained previously at 37°C (3). However, the *r* values obtained for the wild type and the mutants after CAP addition were 0.25 and 0.21, respectively (t_R, 9.9 and 7.9 min, respectively), and are somewhat shorter than the 18 to 20 min observed for the wild type at 37°C (3). This observation may indicate that at the lower temperature physiological cell separation is delayed with respect to visible septal closure. SPT, the fraction of the cell cycle required to complete a septum, was 0.70 and 0.78 or 27.5 to 30 min (Table 1), somewhat longer than the 19 min observed with wild-type cells grown at 37°C (3).

Using a low concentration of PEN G (2 μg/ml), sufficient to prevent division of the mutants, but not high enough to cause lysis of wild-type cultures, we obtained an *r* value of 1.79 (t_R, 67 min). The data obtained showed some spread (Table 1), but were somewhat longer than the 60- to 65-min value obtained with a lysis-defective

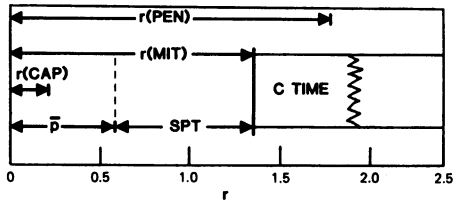

FIG. 1. Example of a cell cycle map for *S. faecium* determined from residual divisions (*r* values) or by frequency of septated cells ($\bar{p}$) and other cell cycle events. The map reads from right to left, where an *r* value of 0 represents division. The product of the *r* × t_D gives the timing of the event in minutes before division. SPT is derived by subtraction of $\bar{p}$ from *r*MIT values as given in Table 1. The map is constructed assuming that certain events in the cell cycle can be inhibited using specific antibiotics. Thus, *r*MIT defines the time between completion of chromosome replication and cell division. *r*CAP defines the time between completion of a requirement for protein synthesis and cell division and should be approximately equivalent, but not necessarily identical, to $\bar{p}$, the time between visible completion of a septum and cell division. Assuming that completion of chromosome replication is required to initiate septum formation, then *r*MIT − $\bar{p}$ gives the time required to complete a septum (SPT). *r*PEN defines the timing between a PEN G-sensitive event and cell division.

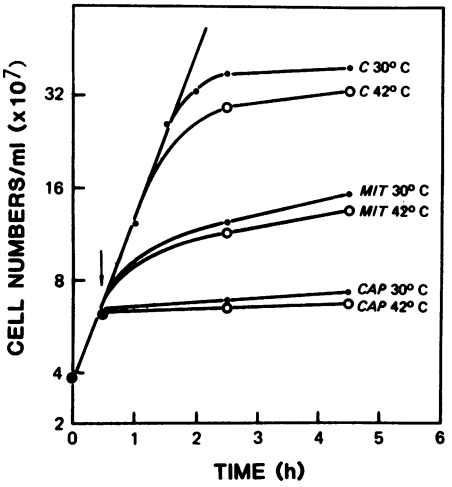

FIG. 2. Determination of r and t_R values for cultures of *S. faecium* ATCC 9790 grown at 30°C for 5 h before the initiation of the experiment. At the arrow the culture was divided into six portions, each of which was incubated at 30 or 42°C either alone (*C*) or with the addition of MIT or CAP. Cell numbers were determined, and r and t_R values were calculated as described in the text.

mutant of *S. faecium* at 37°C (P. Bourbeau and L. Daneo-Moore, unpublished observations).

Also shown in Fig. 3 is an event labeled INIT, representing initiation of chromosome replication (*C* time). The timing of this event was obtained by adding the C time determined for a wild-type culture grown at 30°C to the *D* (or MIT) time of the wild type or mutants. The *C* time value of the 30°C grown cells was 54 min (22; ΔG of 1.56 at t_D of 37.5 min), somewhat longer than the 50- to 52-min value reported elsewhere for cultures growing at 37°C (10, 13). The sequence of events in Fig. 3 should be read from right to left, since the event closest to division has the smallest r value. The events marked DIV in Fig. 3 represent successive divisions at the mean doubling time of the mutants of 37.4 min.

The r values obtained for 107 NT and 72 SP mutants are shown in the histogram in Fig. 3A. A large proportion of the NT and SP mutants (42% for each) appeared affected in a terminal stage of division, with r values of 0.2 or less, corresponding to about 7.5 min of residual division. These mutants are blocked in division almost immediately after the shift to the nonpermissive temperature. Another large group, 53% of the NT and 48% of the SP mutants, were blocked between r values of 0.2 and 1.6 (Fig. 3A), corresponding to the period between completion of chromosome replication (MIT) and completion of physiological cell separation (CAP). A very small group of mutants (1 in the SP series and 4 in the NT series) had r values above 1.6 (Fig. 3A). There seemed to be no difference in the distribution of NT induced to spontaneous mutants in classes 1, 2, or 3, indicating that it is unlikely that we are dealing with multiple mutations. There were substantially more NT than SP mutants in class 4, but the numbers are too small to be statistically significant.

In the initial screening, an additional group of mutants (25 NT and 5 SP) lysed some time after the temperature shift. The parent strain, ATCC 9790, does not lyse under these conditions or even when reaching the stationary phase of growth. To determine accurately the timing of the block, 14 of these mutants were examined in greater detail. Figure 4 shows the effects of a temperature shift on one of these organisms. Division continued for about 90 min followed by a limited, but noticeable loss in cell numbers and in absorbancy at 650 nm (absorbancy data not shown in Fig. 4). The r value determined from the time of the temperature shift to maximal cell number was 2.18. The r and t_R values for several of these lytic mutants are given in Table 2, together with pseudo-first-order reaction rates (k) for absorbance loss. It is clear that most of the blocks in this class occur early in the cell division cycle, as indicated by the large r and t_R values. At least three of these organisms have a t_R value in the INIT region (Fig. 3B). Figure 3B shows data for the lytic mutants expressed in minutes between the block and division.

The defects in division of the various mutants could be seen clearly after staining the cells with crystal violet (Table 3). Mutants with low r values (class 1) did not change the frequency of septated cells after incubation at 42°C. However, mutants with greater r values (classes 2, 3, and 4; Table 3) exhibited greatly reduced fre-

TABLE 1. Mean r and septation values for wild type and six mutants at 30°C[a]

Strain(s)	t_D	rCAP	rMIT	rPEN	p	SPT
Wild type	39.5 ± 11.8	0.25 ± 0.1	1.24 ± 0.1	ND[b]	0.54 ± 0.12	0.70
Mutants	37.4 ± 10.0	0.21 ± 0.1	1.36 ± 0.5	1.79 ± 0.5	0.59 ± 0.01	0.78

[a] r values were obtained from cultures grown at 30°C and treated with each antibiotic for 4 h and are given ±1 standard deviation. Values were obtained using six randomly selected mutants and six wild-type cultures.
[b] ND, Not determined.

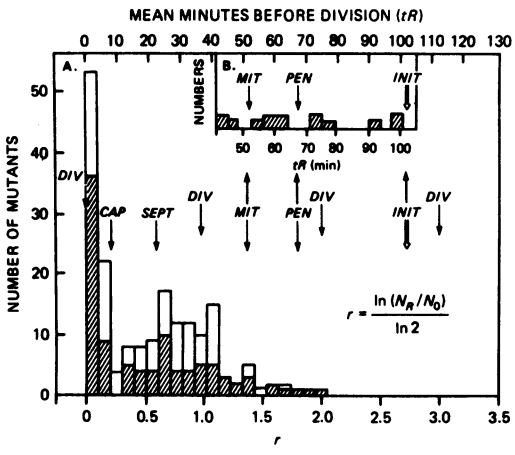

FIG. 3. Numbers of NT (▨) and SP (□) cultures exhibiting various r values. In panel A the various events of cell divisions are also indicated. Also given are mean t_R values in minutes, assuming a mean t_D for mutant cultures of 37.4 min (Table 1). In panel B are shown t_R values for 14 lytic mutants analyzed in some detail (Table 2), where the actual t_D values were determined. The arrows pointing in two directions are for those blocks shared by both panels.

quencies of septated cells after 60 or 120 min at 42°C, indicating blocks before completion of their septa. After 60 min at 42°C, class 3 and 4 mutants contained two to four clearly duplicated nuclei.

In other experiments (data not shown) it was established that when mutants blocked in an event after CAP (Fig. 1) were shifted to 42°C with or without CAP, the residual divisions were identical, e.g., 7 to 8 min. In contrast, when mutants blocked before CAP (Fig. 1) were shifted, the residual divisions obtained in the presence of CAP were always reduced to 7 to 8 min. These results indicate that the CAP event remains operative in mutants blocked at earlier stages.

DISCUSSION

The clusters of mutants shown in Fig. 3 fit reasonably well with our information on cell division cycle events, as determined in *S. faecium* with antibiotics (3, 7) and in synchronously dividing cultures (12, 13). We have discussed elsewhere (7, 20) a model where some 30 min after initiation of chromosome replication there is initiation of a cycle of cell wall biosynthesis in conjunction with activation of the organism's autolytic system. This cell cycle phase, which could correspond to PEN in Fig. 3, contains two types of mutants: a limited number of the mutants shown in Fig. 3A and most of the lytic mutants shown in Table 2 and in Fig. 3B. This group of mutants might include some of the DIV I mutants proposed for *Bacillus subtilis* (16). These are mutants blocked in initiation of cell division.

It would be expected that mutants capable of lysis at the nonpermissive temperature (class 4 in Table 3) would be blocked due to the inability to initiate a site of wall growth at a stage of the division cycle when the autolytic system is activated and before its inhibition at termination of chromosome replication. Only a few cells in the population are affected, as indicated by the only partial lysis observed (see Fig. 4). The rates of lysis of these orgamisms are extremely slow, about 10 to 50 times slower than those observed when *S. faecium* cells are lysing in buffer or in the presence of minimal lytic concentrations of PEN (E. T. Hinks and L. Daneo-Moore, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, A5, p. 1). The slow and limited lysis of these mutants probably accounts for their recovery from the penicillin selection technique. The nonlytic mutants in class 3 (Table 3) may possess a defect in autolysin activation or function.

The cluster of mutants isolated between the MIT-sensitive event (completion of chromosome replication) and the CAP-sensitive event (class 2 in Table 3) appears to be defective in septum closure. However, as might be expected, other mutants blocked at earlier events also fail to complete septa (Table 3). The substantial change in frequency of septated cells seen after incubation at the nonpermissive temperature argues that their defect coincides with, or precedes, septal closure. In Mendelson's classifica-

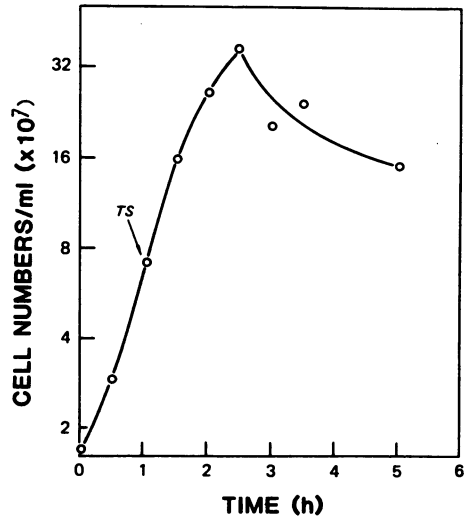

FIG. 4. Effect of a temperature shift from 30 to 42°C (TS; arrow) on cell numbers in a lytic mutant (NT1/119). The r and t_R values calculated were 2.18 and 60 min, respectively.

tion (16), these mutants would be DIV II or DIV III.

Finally, one of the largest classes of mutants (class 1 in Table 3) appears to be blocked in a terminal event of the cell division cycle, after the CAP-sensitive event (Fig. 3). These mutants are equivalent to the DIV VI classification (16), which represents cells with defects in separation. After prolonged incubation at 42°C, a substantial change in the frequency of septated cells was not observed (Table 3). Therefore, the defect appears to be after completion of septum formation, at the stage of daughter cell separation. These mutants may have a temperature-sensitive defect in the expression of an autolytic system that is involved in daughter cell separation. Another possibility, suggested by the reported contractile properties of elongation factor Tu (1, 2), is that a terminal event in bacterial cell division requires a contractile function of Tu. Supporting this view is the large excess of Tu over Ts in bacteria (8); its reported location in association with surface elements (15); and the inhibition of cell division by kirromycin, a specific inhibitor of Tu, late in the cell cycle (L. Daneo-Moore, P. Bourbeau, and D. Carson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I66, p. 95).

The approach used here permits classification of division mutants based on timing of the temperature-sensitive division block. Three of the four classes of mutants reported here appear to be similar to four classes of division mutants found in another gram-positive organism (16). There are three sources of possible inaccuracies in the approach used here. First, cell numbers do not always increase exponentially at 30°C for the 2 to 4 h used in the screening procedure (Fig. 2). A more precise determination of t_D would yield more reliable t_R values, and a timing of cell cycle events in minutes before division for each mutant (r and t_R values presented in Table 2). A second source of inaccuracy arises from the nature of the approach, since the timing at the actual block is determined, rather than the time at which a biochemical event (e.g., inhibition of synthesis of a temperature-sensitive division element) begins. Finally, the method detects only terminal division blocks. If a mutant contains more than one temperature-sensitive block, only the one closest to division will be identified by the screening procedure.

TABLE 2. r and t_R values for 14 lytic mutants

Mutant	t_D (min)[a]	r	t_R (min)	k (h^{-1})[b]
NT1/45	32.70	1.93	63.13	0.211
NT1/83	46.85	1.53	71.80	0.074
NT1/96	29.20	1.83	53.58	0.101
NT1/105	52.80	1.12	59.13	0.077
NT1/106	55.95	1.65	92.30	0.077
NT1/119	27.05	2.18	60.00	0.091
NT1/124	49.27	2.00	98.50	0.178
NT1/130	59.54	1.28	76.20	0.137
NT1/143	33.28	1.30	43.23	0.131
NT2/3	31.30	1.47	46.01	0.061
NT2/13	47.80	0.90	43.03	0.117
NT2/15	56.03	1.09	61.07	0.063
NT2/23	45.40	1.55	70.37	0.172
NT2/29	47.20	2.08	98.17	0.094

[a] The values were obtained every 15 min for a period of several hours. The t_D values were obtained at 30°C. The cultures were then shifted to 42°C to obtain the r, t_R, and k values. The mean t_D for these lytic mutants (48.38 ± 14.14 min) was slower than for class 1, 2, or 3 mutants.

[b] Rate constants for lysis determined from change in the absorbancy at 675 nm (Abs) from the steepest portion of the lysis curve where $Abs/Abs_0 = e^{-k(t - t_0)}$.

TABLE 3. r values and change in the frequency of septated cells (s) in representative mutants after temperature shift[a]

Class	r	s_0	s_{60}	s_{120}
1 (DIV VI)[b]	0.027	0.477	0.353	ND[c]
	0.118	0.460	0.547	0.516
2 (DIV II and III)	0.667	0.533	0.143	ND
	0.845	0.461	0.247	ND
3 (DIV I)	1.827	0.503	0.267	ND
	1.621	0.530	0.127	ND
4 (lytic)	2.180	0.372	0.186	ND
	1.300	0.553	0.535	0.218

[a] s values were determined at 0, 60, and 120 min after the shift from 30 to 42°C.

[b] The DIV assessments are according to reference 16.

[c] ND, Not determined.

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