LEPTOMYCINS A AND B, NEW ANTIFUNGAL ANTIBIOTICS

III. MODE OF ACTION OF LEPTOMYCIN B ON *SCHIZOSACCHAROMYCES POMBE*

TETSUO HAMAMOTO, TAKESHI UOZUMI and TERUHIKO BEPPU

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Mode of action of leptomycin B (LMB), a new antifungal antibiotic, was studied with *Schizosaccharomyces pombe*. A low concentration of LMB caused inhibition of cell division, producing elongated cells with morphologically altered nuclei and several cell plates, while it inhibited nucleic acid synthesis in intact cells at 100-fold higher concentration. Addition of LMB during G2 phase in synchronous culture blocked following events in cell cycle. Analysis of the effect of LMB on *cdc* mutants suggested the antibiotic inhibited some specific step, possibly in M phase just prior to nuclear division.

Leptomycin B (LMB, Fig. 1) is a new antifungal antibiotic found in the course of a screening program for activities inducing morphological abnormalities in fungi. This antibiotic induces cell elongation in fission yeast, *Schizosaccharomyces pombe* and hyphal swelling in fungus, *Mucor rouxianus*[1]. The physico-chemical and biological properties[2], chemical structure[3], biosynthesis[4] and antitumor activity[5] are already described. This paper describes the mode of action of LMB on fission yeast, *S. pombe*.

Materials and Methods

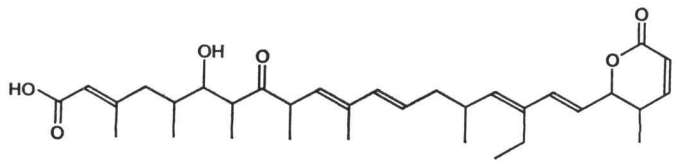
Strains and Culture Conditions

S. pombe L972 h$^-$ [6], cell cycle, temperature sensitive mutants *cdc* 2 (nuclear division blocked) and *cdc* 7 (early cell plate formation blocked)[7] were the generous gifts from Dr. M. YAMAMOTO, Institute of Medical Science, the University of Tokyo. These strains were maintained on malt agar slants containing malt extract (Difco) 1%, yeast extract (Difco) 0.3% and glucose 2%, pH 6.0. The wild type L972 h$^-$ was grown in EDINBURGH's minimal essential medium (EMM2)[8] at 30°C on a reciprocal shaker. The *cdc* mutants were cultured in EMM2 at 26°C (permissive temperature) or at 35°C (restrictive temperature).

Measurements of Growth

To test the effect of LMB on growth of *S. pombe* L972 h$^-$, a logarithmic growing culture (OD_{550} = 0.2) was divided into appropriate volume in test tubes and various amounts of LMB dissolved in ethanol were added. Viable cell number was measured by counting colonies appearing on a EMM2 plate on which appropriately diluted cell suspension was spread and incubated for 3 days at 30°C.

Fig. 1. Chemical structure of leptomycin B.



Total cell number was measured by counting cell number using Thoma hemocytometer, and OD_{550} was measured using Hitachi 200 Spectrophotometer.

Macromolecular Synthesis

D-[*U*-^{14}C]Glucose (1 Ci/mmol), sodium [1-^{14}C]acetate (57 mCi/mmol), [*U*-^{14}C]leucine (1 Ci/mmol) and [8-^{14}C]adenine (50 mCi/mmol) were purchased from New England Nuclear. These compounds were diluted with 0.01 mM carrier solution and used as precursors for synthesis of carbohydrate, lipid, protein and nucleic acid, respectively.

S. pombe L972 h$^-$ was cultured to the log phase (OD_{550}=0.6) in 100 ml of EMM2 medium in a 500-ml Sakaguchi flask. The growing cells were harvested by centrifugation, washed and suspended in the same volume of the prewarmed fresh medium containing various concentration of LMB.

In the experiments for glucose and leucine incorporation, fresh medium free from the precursors were used. After 30 minutes cultivation, radioactive precursors were added to the medium at final concentration of 0.1 μCi/ml and further incubated. To obtain cold acid-insoluble fraction, cells were taken at appropriate intervals into equal volume of ice cold 10% trichloroacetic acid (TCA) and kept on ice for 1 hour. To determine incorporation of [^{14}C]adenine into DNA (alkali-resistant fraction), cells were incubated with 0.1 N NaOH for 30 minutes at 30°C before TCA precipitation. Incorporation into RNA fraction was determined as the difference of radioactivities in the acid-insoluble fraction and those in the alkali-resistant fraction. The TCA precipitates were collected on glass fiber filters (Whatman GF-C) and radioactivities on the dried filters were counted by liquid scintillation counter with a 5-ml of toluene-based scintillator consisting of 0.3% 1,4-bis-2-(5-phenyloxazolyl)-benzene (dimethyl-POPOP) and 1% 2,5-diphenyloxazole (PPO).

Nucleic Acid Synthesis in Isolated Nuclei

Nuclei of *S. pombe* were isolated by the method of SIPICZKI and FERENCZY[9] with slight modification. *S. pombe* cells were washed twice in EMM2 medium and incubated in EMM2 containing 2% D-glucose, 0.05% 2-deoxy-D-glucose at 30°C for 1 hour. The cells were then washed twice, resuspended in a solution containing 20 mg of Novozyme 234 (Novo Co., Denmark) in 5 ml of 0.8 M $MgSO_4$ at the cell density of 2×10^8 cells/ml and incubated at 30°C for 2 hours. The resulting spheroplasts were collected and homogenized with Potter-Elvejem glass homogenizer at 15 strokes. The suspension was centrifuged at $3,000\times g$ for 5 minutes. The supernatant fraction was resuspended in glycerol buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM 2-mercapthoethanol, 20% (w/v) glycerol and 1 mM phenylmethyl sulfonylfluoride and centrifuged at $15,000\times g$ for 20 minutes. The resulting precipitate was suspended in 5 ml of glycerol buffer and used as isolated nuclei.

DNA synthesis in isolated nuclei was measured in the modified reaction mixture described by CHANG[10]. The mixture contained in 0.1 ml glycerol buffer, 15 mM ATP, 20 mM $MgCl_2$, 300 mM NaCl, 0.3 mM dATP, dCTP, dGTP and dTTP, 0.8 mM ATP, CTP, GTP and UTP and 10 μCi [*methyl*-^{3}H]dTTP (0.33 mCi/mmol, NEN) for DNA synthesis or 10 μCi [5-^{3}H]UTP (0.67 mCi/mmol, NEN) for RNA synthesis. The reaction was started by adding 2×10^8 nuclei in 1 ml glycerol buffer and incubated for 2, 10 and 20 minutes at 30°C. RNA synthesis and DNA synthesis were measured as described above.

Staining of Nuclei

Cells of *S. pombe* were collected and suspended in distilled water containing 1% formaldehyde. DNA specific fluorescent dye, 4′,6-diamidino-2-phenylindole (DAPI, Sigma Chemical Co.)[11,12] was diluted to 1 μg/ml. Five μl of the cell suspension was mixed with equal volume of the DAPI solution and placed on a glass slide with coverslip, and observed under the fluorescent microscope (Olympus fluorescent microscope, BHS-2) with ultraviolet illumination[13]. The absorption filter was an Olympus L435.

Synchronous Culture

Synchronous culture of *S. pombe* L972 h$^-$ was established by the method of MIYATA *et al.*[14]. To logarithmically growing culture of *S. pombe*, 16 mM hydroxyurea was added to arrest the cells in G1-S boundary. After 4 hours incubation, the culture was filtered with a membrane filter (Millipore Co.,

HA type, pore size 0.45 μm), washed three times with equal volume of 30°C prewarmed EMM2 medium and then suspended in equal volume of prewarmed EMM2 medium. The synchronous growth was monitored by cell number counted with Thoma plate, cell plate index observed with microscope and nuclear division index with DAPI staining.

In order to obtain synchronous cultures of *cdc* mutants, *S. pombe cdc* 2 and *cdc* 7 were incubated at 26°C (permissive temperature) for 18 hours in EMM2. Then, the culture was filtered and resuspended immediately in EMM2 prewarmed at 35°C (restrictive temperature). After incubation at 35°C for 3 to 4 hours (one generation time), the culture was filtered and resuspended in 26°C prewarmed EMM2. The synchronous growth after their removal from the restrictive temperature was monitored by measuring cell number and cell plate index.

Results

Effect of LMB on Growth and Cell Division of *S. pombe*

Growth of *S. pombe* treated with various concentrations of LMB was measured (Fig. 2). LMB markedly inhibited increase in viable cell number and total cell number without significant effect on turbidity increase. Morphological change of *S. pombe* L972 h$^-$ by 10 ng/ml of LMB for 8 hours was shown in Fig. 3. Almost all the treated cells elongated and had one or several cell plates. These results indicated that the effect of LMB was static and caused inhibition of cell division along with cell

Fig. 2. Effect of LMB on growth of *Schizosaccharomyces pombe*.
○; Control, ●; +0.01 μg/ml, ■; +0.1 μg/ml, □; +1 μg/ml.

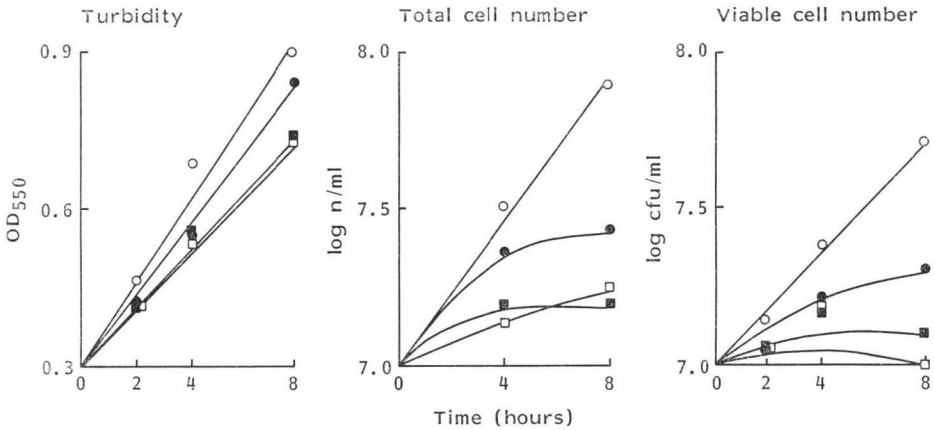


Fig. 3. Effect of LMB on growth of *Schizosaccharomyces pombe*.

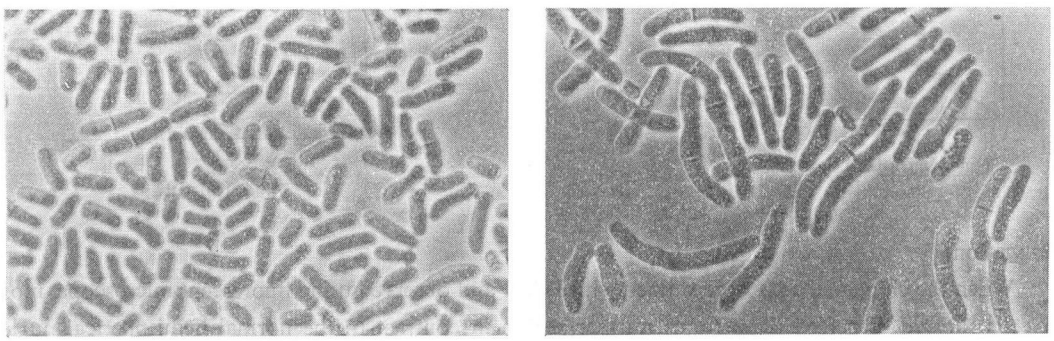


Fig. 4. Effect of LMB on DNA region of *Schizosaccharomyces pombe*.
The logarithmically growing culture of *S. pombe* in EMM2 medium was treated with 10 ng/ml fo LMB and further incubated for 12 hours.

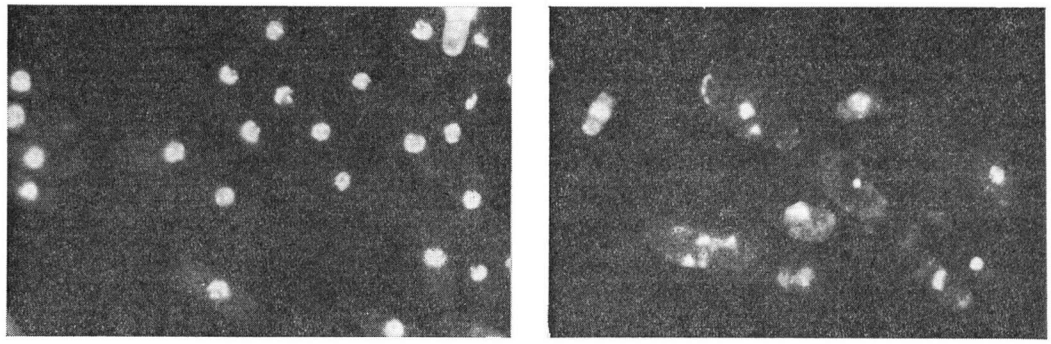


Fig. 5. Effect of LMB on macromolecular synthesis of *Schizosaccharomyces pombe*.
○; Control, ●; +0.01 μg/ml, ■; +1 μg/ml.

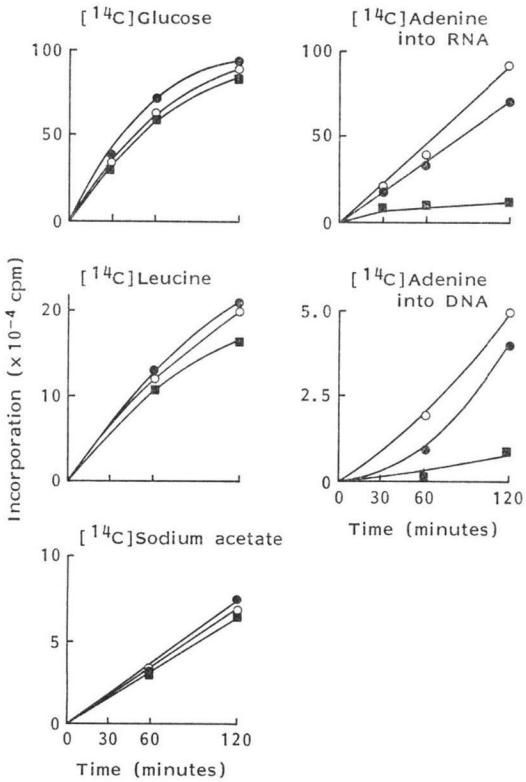
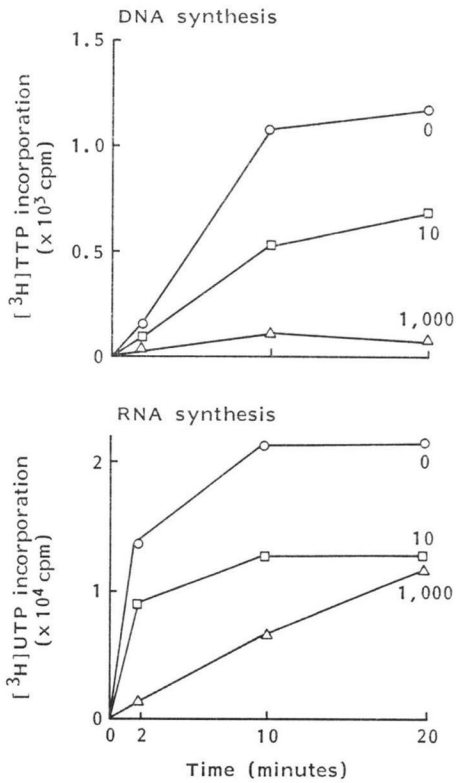


Fig. 6. Effect of LMB on nucleic acid synthesis of *Schizosaccharomyces pombe* nuclei.
Numbers in figure indicate the concentration (ng/ml) of antibiotic.



elongation.

When the elongated cells were stained with DAPI and observed using fluorescent microscope, most of cells showed dispersed filamentous nuclear region in contrast to the normal hemispherical nuclei in the control culture (Fig. 4). These observations indicate that LMB inhibits nuclear division.

Effect of LMB on Macromolecular Synthesis

The effect of LMB on macromolecular synthesis in growing *S. pombe* cells is shown in Fig. 5. Incorporation of [8-^{14}C]adenine into RNA and DNA fractions was significantly inhibited by 1 μg/ml of LMB, while those of [*U*-^{14}C]glucose, sodium [1-^{14}C]acetate and [*U*-^{14}C]leucine into acid-insoluble fraction were not inhibited. LMB caused specific inhibition of nucleic acid synthesis in *S. pombe* at the concentration of 1 μg/ml, however, the lower concentration (10 ng/ml) which was enough to induce cell elongation and inhibition of cell division showed little effect on nucleic acid synthesis.

Similar inhibition of nucleic acid synthesis by LMB was observed in isolated nuclei of *S. pombe*. As shown in Fig. 6, both DNA synthesis from [*methyl*-^{3}H]TTP and RNA synthesis from [5-^{3}H]UTP were partially inhibited by 10 ng/ml of LMB. The higher concentration (1 μg/ml) caused complete inhibition of DNA synthesis while permitting slow RNA synthesizing activity.

Effect on Synchronous Culture

Hydroxyurea was known to inhibit DNA synthesis and thus cause arrest of cell cycle at the boundary between G1 and S phases. Effect of LMB on cell cycle of *S. pombe* was analyzed with cultures synchronized by releasing from the hydroxyurea inhibition. The first synchronous cell division occurred at 2.5 hours after the release following previous sharp increase in nuclear division index and cell plate index at 2 and 2.5 hours, respectively (Fig. 7). Addition of LMB (1 μg/ml) immediately and 1 hour after the release blocked further progress of the cell cycle of *S. pombe*

Fig. 7. Effect of LMB on synchronous culture of *Schizosaccharomyces pombe* by hydroxyurea (HU). Arrows indicate the time of addition of LMB. ○; Cell number, △; nuclear division index, □; cell plate index.

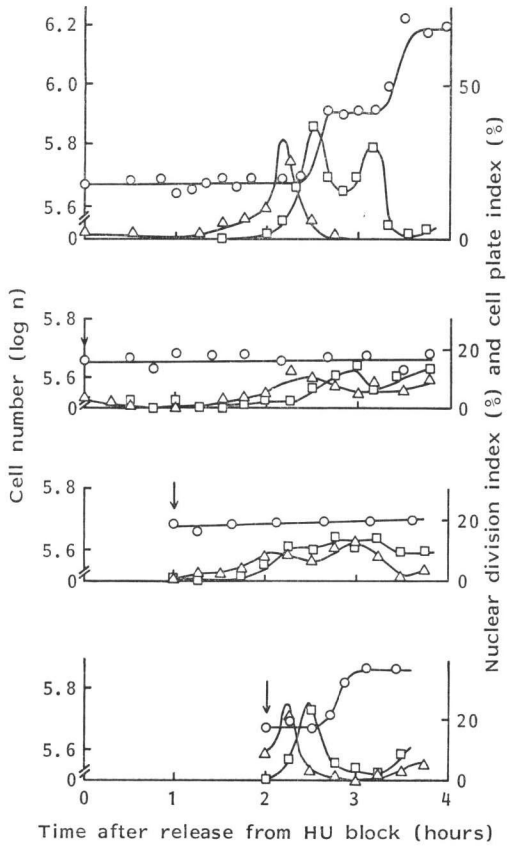


Fig. 8. Effect of LMB on synchronous growth of *Schizosaccharomyces pombe cdc* 2. ○; Cell number and △; cell plate index of control culture. ●; Cell number and ▲; cell plate index of culture added LMB at second temperature shift.

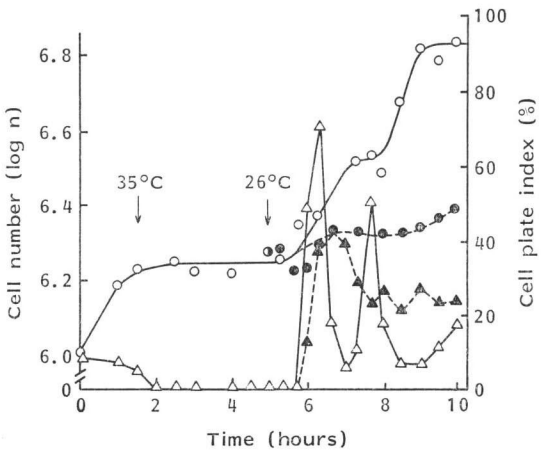
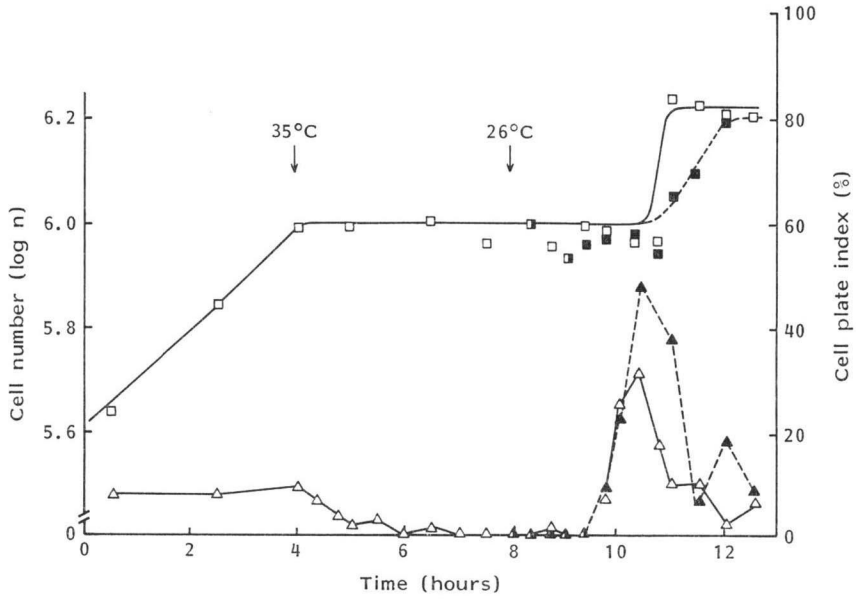


Fig. 9. Effect of LMB on synchronous growth of *Schizosaccharomyces pombe cdc* 7.
□; Cell number and △; cell plate index of control culture. ■; Cell number and ▲; cell plate index of culture added LMB at second temperature shift.



completely. On the other hand, addition at 2 hours after the release allowed cell division at least once. These results indicate LMB blocked the cell division cycle of *S. pombe* at the specific time of 1~2 hours after the release from hydroxyurea block.

Effect of LMB on *cdc* Mutants

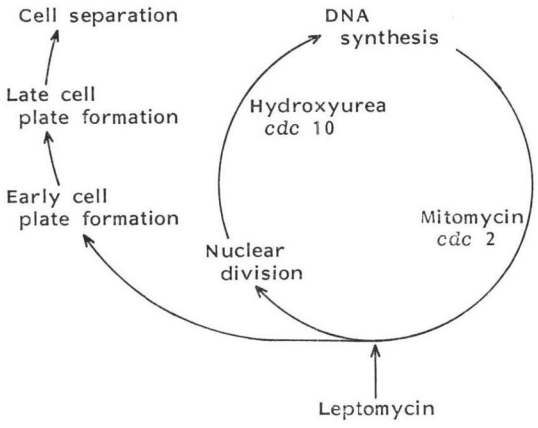
In order to characterize the block point by LMB, effects on *S. pombe cdc* 2 (temperature sensitive nuclear division mutant) and *cdc* 7 (temperature sensitive cell plate formation mutant) were examined. Both mutants started their growth synchronously by temperature shift from 3 hours treatment in the restrictive temperature (35°C) to the permissive temperature (26°C). In the case of *cdc* 2, addition of LMB (1 μg/ml) at the time of temperature shift inhibited the following cell division significantly (Fig. 8). But the same treatment of LMB had no effect on the synchronous growth of *cdc* 7 at least for one generation (Fig. 9). These results clearly indicate that LMB specifically inhibits the cell cycle progression from nuclear division to DNA synthesis corresponding to the period from M phase to S phase, without significant blocking cell plate formation pathway.

Discussion

Addition of a low concentration (10 ng/ml) of LMB to the random culture of *S. pombe* caused inhibition of cell division along with formation of elongated cells with several cell plates and altered nuclear morphology. Cell cycle of *S. pombe* is known to be composed of two independent pathways branched from the step of nuclear division, one for DNA synthesis and the other for cell division *via* cell plate formation[7]. Experiments with *cdc* 7 (temperature sensitive cell plate formation) indicate that LMB has no effect on the pathway of cell plate formation. On the other hand, LMB effectively prevented cell division after the *cdc* 2 arrest (nuclear division), suggesting that the antibiotic inhibits

Fig. 10. Cell division cycle of *Schizosaccharomyces pombe*.

Summary of the cell division cycle of *S. pombe* and the step that LMB attacks (indicated by the arrow). The *cdc* genes and the cell cycle inhibitors are placed just before the event in which they were involved.



some step later than the *cdc* 2 blocked point, possibly just before nuclear division. Experiments with cultures synchronized by hydroxyurea arrest showed that addition of LMB just prior to nuclear division failed to inhibit further events in cell cycle. It seems reasonable to assume that LMB might inhibit some specific step at the branching point of cell plate formation possibly in M phase (Fig. 10).

Nucleic acid synthesis, especially DNA synthesis, in growing cells and isolated nuclei of *S. pombe* is selectively inhibited by the higher concentration of LMB, however, the distinct difference from the inhibitory concentrations for cell division suggests that the effect on nucleic acid synthesis is not a primary effect of LMB. On the other hand, we have observed that LMB caused G1 specific arrest of the cultured Fisher rat kidney fibroblast, 3Y1 cells with inhibition of the DNA replication complex formation, which will be reported elsewhere[15]. This apparent discrepancy may be related to the fact that a pleiotropic control determined by *cdc* 2 acts in *S. pombe* at both nuclear division and DNA synthesis[16]. It is also noticed that G1 phase in cell cycle of *S. pombe* is very short in contrast to the long duration of G2 phase. It seems possible that LMB inhibits an essential step for the initiation of DNA synthesis which occurrs at the end of G2 or the early M phase in *S. pombe*. Further studies to identify the molecular target of LMB will be useful to elucidate the mechanism of eukaryotic cell cycle control.

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