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A MINICELL-PRODUCING MUTANT OF *BACILLUS SUBTILIS*

TAN-CHI HUANG and MIAU-CHAN CHANG

Institute of Botany, Academia Sinica, Nankang, Taipei Taiwan 115, Republic of China(1)

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Abstract

A minicell-producing mutant, ***Bacillus subtilis*** M-37, was isolated from ***B. subtilis*** N-210. Minicells were formed by an unequal cell division occuring near one pole of the rod-shaped parental cells. The spherical minicells with diameter ranged from 0.5 to 0.8 μ were purified from culture by sucrose gradient centrifugation. The minicells contained protein and RNA, but the content of DNA was negligible. The ratio of protein to RNA in minicells was similar to the normal cells. Production of minicells in culture was affected by cultural conditions and the aging of the culture. Thiamine enhanced its formation in synthetic medium, but pentothenic acid, biotin or pyridoxamide decreased the population. Minicells were stable in phosphate buffer at room temperature for about 6 hours after purification, and then they started to decay at a slow rate. The decay process was delayed at low temperature or by treating with chloramphenical.

Introduction

Mutation or environmental stress causes abnormal division of bacteria. One type of abnormal division which happens in rod-shaped bacteria is unequatorial cell division occuring near one pole of cell to form a small spherical "minicell" (Alder *et al.*, 1967). Minicell-producing strains have been isolated from *Escherichia coli* (Alder *et al.*, 1967), *Bacillus subtilis* (Reeve *et al.*, 1973), *Salmonella typhimurium* (Sheehy *et al.*, 1973) and *Haemophilus influenza* (Sedgwick *et al.*, 1975). Based on these studies, minicells have following properties in common: (1) They are deficient in chromosomal DNA and can not grow or divide. (2) They have normal envelope structure and transport system. (3) They have respiratory activity and may have transcription and translation activities if plasmid is present.

Although minicells contain no chromosomal DNA, it is possible to obtain plasmid-containing minicells if their parent cells carry plasmid. It is also possible to introduce a virus genome into minicell by normal bacteriophage

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infection process. Minicells without chromosomal DNA, but having plasmid or virus genome provide a valuble system to study the gene expression. Since studying of gene expression of plasmid or virus genome in normal cell is usually complicated by the gene products directed by chromosomal DNA, minicells have been established as a tool for gene expression of drug resistance plasmids (Levy, 1974; Shivakumar *et al.*, 1979), bacteriocinogenic plasmids (Kool *et al.*, 1974; Inselburg and Applebaum, 1978), recombinant DNA (Alden and Roger, 1977; Meagher *et al.*, 1977) and bacteriophages (Lipinska *et al.*, 1978; Reeve *et al.*, 1978; Smits *et al.*, 1978).

Minicell-producing strain of *B. subtilis* was first reported in 1973 (Reeve *et al.*). It is a filamentous mutant drived from strain of *B. subtilis* div 1V. In our laboratory, a new minicell-producing strain of *B. subtilis* was isolated. This isolate is morphologically similar to normal bacilli, but it can be distinguished from other strains of *B. subtilis* by its ability to produce xanthobacidin and yellowish pigments (Huang and Chang, 1975). In this paper, properties of the new isolate and the production and purification of its minicells are reported.

Materials and Methods

Isolation and enumeration of bacteria

Bacillus subtilis N-210 differentiated from other bacilli by its ability to produce xanthobacidin (Huang and Chang, 1975) is a wild type isolated from rice paddy field. For isolating minicell-producing mutant, *B. subtilis* N-210 was cultivated in liquid PS medium at 30°C for 10 hr with rotatory shaking, and then adding acridine orange at a final concentration of 20 μg/ml. The culture was kept shaking for another 12 hr. Survived bacterial cells were then plated on PS agar. The minicell-producing mutant among survivals was selected by checking each single colony under microscope. Minicell-producing mutant thus obtained was designed M-37.

Concentration of normal cells was measured by colony counting on PS agar plate. To prevent the bacterial colonies from spreading, the agar plates were kept in 60°C oven for 1 hr to evaporate the moisture of plates before plating. For measuring the number of minicells in culture, a loopful of sample taken from the culture was smeared on slide, fixed and stained, and then counting under microscope. The number of minicells and normal cells within the microscopic field were counted, and their ratio was then calculated by dividing the number of minicells with normal cells.

Media

Bacteria were routinely maintained on potato-sucrose (PS) agar which

contains potato extract (200 g of potato per liter), 1.5% of sucrose, 0.5% of peptone, 0.2% of Na_2HPO_4, and 1.5% of agar.

For the production of minicells, *B. subtilis* M-37 was cultivated in a thiamine containing Vogel-Bonner synthetic medium (Thiamine VB medium) consisting 0.2% of $(NH_4)_2SO_4$, 1.4% of K_2HPO_4, 0.6% of KH_2PO_4, 0.1% of sodium citrate $2H_2O$, 0.2% of $MgSO_4 \cdot 7H_2O$, 0.2% of glucose, and thiamine 10 mg/l, pH 7.2.

Culture conditions and purification procedures for minicells

B. subtilis M-37 maintained on PS agar was renewed on a new PS slant and then incubated for 16 hr. The growing young culture in slant was then transfered to liquid "thiamine VB medium" to have an initial concentration of about 2×10^7/ml, and then incubated at 30°C with rotatory shaking for 12 hr. Minicells and normal cells in culture were then centrifuged at 9,000 rpm for 10 min in a SS-34 rotor of Sorvall centrifuge. The pellet was suspended in 0.02 M phosphate buffer, pH 7.2 to have concentration of about 10^{10}/ml. One and a half milliliter of the suspension were layered on the top of 20 ml of 6% sucrose solution and then centrifuged in a HS-4 rotor of Sorvall centrifuge at 3,000 rpm for 5 min. The pellet contained normal cells was discarted. Cells left in supernatant fraction were collected by centrifugation and then resuspended in phosphate buffer. Half miililiter of the cell suspension (about 10^{10}/ml) was layered on top of a 5 to 15% continuous sucrose gradient solution containing in a 29×102 mm tube and spun at 3,000 rpm for 20 min in a HS-4 rotor. After centrifugation, 1.5 ml fractions were taken from top of the gradient. The second and third fractions, which contained minicells, were pooled and collected by centrifugation. Minicells were then washed with phosphate buffer. All procedures of the purification were performed at about 4°C.

Microscopic observation of minicell formation

Hanging block microculture technique was used to observe the production of minicells from a single cell. Three milliliters of melted solid medium were poured in a sterilized slide. After hardening, an agar medium block about 0.5 cm^2 was removed from the slide to a sterilized coverglass. A loopful of bacterial solution in a concentration of about 1×10^7/ml was put on the agar medium block. Smali amount of vaseline was put on four cornors of the coverglass. A hollow-ground slide was attached on the vaseline to have the agar block accommodeted by the "hollow" of the slide, and then inverted the slide. The microculture was then observed with a phase microscope at different period of incubation.

Determination of macromolecule

Amount of DNA, RNA and protein contained in normal cells and minicells were analysed according to the procedures described by Reeve *et al.* (1973).

Results and Discussion

A minicell-producing mutant, *Bacillus subtilis* M-37, was isolated from *B. subtiiis* N-210 after mutagenesis with acridine orange. The mutant appeared either single or double with an average size of 1.0×3.0 μ. Endospores were formed in 50 hr in PS medium at 30°C. The spore was ellipsoidal, central to paracentral with an average size of 0.9×2.0 μ. Pellicle was formed on surface of the liquid bacterial culture when it was grown without shaking. Slight sediment was seen in shaking culture. The colony formed on PS agar plate was dull, flat with rough surface. The bacterium produced slight yellow-green soluble pigment in PS medium. It growed well at a pH range between 5.0 to 8.0 with the optimum at pH 8.0. Optimum temperature for growth was 30° to 40°C. There was acid but no gas formed from sucrose, glucose and mannitol in fermentation test. It hydrolyzed starch, peptonized milk, produced nitrite from nitrate and formed acetyl-methyl carbinol from glucose-peptone water. It contained catalase and urease, but no oxidase or lecithinase.

Bacillus subtilis M-37 formed minicells by an unequatorial cell division near one pole of the cell. Formation of minicells occured simultaneously with normal division or independently (Fig. 1). The population of minicells in liquid culture maintained at low level in the early log phase, and then increased steadily when the cultivation was lasted. It reached the maximum concentration when the culture got into stationary phase (Fig. 2). Observation of the production of minicells from a single cell with microculture technique revealed that minicell usually did not form in the early developing stage (Fig. 3).

Minicells purified from the culture by sucrose gradient centrifugation were spherical with diameter ranged from 0.5 to 0.8 μ (Fig. 4). They did not grow, divide or form spore. They contained protein and RNA, but the content of DNA is negligible. The ratio of protein to RNA content in minicells was similar to normal cells. As indicated in Table 1, the ratio of protein to RNA in minicells was 2.6:1, and in normal cells, 2.7:1. Minicells maintained their integrity in phosphate buffer for about 6 hr at room temperature after

Table 1. *Macromolecular components in normal cells and minicells of* ***Bacillus subtilis*** *M-37*

Type of cells	Cell conc./ml[a] (550 nm)	Macromolecular components (μg/ml)		
		Protein	RNA	DNA
Normal cells	2.54	1,080	400	82
Minicells	2.43	740	288	10

[a] Cell concentration using for this determination was indicated by optical density.

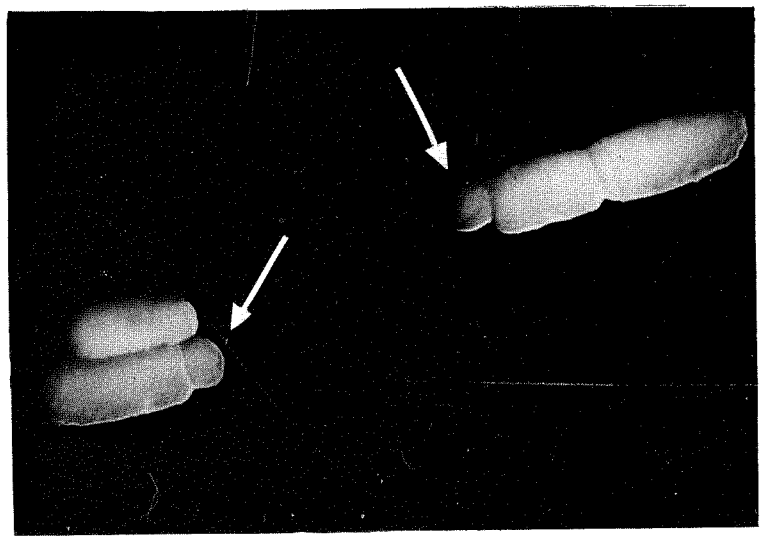

Fig. 1. Electron micrograph showing the formation of minicells (arrow) of ***Bacillus subtilis*** M-37 (14,400 ×).

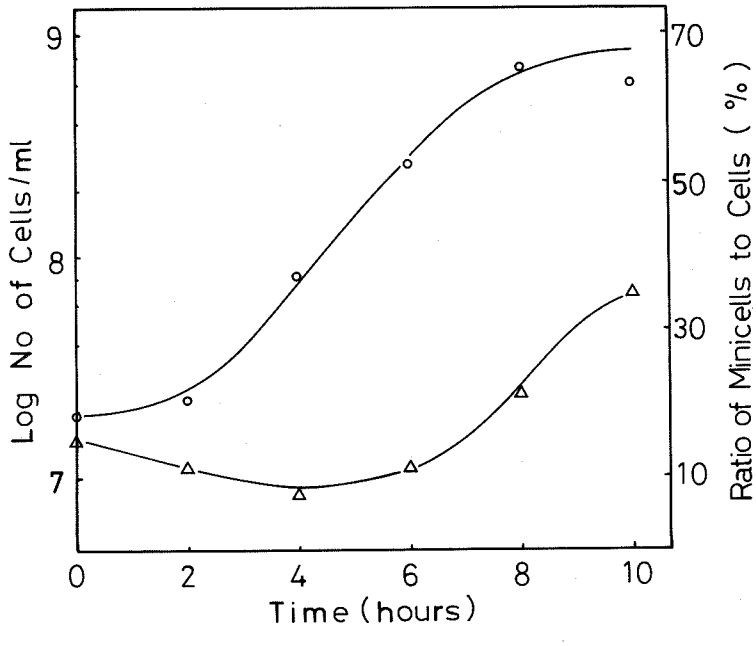

Fig. 2. Growth curve of *Bacillus subtilis* M-37 and the ratio of minicells to normal cells in the culture. The open circles depict the growth of normal cells and the open triangles represent the ratio of minicells to normal cells.

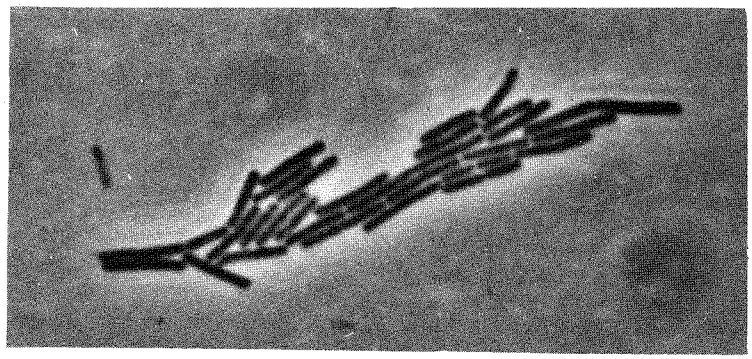

Fig. 3. Photomicrograph of a colony developed from a single cell of ***Bacillus subtilis*** M-37 growing on microculture environment (1,800 ×). Microculture technique was performed as described in "Materials and Methods".

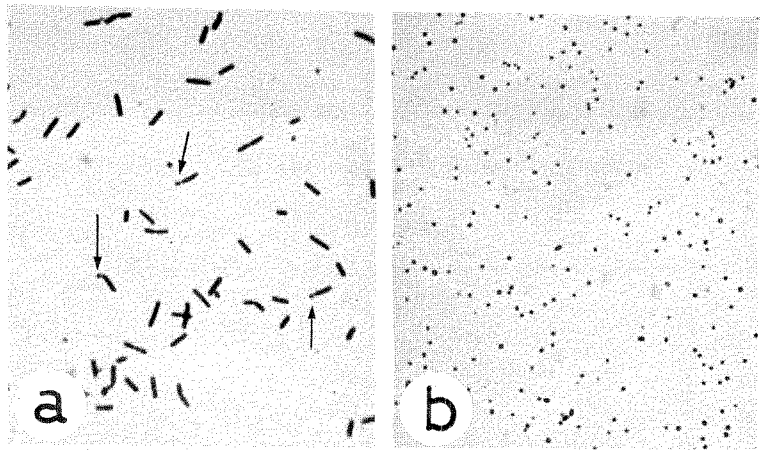

Fig. 4. Photomicrograph of ***Bacillus subtilis*** M-37 and its minicells (1,800 ×). (a) showing the formation of minicells (arrow) in liquid culture; (b) showing the morphology of minicells purified with sucrose gradient centrifugation.

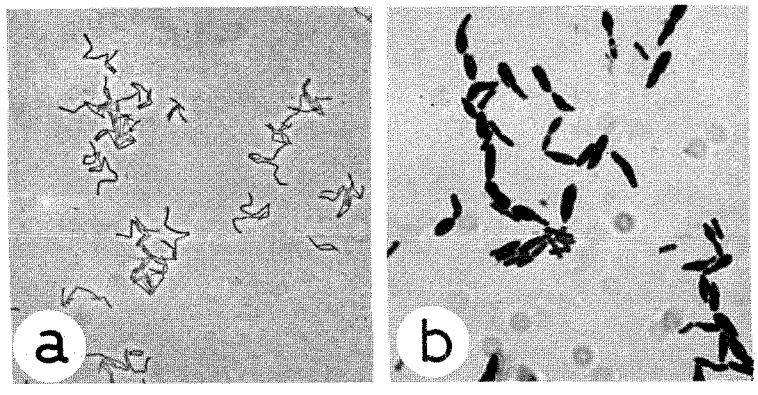

Fig. 5. Effects of environmental stress on the morphology of ***Bacillus subtilis*** M-37. (a) photograph showing the morphological change of ***B. subtilis*** M-37 in the presence of 2 μg/ml of chloramphenical (1,000 ×); (b) ***B. subtilis*** M-37 in the absence of Mg^{++} (2,500 ×).

Table 2. *Effect of growth factors on the population of minicells in the* ***Bacillus subtilis*** *M-37 culture*

Growth factors[a]	Concentration of growth factors (μg/ml)	Ratio of minicells to cells (%)[b]
No growth factor	—	22
Riboflavin	1	22.5
	5	24.5
	10	17.0
Niacinamide	1	26.3
	5	27.5
	10	23.7
Thiamine	1	31.6
	5	34.2
	10	39.1
Pyridoxamide-HCl	1	11.6
	5	11.2
	10	10.5
Pyridoxal-HCl	1	23.2
	5	20.0
	10	20.1
Pantothenic acid	1	13.7
	5	12.0
	10	11.2
Ascorbic acid	1	20.0
	5	21.1
	10	20.5
Biotin	1	14.1
	5	12.5
	10	11.1

[a] *Bacillus subtilis* M-37 on agar slant was transfered to thiamine free VB medium to have a concentration of about 10^7/ml. Growth factors were added to the culture and then incubated at 30°C with rotatory shaking.

[b] Ratio of minicells to normal cells in culture was determined after 6 hr of incubation.

purification. After that, the minicells began to decay and finally lysed. The process of decaying was delayed by keeping them at 4°C or by adding chloramphenical (20 μg/ml) into culture after harvasting.

B. subtilis M-37 produced minicells in a rather widely culture conditions. For example, it formed minicells in minimum media as well as complex media. The values of pH ranged from 5.0 to 8.0, or temperature varied from 25 to 40°C, did not have significant influence on the population of minicells in culture. However, the ratio of minicells to normal cells in culture will be affected by the

presence of certain chemicals. As shown in Table 2, the presence of thiamine in VB medium doubled the ratio of minicell population, but pyridoxamide, pantothenic acid, or biotin caused about 50% of inhibition.

Chloramphenical, mitomycin C, ultraviolet irradiation or thymidine starvation has been reported to induce minicell formation (Chang and Lin, 1974; Sedgwick *et al.*, 1975). However, to enhance minicell production of *B. subtilis* M-37 in restrictive growth condition, such as in the presence of antibiotic or under nutrient limiting condition was unsuccessful. For example, in the presence of chloramphenical, or in the absence of magnesium ion, the formation of minicell was inhibited and its morphology was changed (Fig. 5). Penicillin, although has been used in *E. coli* system to enrich the ratio of minicells during purification steps (Levy, 1970), decreased the stability of minicells of *B. subtilis* M-37.

Minicell-producing mutant of *B. subtilis* was first established and studied by Reeve *et al.* (1973). Since then, no second minicell-producing system has been reported. The new minicell-producing isolate *B. subtilis* M-37, based on morphological and physiological properties, is different from the system established by Reeve *et al.* The new mutant produces large quantities of uniform and stable minicells in a simple culture condition. Therefore, it has potential to be a system for further studies.

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枯草菌之迷你細胞生產菌株

黃檀溪 張妙璨

中央研究院植物所

利用 acridine orange 處理枯草桿菌 N-210，分離出具有產生迷你細胞之變異株——枯草桿菌 M-37。此變異株除了正常分裂外，另有不正常的分裂現象，即在靠近桿狀細胞頂端之處發生分裂而產生一個圓形的小細胞，稱迷你細胞。迷你細胞的大小介於直徑0.5 到 0.8 微米之間，細胞內缺乏 DNA，但是含有 RNA 和蛋白質；RNA 和蛋白質兩者之重量比與正常細胞相似。迷你細胞的產生受環境及培養時期的影響，在合成培養基中，thiamine 增加它的產生，而 pantothenic acid，biotin 以及 pyridoxamide 減少它的產生。迷你細胞純化之後，在磷酸鹽緩衝溶液中可以在室溫維持大約 6 小時的安定，然後開始有衰敗的現象，此衰敗的過程利用降低溫度或氯黴素處理可以減緩。