Thermosensitive cell growth mutants of *Enterococcus hirae* that elongate at non-permissive temperature are stimulated to divide by parental autolytic enzymes

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A series of thermosensitive cell growth mutants of *Enterococcus hirae* have been isolated. Most of these mutants elongate and some show reduced autolytic activity when incubated at the non-permissive temperature (42 °C) in comparison to the wild-type incubated at the same temperature. When mutants were incubated for longer than 15 min at 42 °C and were then shifted to 30 °C, a lag proportional to the time of preincubation at 42 °C was observed before division, indicating that a certain time is necessary to restore normal levels of an active molecule(s) needed for septum formation and division. The addition of wild-type muramidase-1 permitted the immediate formation of septa and a single cell division; further addition of the enzyme stimulated the cells to divide once again. The other *E. hirae* autolytic enzyme, peptidoglycan-hydrolase-2, which is found in the culture medium, seemed to be involved in separation of daughter cells but may also take over the function of muramidase-1. A key role of both enzymes in septum formation and division is postulated.

Introduction

Over the past few years, studies on the role of penicillin-binding proteins (PBPs) in cell physiology and on the effect of β -lactam antibiotics which specifically inhibit septum formation, have demonstrated that a number of bacteria are capable of elongating when septum formation is inhibited (Fontana *et al.*, 1983; Spratt, 1975). These observations are consistent with a model which proposes the existence in rods and some cocci of two different sites for cell wall growth whose activities are balanced under normal conditions (Sattà *et al.*, 1979). Inhibition of the activity of one of these sites implies the prevalence of the other, giving rise to filaments when septum formation is inhibited, or to coccoid cells if elongation of lateral wall is blocked (Lleò *et al.*, 1990; Sattà *et al.*, 1983).

Isolation of thermosensitive (ts) cell growth mutants with one or more defects in septum formation at non-permissive temperatures could provide an excellent system for studying the mechanism of cell wall growth in

those Gram-positive cocci, such as streptococci, which, according to the model, have two sites for cell wall assembly (Lleò *et al.*, 1990).

In this study *Enterococcus hirae* ts cell growth mutants, which elongate after shifting the cultures to the non-permissive condition, have been characterized. Since some of the elongating mutants showed reduced autolytic activity, with respect to wild-type, when incubated at 42 °C, we considered the possibility that alterations in these enzymes could be the cause of the blockage of cell division in the mutants in the non-permissive condition, as has been previously suggested (Daneo-Moore & Shockman, 1977; Fan & Beckman, 1971; Fan *et al.*, 1972; Tomasz & Waks, 1975; Koch & Burdett, 1986).

E. hirae contains two endogenous autolytic enzymes, both of which hydrolyse only the β -1,4 link between *N*-acetylmuramic acid and *N*-acetylglucosamine (Barrett *et al.*, 1984*b*; Barrett & Shockman, 1984). One enzyme, muramidase-1, is synthesized in a latent form (130 kDa) which is transported to specific sites in the cell wall where it is then activated, generating the active form (87 kDa). Muramidase-1 is active on *E. hirae* walls inactivated by sodium dodecyl sulphate (SDS-walls) (Barrett *et al.*, 1984*a*; Shockman & Barrett, 1983). The second enzyme, peptidoglycan-hydrolase-2 (Pg-hydrolase-2), comprising two proteins (125 and 75 kDa), is active on *Micrococcus luteus* SDS-walls and on purified Pg of *E. hirae* and is

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Abbreviations: ts, temperature sensitive; Pg, peptidoglycan; SDS-walls, sodium dodecyl sulphate-treated cell walls; PBP, penicillin-binding protein.

Fig. 1. (a) Cell number ($\diamond$, $\blacklozenge$) and mass ($\square$, $\blacksquare$) increase at 30 °C (open symbols) and at 42 °C (filled symbols) of a ts cell-growth mutant that retains coccoid morphology (NON-10) and of one that elongates (NON-11) at 42 °C. (b) DNA ($\diamond$, $\blacklozenge$), protein ($\triangle$, $\blacktriangle$) and peptidoglycan ($\square$, $\blacksquare$) synthesis at 30 °C (open symbols) and at 42 °C (filled symbols) of the same mutants. At time 0 the absolute values were: NON-10: 1.8×10^7 cells ml^{-1} at an OD_{650} of 0.1, and 3930 c.p.m. ml^{-1} (DNA), 4290 c.p.m. ml^{-1} (proteins) and 2725 c.p.m. ml^{-1} (peptidoglycan). NON-11: 1.4×10^7 cells ml^{-1} at an OD_{650} of 0.1, and 3650 c.p.m. ml^{-1} (DNA), 4180 c.p.m. ml^{-1} (proteins) and 2940 c.p.m. ml^{-1} (peptidoglycan). The data are from an experiment that was repeated twice with no significant differences.

found in the culture medium during growth (Kawamura & Shockman, 1983b).

Preliminary experiments performed to study the recovery of division in some of the mutants in the presence of culture medium in which the wild-type had been grown permitted the identification of a factor allowing division at 42 °C (Lleò *et al.*, 1984, 1990). This compound also showed a lytic activity on SDS-walls of *E. hirae* and *M. luteus*. The effect of wild-type purified muramidase-1 and Pg-hydrolase-2 on cell division of the mutants incubated at the non-permissive temperature was also studied.

Methods

Bacterial strains and growth conditions. *Enterococcus hirae* ATCC 9790 and 13 thermosensitive cell growth mutants derived from it were used in this study (see Tables 1 and 2). Bacterial strains were grown in Brain Heart Infusion broth (BHI, Difco) at 30 °C or 42 °C. Growth

was measured as OD_{650} in a Beckman DU6 spectrophotometer. Cell numbers were determined with a Coulter counter model ZBI equipped with a 30 μm orifice, as previously described (Canepari *et al.*, 1984b).

Selection procedure. An exponential phase culture of *E. hirae* was mutagenized with ethylmethanesulphonate (EMS) as described by Lleò *et al.* (1990). Briefly, exponentially growing cells were incubated with EMS at 30 °C for 60 min. Then cells were washed twice in 10 mM-phosphate buffer (pH 7) and incubated overnight in BHI at 30 °C. Penicillin enrichment, which is usually used to increase mutant selection, was avoided in this case in order to obtain that part of the population capable of changing their morphology. The mutagenized culture was plated on BHI agar, and plates were replicated and incubated at 30 and 42 °C. Clones which formed colonies at 30 °C but not at 42 °C were considered to be thermosensitive cell growth mutants.

Morphological examinations. Slides for microscopic examination and micrographs were prepared as described by Canepari *et al.* (1984a) and examined with a Leitz Orthoplan microscope equipped with an automatic camera.

Macromolecular synthesis. DNA, protein and Pg syntheses were determined by continuous incorporation of specific radiolabelled precursors (respectively [^3H]thymidine, 2 μCi (74 kBq) ml^{-1} ; [^3H]leucine, 2 μCi ml^{-1} and [^3H]lysine, 6 μCi ml^{-1}) into trichloroacetic-acid-precipitable fractions. Briefly, cells were grown at 30 °C for at least three generations in the presence of radiolabelled precursors and then (when $\text{OD}_{650} = 0.1$) the culture was subdivided into two parts. One was reincubated at 30 °C and the other was shifted to 42 °C. At 30 min intervals, 1 ml samples were taken, precipitated with 5 ml ice-cold 10% (w/v) trichloroacetic acid and maintained for 30 min on ice. The precipitated material was collected on 0.45 μm filters and radioactivity was then measured in a Beckman LS 7000 counter. Before measuring radioactivity, samples for Pg synthesis were incubated with pronase to remove radioactivity bound to proteins, as described by Boothby *et al.* (1971).

Cellular autolysis. After incubation for 75 min at 42 °C, cells were rapidly collected by centrifugation, washed twice with ice-cold 0.01 M-phosphate buffer and resuspended in one-third of the original volume of 0.3 M-phosphate buffer (pH 7). Samples were incubated at 30 °C and 42 °C and lysis was monitored turbidimetrically and expressed as percentage decrease in OD_{650} .

Extraction and purification of autolytic enzymes. Muramidase-1 was obtained from cell-wall autolysates as described by Shockman *et al.* (1967). Purification of enzyme was performed by affinity chromatography (Kawamura & Shockman, 1983a). Pg-hydrolase-2 was obtained from culture supernatants and purified by binding these supernatants to SDS-inactivated walls of *M. luteus* as described by Kawamura & Shockman (1983b). Enzymes were renatured before use (Hager & Burgess, 1980).

Assay for autolytic enzyme activity. Activity of muramidase-1 and of Pg-hydrolase-2 was measured by the rate of dissolution of SDS-walls of *E. hirae* or *M. luteus*, respectively, in 10 mM-phosphate buffer, pH 7, at 37 °C (Cornett *et al.*, 1978). SDS-walls were prepared by treatment of walls of *E. hirae* and *M. luteus* with 2% (w/v) SDS as described by Shockman *et al.* (1967). One unit of lytic activity corresponds to a 1% decrease in OD_{450} in 20 min at 37 °C.

Analysis of penicillin-binding proteins (PBPs). Analysis of PBPs was performed as previously described (Coyette *et al.*, 1980). After binding with benzyl[^{14}C]penicillin, membranes were solubilized with 2% SDS and proteins separated by SDS-PAGE (Laemmli, 1970). PBPs were visualized by fluorography.

Antibiotics and radiochemicals. Penicillin G was from Squibb. [^3H]Thymidine [sp. act. 20 Ci (740 GBq) mmol^{-1}], [^3H]leucine [sp. act.

Fig. 2. Cell morphology of NON-11 ts cell growth mutant at different times during incubation at 42 °C.

^{146}Ci (5.4 TBq) mmol^{-1}], [^3H]lysine [sp. act. 85 Ci (3.15 TBq) mmol^{-1}] and benzyl- ^{14}C penicillin [sp. act. 54 mCi (2.0 GBq) mmol^{-1}] were from Amersham.

Results

Morphological changes and cell division in mutants incubated at 42 °C

A series (series NON) of ts cell growth mutants of *E. hirae* unable to form colonies at 42 °C has recently been isolated after mutagenesis with EMS and without selection with penicillin (Lleò *et al.*, 1990). Of the 13 mutants used in this study, 10 elongated and 3 maintained coccoid morphology when incubated at the non-permissive temperature (42 °C).

Fig. 1 shows the divisional behaviour of one (NON-11) of the mutants which elongates when incubated at 42 °C. All the other mutants that elongated at 42 °C showed similar behaviour with regard to morphology and division. A slight increase in cell number was

observed over the first 15 min and then cell division stopped. After 15 min, cells began to elongate (Fig. 2) reaching a maximum length in 60–90 min. Moreover, during elongation, the cell number remained constant, but cell mass increased about fivefold. Between 90 and 120 min of incubation at 42 °C apparently normal septa began to be formed, and after 150 min the septum formation was complete (Fig. 2). The cells formed (on average 3–4 cells per elongated element), each of which had a nucleus, could not separate or separated only partially (Fig. 2). A doubling or less of the cell number occurred. This is attributed to the growth of the organism in chains of unseparated cells; the Coulter counter underestimates cell numbers in such cases. Further divisions were not observed (Fig. 1).

In the case of NON-10 (and of the three strains retaining their coccoid shape at the non-permissive temperature) cell number increased over 60 min of incubation at 42 °C, after which no further divisions were observed (Fig. 1).

Macromolecular synthesis in the mutants incubated at the non-permissive temperature

DNA, protein and Pg synthesis were measured in the mutants that elongate and in those that retain their coccoid shape at 42 °C. As shown in Fig. 1, in a mutant (NON-11) which elongates at 42 °C, DNA and protein synthesis continued during elongation and then declined and stopped; Pg synthesis continued for at least 120–150 min. Table 1 shows the values of cell number increase and macromolecular synthesis of the mutants used in this study. It is also evident that the mutants show a wide variation in the pattern of macromolecular synthesis suggesting that the mutants may be disturbed in a number of quite different ways. In some of the elongating mutants (NON-11, NON-19, NON-39, NON-43, NON-48 and NON-76) we observed partial release of radioactivity bound to the cell wall polymer (which we will call Pg turnover) at the time corresponding to septum formation at 42 °C (Fig. 1 and Table 2). Although Pg continued to be synthesized after 120 min it appears to represent resynthesis of the Pg lost through degradation between 90 and 120 min. In the mutant NON-10 and the other two mutants (Fig. 1 and Table 2) which maintained their coccoid morphology at 42 °C, DNA and protein synthesis were inhibited immediately after the block of cell division, while Pg synthesis continued for some minutes after inhibition of cell division.

Evaluation of cell division and Pg synthesis in mutants grown at 30 °C after different periods of incubation at the non-permissive temperature

In order to analyse whether a period of synthesis is required for cell division at the non-permissive temperature, or whether the material synthesized at 42 °C may need to be held for some time at 30 °C for its expression, as has been demonstrated for *ts* mutants of *Esch. coli* and other Gram-negative rods (Ahmed & Rowbury, 1971; Reeve & Clark, 1972), mutants were incubated at 42 °C for different periods of time before shifting the cultures to the permissive temperature.

Fig. 3(a) shows that if mutant NON-11 was incubated at 42 °C for less than 15 min, cell division continued normally after the shift to 30 °C: elongation did not begin and cells maintained their normal coccoid shape and their ability to divide. After incubation at 42 °C for longer than 15 min (30, 45 and 60 min) cell division started at 30 °C after a lag proportional to the time of preincubation at the non-permissive temperature (Fig. 3a). Moreover, if elongation had already begun at 42 °C, it continued after shifting to 30 °C until reaching the maximum and only then did septa begin to be formed

Table 1. *Morphology and increase in cell number and macromolecular synthesis after 3 h incubation at 42 °C in elongating and non-elongating E. hirae ts cell growth mutants*

Data are representative results of an experiment that was repeated twice with no significant differences.

Strain	Morphology at 42 °C	Increase in cell number (%)	Percentage increase in synthesis of:		
			DNA	Protein	Pg
Wild-type	Coccus	470	450	485	480
NON-5	Coccus	70	60	75	150
NON-9	Coccus	85	90	95	150
NON-10	Coccus	50	50	60	80
NON-11	Rod	40	50	100	50
NON-29	Rod	30	130	210	220
NON-39	Rod	40	110	220	210
NON-43	Rod	50	120	200	110
NON-44	Rod	80	170	185	120
NON-48	Rod	90	110	195	220
NON-56	Rod	20	54	130	440
NON-76	Rod	50	70	140	420
NON-90	Rod	35	95	140	250

Table 2. *Autolytic activity of whole cells from ts cell growth mutants of E. hirae*

The data are from an experiment that was repeated three times with no significant differences.

Strain*	Percentage decrease in OD ₄₅₀ in 90 min at:		Ratio wild type: mutant autolytic activity at:		Pg turnover† at 42 °C
	30 °C	42 °C	30 °C	42 °C	
Wild-type	45	61	1.00	1.00	—
NON-5	35	59	1.25	1.03	—
NON-9	40	51	1.10	1.19	—
NON-10	37	21	1.20	2.90	—
NON-11	32	9	1.45	6.70	+
NON-19	43	55	1.04	1.30	+
NON-29	31	61	1.45	1.00	—
NON-39	29	50	1.50	1.22	+
NON-43	36	36	1.20	1.70	+
NON-44	30	61	1.55	1.00	—
NON-48	38	21	1.20	3.00	+
NON-56	29	60	1.55	1.01	—
NON-76	32	40	1.45	1.52	+
NON-90	39	66	1.16	0.92	—

* Morphology of strains after 75 min incubation at 42 °C: *E. hirae* wild-type, NON-5, NON-9 and NON-10 were cocci; all other strains were rods.

† Pg turnover indicates the release of radioactivity bound to Pg at 42 °C between 90 and 120 min, corresponding to the time of septum formation shown in Figs 1 and 2.

and division occurred. On the contrary, the mutants that did not elongate at 42 °C, divided 15 min after the shift to 30 °C regardless of the length of preincubation at 42 °C (Fig. 3b).

Fig. 3. Cell morphology and regrowth at 30 °C of NON-11 (a) and NON-10 (b) mutants after different periods of incubation at 42 °C. Div., cell division.

Pg synthesis was evaluated during regrowth at 30 °C after periods of 15, 30, 45 and 60 min of incubation at 42 °C in mutants such as NON-11 that elongated at the non-permissive temperature. Degradation (turnover) of the wall polymer was observed. It should be noted that about half the cell wall synthesized is degraded (Fig. 4*b, c, d*). The duration and intensity of this turnover were proportional to the time the mutants were held at the non-permissive temperature and it is also apparent that degradation became progressively slower with increasing periods at 42 °C (Fig. 4*a–d*). Pg synthesis continued normally at the end of the turnover and cell division always started 30 min after that time. Pg synthesis started immediately after the shift to 30 °C when NON-10 and the other mutants that did not elongate at 42 °C were analysed (Fig. 4*e, f*).

Analysis of penicillin-binding proteins

The PBPs of all 13 mutants used in this study were analysed, but no alterations of the electrophoretic PBP patterns were observed (data not shown).

Autolytic activity in *ts* cell-growth mutants during incubation at the non-permissive temperature

Table 2 shows that cellular autolysis tested at the non-permissive temperature was significantly decreased in some of the mutants compared to wild-type *E. hirae* grown at the same temperature. These data suggested

that, at least in some of the mutants, an alteration of the autolytic enzymes might be correlated with inhibition of septum formation and with the inability of the cells to separate when the septa were complete. There was also a correlation in the elongating mutants but not in those conserving their coccal shape, between reduced autolytic activity and peptidoglycan turnover at 42 °C (Table 2).

Table 3 presents the values of muramidase-1 activity from the wild-type and a *ts* cell-growth mutant (NON-11) grown at 42 °C; muramidase-1 showed at least a five- to sixfold reduction in the mutant compared to the wild-type, while values were similar when both strains were grown at 30 °C. Marked differences in the susceptibility to muramidase-1 of the walls from the mutant NON-11 and from the parent were also observed. Similar, but small differences, were found with muramidase-1 extracted from cells grown at 42 °C. The data in Table 4 demonstrate that the activity of Pg-hydrolase-2 (when tested on SDS-walls of *M. luteus*) was also reduced by about half in the mutant grown at 42 °C compared to the wild-type grown at the same temperature.

Effect of purified muramidase-1 and Pg-hydrolase-2 from wild-type on cell division of *ts* cell-growth mutants incubated at 42 °C

Muramidase-1 and Pg-hydrolase-2 were purified as described in Methods. Electrophoresis on SDS-polyacrylamide gel revealed a single band at 87 kDa corresponding to the active form of muramidase-1 and

Fig. 4. Cell number (■) and peptidoglycan synthesis (●) increases at 30 °C of NON-11 mutant after 15 (a), 30 (b), 45 (c) and 60 (d) of incubation at 42 °C and of NON-10 mutant after 30 (e) and 60 (f) min of incubation at 42 °C. The data are from an experiment that was repeated twice with no significant differences. The experiment was also done with three other mutants with similar results.

Table 3. Enzymic activity of muramidase-1 from wild-type and from a *ts* cell growth mutant (NON-11) on SDS-walls of both wild-type and the same mutant

Both wild type and NON-11 were incubated at 42 °C for 75 min before SDS-walls preparation. It is important to note that the wild-type had a higher growth rate at 42 °C than at 30 °C (Fontana *et al.*, 1983). Activity is expressed as units of lytic activity at 37 °C (mg protein^{-1}). One unit of lytic activity corresponds to a 1% decrease in OD_{450} in 20 min at 37 °C. The data are from an experiment that was repeated four times with no significant differences.

Muramidase-1 from:	SDS-walls of:			
	<i>E. hirae</i>		NON-11	
	grown at:		grown at:	
	30 °C	42 °C	30 °C	42 °C
Wild-type grown at 30 °C	83	83	584	280
NON-11 grown at 30 °C	57	98	771	340
Wild-type grown at 42 °C	866	1068	2533	1723
NON-11 grown at 42 °C	133	200	650	525

Table 4. Enzymic activity of Pg-hydrolase-2 from wild-type and from a *ts* cell growth mutant (NON-11) on SDS-walls of both *E. hirae* and *M. luteus*

Activity is expressed as units of lytic activity at 37 °C (mg protein^{-1}). One unit of lytic activity corresponds to a 1% decrease in OD_{450} in 20 min at 37 °C. The data are from an experiment that was repeated three times with no significant differences.

Pg-hydrolase-2 from:	SDS-walls of:	
	<i>E. hirae</i>	<i>M. luteus</i>
Wild-type grown at 30 °C	36	750
NON-11 grown at 30 °C	40	735
Wild-type grown at 42 °C	70	990
NON-11 grown at 42 °C	23	501

Fig. 5. Cell number (a) and peptidoglycan synthesis (b) increases of a *ts* cell-growth mutant (NON-11) incubated at 30 °C (□), at 42 °C (■) and at 42 °C in the presence of one unit of muramidase-1 (Δ). The arrow indicates the addition of a second equal dose of the same enzyme. At time 0 the absolute values were: 1.5×10^7 cells ml^{-1} at an OD_{650} of 0.11, and 2105 c.p.m. ml^{-1} for peptidoglycan synthesis. The results are from an experiment that was repeated four times using different enzyme preparations. The experiment was also done with three other mutants with similar results.

two bands at 123 and 75 kDa corresponding to Pg-hydrolase-2 (data not shown).

When purified enzymes were added to cultures of the mutants and immediately incubated at 42 °C, complete inhibition of cell elongation was observed when muramidase-1 was tested, while only partial inhibition of elongation was observed when Pg-hydrolase-2 was added (data not shown). In both cases, at least 100% increases in cell number and in Pg synthesis (Fig. 5) were observed in the NON-11 culture compared to the control without enzymes. The best stimulatory activity of both enzymes was obtained with about $0.01 \mu\text{g enzyme (}10^8 \text{ cells)}^{-1}$ for

muramidase-1 and 0.02 µg enzyme (10⁸ cells)⁻¹ for P_g-hydrolase-2 corresponding approximately to the physiological amounts of these enzymes in the wild-type (Kawamura & Shockman, 1983*a, b*). These amounts of enzymes did not cause any lytic effect on cell cultures, as reported by Tomasz & Waks (1975) and Fan & Beckman (1971). When chloramphenicol at 10 µg ml⁻¹ (the minimal concentration inhibiting protein synthesis) was added together with muramidase-1 during a shift to 42 °C, no changes in shape or in cell division were observed (data not shown) suggesting that active protein synthesis was needed.

When, after doubling of the cell number, a further dose of enzyme was added, a further 100–150% increase in cell number and P_g synthesis was observed. In addition, when each of the two enzymes was added to the culture incubated at 42 °C, the decrease in P_g synthesis occurring at 90 min was not observed (Fig. 5).

If autolytic enzymes were added to the cultures after 90 min of incubation at 42 °C, when cells were already elongated, separation of daughter cells was observed with a consequent increase in cell number higher than that shown by control cultures incubated without enzymes. Moreover, when a NON-11 culture with added autolytic enzymes was maintained for 45 min at 42 °C and then shifted to 30 °C, cell division started immediately, while control culture without enzymes showed a 120 min lag before cell division. Again, addition of chloramphenicol inhibited recovery of cell division, thus indicating that *de novo* protein synthesis was needed.

Discussion

We have previously demonstrated that in coccoid bacteria two distinct biochemical reactions that we call 'sites', responsible for lateral wall extension and septation, may exist for peptidoglycan assembly but that cells present a coccal morphology because the septum formation site always prevails over the lateral wall elongation site (Lleò *et al.*, 1990). When septum formation is inhibited, elongation may be expressed so that some cocci change to rods. In this study, we have demonstrated the validity of this hypothesis in a number of *ts* cell-growth mutants of *E. hirae* which elongate when incubated in the non-permissive condition. Even if these mutants are capable of forming a series of septa on continuous incubation at 42 °C, cells separate only partially and further septum formation and division do not occur.

Analysis of macromolecular synthesis in *E. hirae* mutants which elongate and in those retaining their coccal morphology at 42 °C confirm our previous hypothesis that the envelope controls the increase in cell mass, so that when the envelope is able to extend, new

macromolecules can be synthesized (mutants elongating at 42 °C), but when the extension of the envelope is inhibited, macromolecular synthesis is also blocked as no available space is created (mutants retaining coccal morphology) (Lleò *et al.*, 1990).

Since septum formation is a complex event in the cell cycle requiring different genetically controlled steps (Ishino *et al.*, 1989), a mutation causing the block of septum formation may affect one or more of the different proteins involved in this process. As has been previously postulated, at least one PBP is directly involved in septum formation in *E. hirae* (Coyette *et al.*, 1980; Pucci *et al.*, 1986; Canepari *et al.*, 1987). A mutation in this protein could cause the inhibition of septum formation, as has been clearly demonstrated in other bacteria (Ishino & Matshuashi, 1981; Spratt, 1977). Nevertheless, no apparent defect in PBP patterns was observed in any of the 13 *ts* cell-growth mutants isolated from *E. hirae* and analysed here.

On the contrary, analysis of autolytic activity revealed a reduction in such activity in some of the mutants when grown at the non-permissive temperature. As autolytic enzymes seem to be necessary for septum formation and cellular separation (Daneo-Moore & Shockman, 1977), a defect in these proteins may interfere with septation. Among the elongating mutants which show a reduced autolytic activity we chose mutant NON-11 for further studies because of its more marked reduction in activity. Data presented (Table 3) show that cell walls of NON-11 are more sensitive to muramidase-1 activity than cell walls of wild-type. This fact could (in part) explain the reduced amount of the enzyme present in the mutant. However, only definition of the chemical composition of the P_g will clarify whether the reduced amount of muramidase-1 is the primary defect of the mutant or a consequence of the different chemical composition of its walls.

The regrowth of NON-11 after different periods of incubation at 42 °C demonstrated the need for the completion of elongation before septa can be initiated, as previously suggested (Satta *et al.*, 1983), and indicates involvement of *ts* defects in one or more molecules needed for cell division, as is the case with *Esch. coli* BUG-6 (Reeve & Clark, 1972) and other strains (Ahmed & Rowbury, 1971). Longer periods of incubation at 42 °C caused longer delays in initiation of division at 30 °C, indicating that longer times are needed to restore normal levels of the active molecule(s) for septum formation and division. These data are very similar to those on recovery of autolytic activity of *E. hirae* cells from starvation (Pooley & Shockman, 1969).

Probably because the defective molecule(s) is synthesized at 42 °C in an altered form, the longer the incubation at the non-permissive temperature the higher

will be the proportion of defective molecules compared to the active form and the longer will be the time necessary to restore normal levels of the functional molecules at 30 °C. Only when these levels are raised, do cells begin to form septa and divide. It is suggested that this molecule(s) may be an autolytic enzyme whose role in septum formation has often been postulated (Daneo-Moore & Shockman, 1977; Pooley *et al.*, 1972; Shockman *et al.*, 1974), but never demonstrated.

Pg turnover was observed during regrowth and was proportional to the time of preincubation of the cultures at 42 °C. Turnover (the massive release of radioactivity from Pg during its synthesis) was also observed in some strains when Pg synthesis was analysed at the time of septum formation at 42 °C. However, the rate of turnover was not measured directly. Although Pg turnover has never been described in *E. hirae* (Boothby *et al.*, 1973), we observed a turnover of wall material in the wild-type when cells were grown in a rich medium (BHI) but not in the chemically defined medium used by others (Boothby *et al.*, 1973) (data not shown).

This Pg turnover occurring during regrowth at 30 °C and before cell division also suggests involvement of a lytic enzyme as the cause of alterations in the mutants. We suggest that this turnover, whose duration is proportional to the time the cultures are held at 42 °C, may represent a rearrangement of the cell wall in order to localize the new sites for septum initiation in an elongated bacterium. Similarly, the same explanation may be given for the turnover observed in rods at 42 °C between 90 and 120 min when it is needed to localize the new sites for septum formation. Moreover, there is a good correlation between reduced autolytic activity (compared to the wild-type) and Pg turnover at the time of septation in the case of rod-shaped mutants, while mutants which retain coccoid morphology never exhibit Pg turnover during the cell cycle at 42 °C (see Table 2).

We suggest that these *E. hirae* mutants are defective in septum formation and that, at least in some of the mutants examined, the molecule(s) involved in the cell division alteration may be autolytic enzymes whose activity is reduced at the non-permissive temperature. Thermosensitivity of the enzymes at 42 °C has not been observed (unpublished observations), thus suggesting that synthesis is reduced at the restrictive temperature.

This hypothesis is supported by several results obtained in this study. The addition of wild-type muramidase-1 to the NON-11 mutant permitted the immediate formation of septa and a single division of the cells. In this case no turnover was observed because the cell maintained its normal morphology (coccus) and septa were formed at the due time as in the control. Further additions of the enzyme stimulated the cells to divide again. Similarly, the recovery of the different autolysin functions in deficient

mutants was observed when exogenous enzyme was added to cell cultures (Fan & Beckman, 1971; Tomasz & Waks, 1975; Koch & Burdett, 1986). If, on the other hand, the enzyme was added when cells had already begun to elongate, no immediate effects were observed and only when cells were completely elongated, did septa form and division occur. This provides further support for our hypothesis that elongation and septation are two alternating processes (Satta *et al.*, 1980).

Similarly, when the mutants were incubated briefly at the non-permissive temperature in the presence of the autolytic enzyme, the return to the permissive condition caused the immediate division of the cells. On the contrary, when preincubation at 42 °C was done without enzyme, a lag was observed before division, indicating that time at 30 °C was needed before cells could synthesize a sufficient quantity of normal autolytic enzyme to form septa.

These data demonstrate the fundamental role of autolytic enzymes in septum formation. Pg-hydrolase-2, an enzyme which is not active on *E. hirae* walls, causes only partial inhibition of the elongation process, indicating that this enzyme may take over, albeit not completely, the function of muramidase-1. Unfortunately, to date it has not been possible to isolate mutants which completely lack the autolytic system, and thus it is very difficult to establish the precise role of each of the lytic enzymes in the cell cycle. However, we have seen that the cells which remain attached to one another forming chains in the control culture without enzyme can undergo separation when Pg-hydrolase-2 is added, giving rise to a doubling of the cell number. Although muramidase-1 may also stimulate this separation, the fact that only Pg-hydrolase-2 is found in the culture medium (Kawamura & Shockman, 1983*b*) makes it more likely that the latter enzyme is mainly involved in the separation of the daughter cells, operating from the outside.

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