African Journal of Biotechnology Vol. 7 (10), pp. 1505-1511, 16 May, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Effect of varying temperature on growth, morphology and soluble protein content of *div IV* and *div V* mutant strains of *Bacillus subtilis*

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Accepted 24 July, 2007

Cell division affecting genes (*div* genes) have been mapped on circular chromosome of *Bacillus subtilis*. In the present work, *div*-gene(s) involved in mini-cell production and temperature sensitive cell division were studied. Effect of different temperatures (25, 37 and 42°C) and varying incubation periods (4, 16, 24, 48, 72 and 96 h) on the colony and cell morphology, staining behavior, growth rate and soluble protein content of PY79 (wild type) and *div* mutant strains (IA197, IA292, IA314, IA315) was studied. PY79 differs from *div*-mutants in colony morphology especially in colony margin. High temperature severely affects cell morphology (cell size, cell types, formation of filaments/minicells and staining behavior of cells). Degeneration and lysis was more in *div* mutant strains when compared with PY79 especially at 42°C. Sporulation was found to be affected at non-permissive temperature and *div*-mutants form defective spores at 25 and 42°C. At 37°C spores were formed by *div*-mutants but were defective. Also spores produced at 42°C by *div*-mutants were defective in spore germination. All *div*-mutants have less protein content when compared with PY79 at all temperatures. Also protein content decreases at sporulation stages. Hence *div*-mutations are multifaceted mutations which cause effects on cell morphology, sporulation and germination processes in *B. subtilis*.

Key words: *Bacillus subtilis*, *div*-genes, *div*-mutants.

INTRODUCTION

Cell division is a highly regulated process, which requires high coordination among events like septum formation, DNA replication and separation of two cells. Any defect or disturbance in any of these mechanisms could lead to drastic effects (Cheung et al., 1999; Zaritsky et al., 1999). In *Bacillus subtilis*, genes known as *div*-genes affect cell division. These can be categorized into four classes; (i) temperature sensitive cell division, (ii) minicell production, (iii) filamentous growth and (iv) septum initiation. At different positions of circular map of *B. subtilis*, twelve *div*-genes have been mapped (Dean and Zeigler, 1994).

Cell division in *B. subtilis* is affected by different environmental factors one of which is the extreme of temperature. Mutations and defects in cell division which are induced by non-permissive temperature in *B. subtilis* have been observed. Septum formation is inhibited by non-permissive temperature resulting in filamentation (Sievers and Errington, 2000a). Non-permissive temperature could lead to the formation of DNA less cells (rod, round cells, filaments, minicells). Increased temperature affects sporulation. The sporulation efficiency decreased at 42°C (Cheung et al., 2004). High temperature is found to cause degradation as well as variation in membrane lipids and also imbalance in cellular protein synthesis. Non-permissive temperature also results in thermal denaturation and finally causes cell death and lysis (Ahmed and Sabri, 2004).

The aim of this work is to study the effect of different temperatures (25, 37 and 42°C) on the cell morphology and staining behavior, growth rates as well as soluble protein contents of PY79 (wild type) and *div*-mutants.

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Table 1. Bacterial strains used in this study.

Bacterial strains	Genotype	Phenotype	Source
PY79	Prototroph	Wild type	Youngman et al. (1983)
1A197	*divIVB1 metB5 Spo⁻ thyA1 thyB*	Minicell producing mutant	Bacillus Genetic Stock Center, Ohio State University, USA.
1A292	*divIVB1 metB5 thyA1 thyB1*	Minicell producing mutant	
1A314	*divV32 thr–5 trpC2*	Temperature sensitive mutant	
1A315	*divV71 trpC2*	Temperature sensitive mutant	

MATERIALS AND METHODS

Bacterial strains and growth conditions

B. subtilis prototroph PY79 was supplied by Youngman et al. (1983) and *div*-mutant strains (IA197, IA292, IA314, IA315) were obtained from *Bacillus* Genetic Stock Centre, Ohio State University, USA (Table 1).

For *B. subtilis* growth, nutrient broth, nutrient agar (Gerhardt et al., 1994), PGYEA (Dring and Gould, 1971), Spizezen minimal media (Anagnostopolous and Spizezen, 1961) were used. Spizezen minimal media was supplemented with glucose (0.5%), casein amino acid (0.02%) and amino acids (50 µg/ml). Thymine, methionine, tryptophan and threonine (50 µg/ml) were added whenever required (Anagnostopolous and Spizezen, 1961). Growth was normally obtained at 37°C. For the study of effect of temperature, bacterial growth was observed at 25, 37 and 42°C on solid cultures.

Strain characterization

Following Gerhardt et al. (1994), colony morphology of wild type and *div* mutant strains was studied after 24 h of incubation at 37°C. Ensuing Cappuccino and Sherman (1996), Gram staining was done. Cell morphology of PY79 and *div*-mutants (IA197, IA292, IA314, and IA315) was observed on nutrient agar after incubating the bacterial strains for different times (4, 16, 24, 48, 72 and 96 h) at different temperatures (25, 37 and 42°C). Bacterial growth rate was estimated by constructing the growth curves for which bacterial cultures were incubated at 25, 37 and 42°C for 4, 16, 24, 48, 72 and 96 h and then optical densities were monitored (at 600 nm) and recorded. Method of Moir (1981) was used for authenticating spore-forming ability.

Soluble protein estimation

Following Laemmli (1970) and Hancock (1994), soluble proteins were estimated after 24 and 96 h of incubation at 25, 37 and 42°C.

RESULTS AND DISCUSSION

B. subtilis is a Gram-positive rod-shaped bacterium. This bacterium replicates by the process of cell division. In *B. subtilis*, a set of ten conserved proteins are important for cell division (Errington et al., 2003). About 12 genes, which affect cell division, have been mapped on *B. subtilis* chromosome (Dean and Zeigler, 1994). Mutations in *B. subtilis* are associated with minicell production (1A197, 1A292) as well as filamentation (1A314, 1A315). Wild type strain PY79 and different strains having different mutations for *div* gene i.e., *div IVB* (1A197-mp247B; 1A292-mp247B) and *div V* (1A314 -mp273D; 1A315-mp273D) were used for this work. As regard the colony morphology, PY79 (wild type) differs from *div*–mutants. In PY79, colony margin was entire but in *div*-mutants it ranged from undulate to lobate conditions. As regards the cell morphology, the effect of different temperatures (25, 37 and 42°C) on PY79, 1A197, 1A292, 1A314, 1A315 was observed by varying time of incubation (4, 16, 24, 48, 72, 96 h). All *div* mutants exhibited pleiotropic effects of mutations especially at higher temperature, which includes variability in Gram staining, cell morphology, sporulation and germination (Feucht and Errington, 2005; Anwar et al., 2007).

In PY79, all rods were Gram-positive and similar in shape/size at all temperatures except the arrangement of cells (in chains, pairs and isolated) which was different at different temperatures and time of incubation. Filaments were absent in PY79. In both *divIVB1* mutant strains (1A197 and 1A292) at 25 and 42°C, both filamentous cells and filaments were obtained which may be Gram positive, Gram negative or Gram variable whereas at 37°C no filaments /filamentous cells (except in 1A292 after 4 h) were present (Tables 2, 3 and 4). In case of *divV* mutant strains (1A314 and 1A315) variability in frequency of filaments/filamentous cells was found at different temperatures and time of incubation (Tables 2, 3 and 4). Increased temperature inhibits septation and many bacilli grow as long non-septate filaments (Feucht and Errington, 2005). According to Reeve et al. (1973), *div* mutants grow as long snakes during early and mid log phase of growth. In general, in different *div* mutants in early stages, filamentous cells were present whereas in later phases long filaments were more frequent. The *fts* genes are involved in filamentation. Fts Z is a cytosolic protein. It is a tubulin homologue and can polymerize like tubulin to form a ring like structure (the Z ring) at the division site. The Z ring plays a key role in constriction of the cell membrane as well as in coordination of the whole process of division (Sievers and Errington, 2000b). The

Table 2. Frequency of different cell types per 100 cells of *B. subtilis* strains PY79 (wild type), 1A197, 1A292, 1A314 and 1A315 (*div*–mutants) at 25°C after incubating for different times on nutrient agar medium.

Time	Cell type	*PY79*	1A197 *div IV*	1A292 *div IV*	1A314 *div V*	1A315 *div V*
24 h	Rods G+ve	99.0	11.50		5.0	
	G-ve		13.80			10.0
	Gv			15.50		
	Filamentous cells G+ve		57.40			
	G-ve		5.80			
	Gv			50.50		
	Long filaments G+ve				86.0	
	G-ve				4.0	
	Gv		4.60			79.0
	Minicells		4.60	13.0	5.0	
	Oval cells			13.0		
	Round cells G-ve					11.0
	Unipolars	1.0				
	Bipolars		2.30			
	Refractile spores			8.0		
48 h	Rods G+ve	100	15.49			
	G-ve					2.67
	Gv			20.0		8.95
	Filamentous cell G+ve		51.55			
	Gv			73.0		
	Long filamentous Cells Gv		25.75			
	Very long filaments Gv				100	71.42
	Minicells			4.0		13.39
	Minicells in chains		7.12			
	Oval cells					3.57
	Defective spores			3.0		
72 h	Rods G+ve	99.90				
	G-ve		1.0			6.54
	Gv		19.0	20.0		
	Filamentous cells Gv		30.0	80.0		
	Filaments G-ve					9.35
	Gv		50.0		100	84.11
	Unipolars	0.07				
	Bipolars	0.03				
96 h	Rods G+ve	7.69				
	G-ve		54.28			50.0
	Gv			5.0	6.96	14.0
	Filamentous cells G-ve					7.0
	Gv				59.94	7.0
	Minicells		19.37	6.70		7.0
	Minicells in chains				29.46	15.0

Table 2. Contd.

	Round cells			2.70		
	Oval cells		1.55			
	Unipolars	34.60	4.65	22.0		
	Bipolars	38.50	4.65	33.0	3.64	
	Spores		15.50	1.67		
	Defective spores	19.20		28.93		

Table 3. Frequency of different cell types per 100 cells of *B. subtilis* strains PY79 (wild type), 1A197, 1A292, 1A314 and 1A315 (*div*–mutants) at 37°C after incubating for different times on nutrient agar medium.

Time	Cell type	*PY79*	**1A197** ***div IV***	**1A292** ***div IV***	**1A314** ***div V***	**1A315** ***div V***
24 h	Rods G+ve		33.40			
	G-ve		11.0	8.62	10.0	
	Gv	4.04	4.41			18.0
	Filamentous cells G-ve				63.63	
	Filaments G-ve				22.74	
	Minicells		10.93	0.01		12.0
	Unipolars		10.20	5.17		20.0
	Bipolars	35.96	17.07	60.34	3.63	20.0
	Spores	60.0	1.09	25.86		30.0
	Defective spores		11.90			
48 h	Rods G+ve		12.73	Lysis		
	G-ve		3.20			33.50
	Gv	4.43	25.50			
	Filamentous cell G-ve				31.20	
	Minicells		5.10		15.66	
	Minicells in chains		2.54			
	Unipolars	13.83	2.54			
	Bipolars	21.93	9.55		31.27	
	Spores	59.81	38.84			
	Defective spores				21.87	66.50
72 h	Rods G-ve		10.0	Lysis	Lysis	10.0
	Gv	2.10	11.0			
	Larger cells (violet)		2.0			
	Unipolars	10.69	10.0			
	Bipolars	5.04	15.0			
	Spores	82.17				
	Defective Spores		52.00			90.0
96 h	Rods Gv			Lysis	Lysis	Lysis
	Oval cells		39.13			
	Spores	100				
	Defective spores		60.87			

Table 4. Frequency of different cell types per 100 cells of *B. subtilis* strains PY79 (wild type), 1A197, 1A292, 1A314 and 1A315 (*div*–mutants) at 42°C after incubating for different times on nutrient agar medium.

Time	Cell type	*PY79*	1A197 *div IV*	1A292 *div IV*	1A314 *div V*	1A315 *div V*
24 h	Rods G+ve	3.84		Lysis		
	G-ve		15.22			
	Gv				61.72	
	Filamentous cells G-ve					81.39
	Gv		8.36		11.12	11.63
	Long filaments G-ve				18.52	
	Minicells		16.15			
	Minicells in chains		56.64			
	Round Oval cells				8.64	
	Unipolars	15.38				
	Bipolars	23.07	3.63			
	Spores	57.71				
	Defective spores					6.98
48 h	Rods Gv	2.32	Lysis	Lysis	Lysis	
	Unipolars	2.20				16.66
	Bipolars	24.40				
	Spores	71.08				
	Defective spores					83.33
72 h	Rods G-ve	4.0	Lysis	Lysis	Lysis	Lysis
	Gv	8.0				
	Spores	88.0				
96 h	Spores	100	Lysis	Lysis	Lysis	Lysis

inactivation of *fts* Z (filamentous temperature sensitive) gene product or its elimination results in the formation of filaments lacking constrictions. The filamentation phenol-type would be the consequence of two events. First, a moderate filamentation would result from the minicell divisions. A second cause of filamentation would be that, without the pilot protein (DivIVA) to direct Min CD to the polar sites, Min CD would be available to block septation at mid cell (Hamoen and Errington, 2003) thus giving rise to filamentous growth. The polar and mid cell inhibitions are somewhat leaky, giving rise to some minicells and some residual cell division.

Minicell production varies in *div*-mutant strains with temperature and time of incubation (Tables 2, 3 and 4). Maximum minicell production in most of cases was observed between 24/96 h (rarely at 4, 16, and 48 h). Sabri and Hasnain (1992) reported that in *divIVA* mutant (1A196) maximum minicells in most of cases are after 8 or 12 h. According to Reeve et al. (1973) minicells are produced at all stages of culture growth but are more numerous during the transition from log phase to stationary phase. Minicells are formed in identical manner, a division close to proximity to pole of a growing cell results in minicell formation (Ahmed and Sabri, 2004).

In *div* mutant strains, maximum number of dark oval cells were found in 1A197 (~ 39%) after 96 h, at 37°C, in 1A292 (~ 13%) after 24 h, at 25°C, in 1A314 (~ 8%) after 24 h, at 42°C, in 1A315 (~ 3%) after 48 h, at 25°C (Tables 2, 3 and 4). From above discussion it is clear that *div* mutants (1A197, 1A292, 1A314, 1A315) had pleiotropic effects of mutations. 1A197, 1A292 in addition to minicells produce filaments, oval cells or other abnormal cell types whereas 1A314, 1A315 produce all other abnormal cell types in addition to filamentous growth. The same pleiotropic effect of *div* mutation was also reported by Sabri and Hasnain (1992, 1994) and by Ahmed and Sabri (2004). Many temperature sensitive markers affect cell division at restricted temperature (45°C) and by altering plane of division, formation of DNA less cells (round cells, filaments, minicells) occur (Migocki et al., 2002; Feucht and Errington, 2005). In general abnormalities in cell shape and staining behaviour and sporulation was more in *div* mutants as compared to wild type (PY79). In different *div* strains variability in cell shapes like filaments with one thicker/swollen end, bloated, curled and curved ends were observed while in vegetative rods either ends were swollen or complete rod became thicker to give the appearance of coccoid form. Further the rods / filaments

Table 5. Effect of temperature (25, 37 and 42°C) on soluble protein content of wild type (PY79) and *div*-mutants (1A197, 1A292, 1A314 and 1A315).

Bacterial strains	Protein contents (mg / gm)					
	24 h			96 h		
	25°C	37°C	42°C	25°C	37°C	42°C
PY79	46.0 ± 1.23	0.96 ± 0.08	16.66 ± 0.09	18.66 ± 0.09	0.57 ± 0.05	11.42 ± 0.07
1A197	9.29 ± 0.04	0.43 ± 0.01	11.00 ± 0.08	10.5 ± 0.06	0.33 ± 0.02	4.00 ± 0.008
1A292	22.66 ± 0.06	0.46 ± 0.02	13.00 ± 0.07	12.00 ± 0.05	0.48 ± 0.03	7.71 ± 0.08
1A314	21.91 ± 1.50	0.81 ± 0.07	14.33 ± 1.37	14.85 ± 0.09	0.55 ± 0.04	6.88 ± 0.05
1A315	10.30 ± 0.90	0.34 ± 0.09	8.00 ± 0.63	13.00 ± 0.06	0.21 ± 0.009	5.40 ± 0.03

arranged themselves in complete circular form, semi-circular, curved, V-shape. These abnormal cell types were more at 25 and 42°C when compared with 37°C. Sometimes (in pairs or in chains) one normal cell and other filamentous cell or one Gram-positive and other Gram-negative (or with variable staining behaviour) were also observed. In few cases alternate cell types (one rod, one filamentous or minicell) as well as variable staining behaviour (one Gram-positive other Gram negative then Gram-positive) in filaments were noticed. These types were rather frequent at high temperature. Change in staining behaviour may be due to change in membrane composition. Variation in membrane lipid of bacteria (Mackey et al., 1991) as well as changes in membrane protein composition has been reported with increased temperature.

An added complexity to septum site selection system in *B. subtilis* is the sporulation process. In *div* mutant strains (1A197, 1A292, 1A314, 1A315) sporulation frequency, shape, staining and germination were affected with varying time and temperature (Ahmed and Sabri, 2004). In PY79 spores appeared as a clear central portion with very faintly stained surface walls at all temperatures. In *div*-mutant strains, normal spores were only formed either at 25°C or at 37°C but their frequency was very low (Tables 2 and 3). In addition to normal spores at 25 and 37°C, defective spores were also observed. The defective spores were darkly stained retaining CV-complex, thick walled, in some cases the spores showed variability in wall thickening. Sabri and Hasnain (1996a) reported that *divIVA* mutant could form germination defective spores at 45°C and they attributed the defect of *divIVA* spores to *ger* B pathway. Hence *div* mutants (1A197, 1A292, 1A314, 1A315) were checked for spore germination ability at 37 and 45°C by tetrazolium overlay test which ensures the germination of spores from heated inoculum. *div*-mutants were defective in spore germination at 45°C. The Spo- may be due to change in series of biochemical and physiological processes which result in loss of resistance of cell to heat (Venkatasubramanian and Johnstone, 1993).

The loss of viability at high temperature is due to inability of cells to divide and form colonies, rather than cell death (Holmquist and Kjelleberg, 1993). Decline in ATP content preceded loss of viability of cells. It also may be due to the temperature effect on biochemical and physiological processes, blocking of major cell structural components, defects in cell envelope and membrane protein (Mengin – Lecreulx et al., 1998). Rise in temperature also cause thermal denaturation, deformation, resolution of cells, degeneration, cell lysis, death and loss of cell viability (Ahmed and Sabri, 2004).

To determine whether there is a relationship between bacterial growth, cell types and sporulation, growth rates of PY79 and *div*-mutant (1A197, 1A292, 1A314, 1A315) strains were recorded at different temperatures by varying time of incubation (4, 16, 24, 48, 72, 96 h). When growth rates of wild type and *div*-mutants were compared, it was concluded that mutants had slow growth rates as compared to wild type.

To probe whether difference in wild type (PY79) and *div* mutants (1A197, 1A292, 1A314, 1A315) are due to change in protein content, soluble protein content was estimated at different temperatures (25, 37 and 42°C) after 24 and 96 h. At all temperatures, protein content was less in *div* mutants when compared with PY79. At 25 and 42°C, the protein content was more as compared to 37°C (Table 5). Exposure of cells to increased temperature triggers the induction of a phenomenon known as the heat shock response which is characterized by the enhanced synthesis of a set of proteins, called heat shock proteins (Sabri and Hasnain, 1996b). Various stressful stimuli in addition to heat shock can result in increased synthesis of heat stable proteins and these proteins most probably protect cells from stress. DNA binding proteins (HU) are also affected by high temperature. The expression of *fts* genes is altered by the HU proteins. HU proteins perturb expression of cell division genes triggering cell filamentation and production of anucleate cells (Ahmed and Sabri, 2004).

From overall discussion it is concluded that high/low temperature cause abnormalities in cell shape, size, staining behaviour, sporulation and germination. Further at high temperature lysis and degeneration of cells is more pronounced. It appeared that *div* mutations (*div IV, V)* are multifaceted mutations which cause developmen-

tal defects in vegetative as well as sporulating stages. Further work will impart the role of different *div* mutations (*div IV*, *V)* in the morphogenesis of *B. subtilis* involving cell division, sporulation and germination processes.

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