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Germination Response of *Bacillus subtilis* *div* Mutants to ALA and AGFK System

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Abstract: The present study deal with the germination analysis of spores of different *div* mutants (*divI*, *divII*, *divIV* and *divV*) of *Bacillus subtilis* in ALA and AGFK systems and the relationship of these mutations with ger genes. Eight *B. subtilis* strains PY79 (wild type) and 07 different *div* mutants were the subject of this study. All *div* mutant strains produce germination defective spores. Their germination response was analyzed in both ALA and AGFK systems as well as in the presence of glucose, fructose and combination of both in ALA system. Inhibition effects of HgCl₂ and NaN₃ and reversal by mercaptoethanol and aqueous washing, respectively, were studied. On the basis of these results 1A292 (*div IVB*), 1A314 (*div V*) and 1A315 (*div V*) were aligned with *gerD*, *gerA* and *gerC* mutations, respectively. Peptidoglycan components (hexoamines, teichoic acid, diamino pimelic acid) of germination defective spores exhibit variation from wild type, hence changes in wall composition leads to defects in cell division and then spore germination. These studies support our earlier finding (the bases of this project) that *div* mutants (previously *div IVA* which was aligned to *gerB*, now *div IVB* and *div V*) exhibit pleiotropic effects.

Key words: Spore germination, *div* mutants, *B. subtilis*, wall composition

INTRODUCTION

Nutrient deficiency/stress conditions induces sporulation that through a well defined series of morphological stages and biochemical processes that involve conversion of a growing vegetative cell into spore^[1,2]. Bacterial spores retain and alter sensory mechanism. However they respond to spore specific germinants, inspite of their extreme dormancy and thermostability. Germinants are the specific molecules that trigger spore germination. Defect in spore germination, hence, could be corroborated by inducing germinants. Spores respond to at least two different types of germination stimuli: i) L-alanine (ALA-system) and ii) combination of L-asparagine, glucose, fructose and KCl (AGFK)^[2-4]. The germination response is initiated by sequential interrelated biochemical events. A number of *ger* mutants (*gerA*, *B*, *C*, *D*, *E*, *F*, *G*, *H*, *I*, *J*, *K* and *M*) have been described. On the basis of germination responses to germinants they have been characterized in three major categories; i) deficient in ALA but normal in AGFK, ii) deficient in both ALA and AGFK, iii) deficient in AGFK but normal in ALA-system^[1,3,4].

The spore coat, cell wall characteristics, structural properties of wall, nature of polymers attached to cell wall greatly determine the morphogenetic pathways of the

bacterial cells^[5,6]. The peptidoglycan cortex of the endospore of *B. subtilis* which constitute 40-80% of the total spore weight is required for maintenance of spore dehydration and dormancy. A significant difference between spore and growing cell peptidoglycan structure is the low peptide cross-linking in cortical PG^[7]; regulation of the degree of this cross-linking is exerted by D, D-carboxypeptidases^[8]. Secretion of specific substances and the hydrolysis of peptidoglycan (PG) in spore cortex are important and crucial events in germination. During germination loss of heat resistance of spores as well as loss of absorbance lead to the regaining of the metabolic activity of vegetative cells^[2,4,9,10]. The content of teichoic acid (TA), diamino pimelic acid (DAP) and peptidoglycan (PG) and their composition vary with growth cycle and cell shape^[1,2].

Many *div* mutants^[11] and temperature sensitive strains of *B. subtilis* are non-sporformer, at 42°C^[12]. Earlier research on *B. subtilis divIVA* mutant strain revealed the formation of spore at 45°C but these spores lack the germination ability and defect in germination is linked with *gerB* mutation. Germination defective spores of *divIVA* mutant exhibits different wall composition. Based upon these findings the present study deals with the germination analysis of spores of different *div* mutants (*divI*, *divII*, *divIV* and *divV*) strains in ALA

and AGFK systems. The wall composition (teichoic acid, diamminopimelic acid and peptidoglycan) of vegetative cells and spores of these mutants was also reported.

MATERIALS AND METHODS

Bacterial strains: Wild type as well as *div* mutant strains of *Bacillus subtilis* were used.

Prototroph: PY79 (13).

Mutant strains: 1A197 (*divIVB1, metB5 spo(-) thyA1, thyB1*), 1A292 (*divIVB1, metB5, thyA1* and *thyB*), 1A314 (*divV32, thr-5 trpC2*), 1A315 (*divV71, trpC2*), 1A316 (*divII55, thr-5 trpC2*), 1A317 (*divI61, thr-5 trpC2*) and 318 (*divI32, trpC2*) were obtained from BGSC, Ohio State University, USA. 1A197 and 1A292 were minicell producing strains while 1A314, 1A315, 1A316, 1A317 and 1A318 exhibit temperature sensitive mutations.

Bacterial growth conditions: Nutrient agar, Penassay Broth (PAB), Potato Glucose Yeast Extract (PGYEA) and spizezen minimal media were used^[14]. Media was supplemented with methionine, thymine, threonine, tryptophan whenever required. Growth was normally at 37 or 45°C.

Morphological studies: Following Gerhardt *et al.*^[14] wild type as well as mutant strains were morphologically studied. Overnight cultures at 37 and 45°C were used for morphological observation. Whereas spores were obtained after incubation for 96 h at 37 and 45°C.

Spore germination test: Spore germination was checked by tetrazolium overlay method of Moir^[15] both at 37 and 45°C.

Germination studies: Spore germination in ALA and AGFK pathways was checked by ensuing Venkatasubramanian and Johnstone^[16]. Spores were prepared by growing bacterial strains (wild type as well as mutant strains) at 37 and 45°C for 96 h. 50 mM phosphate buffer supplemented with 1 mM alanine and 30 mM asparagine (pH 7.4) was used for spore germination response in ALA-system. While for AGFK system, 50 mM Tris/HCl buffer, (pH 7.5), 5.6 mM glucose, 5.6 mM fructose, 67 mM KCl, 30 mM asparagine or 1 mM alanine were added. Spore germination was calculated as percentage loss in OD₆₀₀ of cultures after 60 min. The germination rate was calculated as rate of loss of OD₆₀₀

divided by initial OD₆₀₀. The spore germination was confirmed by observing cultures microscopically after Gram-staining.

Effects of HgCl₂ and NaN₃ on germination of spores: Venkatasubramanian and Johnstone^[16] was followed for studying the overall effects of HgCl₂ (3 mM) and NaN₃ (10 mM) in ALA and AGFK-systems. Inhibitors were added at 37 and 45°C for 15 min before the addition of germinants in respective ALA and AGFK-buffers. The percentage inhibition of OD₆₀₀ was determined after 60 or 80 min, respectively, for ALA and AGFK systems.

Reversal of inhibitory effects of inhibitors: Ensuing Venkatasubramanian and Johnstone^[16] 7 mM β-mercaptoethanol and washing were used for observing reversal of inhibitory effects of HgCl₂ and NaN₃, respectively.

Measurement of resistance of spores to toluene: The germination ability of spores was checked by treating spores with toluene. Samples (1 mL) were treated with toluene (0.025 mL/mL) for 1 min^[17]. After expelling toluene from medium and serial dilutions were plated onto nutrient agar. Tol^R values were expressed as colony forming unit per millilitre.

Cell wall analysis

Preparation of cell walls: Bacterial strains were grown in penassay broth (pH 7) at 37 and 45°C for 24 h. Then 100 μL from these cultures was inoculated in prewarmed PAB and incubated at 37 and 45°C for 24 and 96 h. Following Hancock^[18] cell walls from vegetative cells (after 24 h) and sporulating cells (after 96 h) were purified.

Determination of peptidoglycan: After purifying cell walls, hydrolysate of samples was prepared in 3 N HCl (heated for 4 h at 95°C). For estimation of hexosamines in hydrolysate method of Morgan-Elson, as described by Gerhardt *et al.*^[14], was used. All N-acetylated hexoamines react with ρ-dimethyl-aminobenzaldehyde to produce a red-colored product which was monitored at 585 nm.

Determination of teichoic acid: Method of Chen *et al.*^[19] was used for determination of teichoic acid (as organic acid) in purified cell walls of PY79 and *div* mutants at 37 and 45°C for 24 and 96 h. Bacterial digestion was performed with ethanolic magnesium nitrate^[20]. Blue colored product was monitored at 820 nm.

Determination of diaminopimelic acid: DAP was estimated in purified the cell walls of *div*⁺ and *div* strains, which were grown at 37 and 45°C for 24 and 96 h. Following Work^[21] yellow colored product produced by acidic ninhydrin was read at 440 nm.

RESULTS AND DISCUSSION

Many *div* genes, which affect cell division have been mapped on *B. subtilis* chromosome. Wild type strain and different *div* mutants strains having different mutation for *div* gene, i.e., *divI* (1A317, 1A318), *divII* (1A316), *divIVB* (1A197, 1A292), *divV* (1A314, 1A315) were used for this work. *div*-mutations in these strains encompass the whole range of *B. subtilis* chromosome. They exhibit temperature sensitive (1A314, 1A315, 1A316, 1A317, 1A318) and minicell production (1A292, 1A197) *div*-mutations. The colony and cell morphology of these strains and spore germination response in ALA and AGFK systems of these strains were studied. Defective spore morphology and variation in gram-staining is associated with cell wall components, hence comparison of wild type (PY79) and *div* mutants with respect to cell wall composition was also made.

Morphological studies: All *div*-mutant and wild type resembled with one another but they differ in their margin. In PY79 it was entire but in *div* mutants it ranged from undulate to lobate conditions. Sabri and Hasnain^[22] reported that *divIVA* mutants had highly undulate margin. VanAlstyne and Simon^[23] also described variation in colony characteristics of *div* mutants. As regards the cell morphology, all *div* mutants whether they are minicell producing or temperature sensitive they exhibited pleiotropic effects of mutations especially at 45°C which include variability in Gram -staining, cell morphology, sporulation and germination. In addition to minicells 1A197 and 1A292 also produced unseptate filaments, dark oval cells, round cells. In the same way temperature sensitive strains also produced minicells and other abnormal cell types in addition to filaments. The pleiotropic effects of *div* mutation on cell morphology is attributed due to defect in placement of the septum^[5]. Higher temperatures inhibits septation which leads to extensive filamentation^[24,25]. PY79 and all *div* mutants were also checked for spore forming ability at 45°C and spore germination by using tetrazolium overlay test which ensures the spore germination from heated inoculum. Nevertheless existed reports impart defect in *div* genes hinder the spore forming ability of *B. subtilis*. Many cell division mutants are non-spore former^[11,23].

Sabri and Hasnain^[26] reported *divIVA* mutants could form germination defective spores and they attributed the defect of *divIVA* spores to *gerB* pathway. Hence *div* mutant strains were checked for spore forming and spore germination abilities. *Div* mutants were defective in spore germination at 45°C. Even spores, at 37°C, of 1A197 (*divIVB*), 1A292 (*divIVB*), 1A318 (*divI*) produced but were defective in germination. Microscopic examination of spores also revealed that they were defective spores. Pleiotropism in spore wall thickening and retention of stain in defective spores was microscopically studied. Sabri and Hasnain^[22,27] reported pleiotropism in the appearance of spores.

Spore germination studies

Effects of L-alanine and L-asparagine on spore germination: The germination response of PY79 and 1A197, 1A292, 1A314, 1A315, 1A316, 1A317 and 1A318 spores were analyzed for ALA and AGFK-systems at 37 and 45°C. For ALA-pathway spores were induced to germinate by incubation in the presence of germinant L-alanine (1 mM) and 50 mM potassium phosphate buffer. In ALA system PY79 displayed more germination at 45°C than at 37°C. However in *div* mutant strains germination response differed with strains. 1A197 showed improved germination at both temperatures than wild type. Whereas 1A292 was germination defective at 45°C and failed to germinate at this temperature, but at 37°C more germination than wild type was recorded. 1A314 did not germinate at 37°C but at 45°C germination response was significantly higher than wild type strains. 1A315 was germination defective at both temperatures and yielded 0% germination at 45°C. *divI* and *divII* spores (1A317, 1A318, 1A316) were defective at 37°C in ALA system and slightly less germination than PY79 was recorded but their response was significantly high at 45°C than that of wild type. 1A316 yielded maximum growth at 45°C (Table 1). Sabri and Hasnain^[22,27] reported germination defect in the spores of *divIVA* mutants at 45°C. Two germination responses are well studied in *B. subtilis*. Either alanine alone is enough for spore germination or a mixture of Asn, Glc, Fru and K ions (AGFK). In case of AGFK system in the presence of asparagines (30 mM) spore germination rate of PY79 at 37°C was 8 fold more than its rate in ALA system at the same temperature. 1A314 yielded maximum germination rate at 37°C among the mutant strains. 1A292 spores were defective at 37°C and completely failed to germinate at elevated temperature. Rest of mutants observed significantly high percentages of germination than that of PY79 at 45°C. Spore germination response of 1A318 was slightly reduced at 37°C than the wild type.

Table 1: Spore germination response of *Bacillus subtilis* PY79 and *div* mutant strains in ALA and AGFK systems

Bacterial strain	<i>div</i> mutation	Temperature (°C)	ALA system		AGFK system	
			Alanine	Asparagine	Alanine	Asparagine
PY79		37	5.670	15.370	32.800	44.426
		45	8.390	24.647	30.636	16.110
1A197	IVB	37	7.384	34.740	26.672	45.645
		45	12.070	20.425	66.021	24.847
1A292	IVB	37	11.695	13.870	29.331	29.409
		45	0.000	65.880	23.086	0.000
1A314	V	37	0.000	11.795	9.118	75.546
		45	27.055	31.780	31.618	35.250
1A315	V	37	2.340	0.000	40.584	49.000
		45	0.000	0.000	72.490	85.785
1A316	II	37	4.640	0.000	38.303	46.519
		45	33.729	0.000	17.020	96.290
1A317	I	37	4.720	0.000	33.744	46.295
		45	12.826	13.970	30.810	52.620
1A318	I	37	4.308	1.717	35.098	39.599
		45	30.030	13.070	35.770	67.143

In 1A197, 1A315, 1A316, 1A317 and 1A318 spore germination response was almost the same at 37°C, while at higher temperatures 1A197 yielded different response than other mutant strains. The germination response in 1A197 was relatively less than at 37°C but in other cases it was higher at elevated temperatures. Spore germination. *Ger* mutants have been isolated which are defective in either or both of these responses^[3,4]. These are separate germinant-specific triggers, which later lead to convergence of the germination pathways. A series of critical number of events have taken place, when spores show germination response^[9]. During spore germination spore coat releases lytic enzyme which result in loss of absorbance^[2,28]. Germination Specific Cortex Lytic Enzymes (GSLES) have been reported in cortex hydrolysis^[2]. A number of *ger*-mutations have been identified^[3]. The *gerA* and *gerC* mutants are defective in ALA pathway and showed temperature sensitive spore germination in phosphate buffer supplemented with alanine. *gerB* and *gerK* mutants are defective in the AGFK system, but germinate normally in alanine. Mutants *gerD* and *gerF* are defective in both germination pathways. These mutants germinate slowly in the presence of alanine while they failed to germinate in the presence of asparagine^[29]. Except 1A314, 1A318 (45°C) and 1A197 (37°C), *div* mutants being analyzed are defective in spore germination in AGFK system.

Venkatasubramanian and Johnstone^[16] reported that L-Ala can replace L-Asn in the AGFK pathway (the GFAlaK pathway) and L-Asn can be replaced with L-ALA in ALA-pathway. In ALA-pathway, ALa was replaced with 30 mM Asn while in AGFK pathway spores were induced to germinate in the presence of L-ALA (1 mM) instead of L-Asn. Germination response was recorded after 60 and 80 min in Ala and AGFK

systems, respectively, revealed great variation in observed germination rates of wild type as well as mutant strain's response. In ALA-system the replacement of ALA with Asn resulted in 3 fold increase in spore germination in PY79 than in normal ALA-pathway, at both temperatures. However 1A315, 1A316 at both temperatures and 1A317 at 37 failed to germinate at all. Response of 1A318 was significantly poor in ALA system with replacement. On contrary, in AGFK replacement 1A197 and 1A314 *div* mutants rendered defective spore germination at 37°C. PY79 responded in the same manner at both temperatures. 1A292 was defective in germination response at 45°C in AGFK system, but with the Asn replacement significant germination was recorded. 1A292 did not show any change in %age spore germination at 37°C in normal as well as replacement in AGFK systems. In rest of *div* mutants strains Ala replacement caused some decrease in the germination rate at both temperatures (Table 1). Germination receptors in ALA and AGFK pathways can functionally interact with each other to commence *B. subtilis* spore germination^[4]. Hasnain and Sabri^[26] reported that spore germination was exhibited when L-ALA was replaced by L-Asn or vice versa. There are genetical and physiological differences between ALA and AGFK germination response^[4,9].

Effect of HgCl₂ and NaN₃ on spore germination: Spores of wild type and mutants of *B. subtilis* respond differently to germination inhibitors HgCl₂ and NaN₃^[16], hence the inhibitory effects HgCl₂ and NaN₃ were studied both at 37 and 45°C, in AGFK and ALA systems.

As regard the percentage spore germination in ALA and AGFK systems, PY79 responded both at 37°C as well as 45°C, even after addition of germination inhibitor NaN₃. While addition of 10 mM NaN₃, in ALA system completely inhibited the spore germination in mutant 1A315, 1A316 and 1A318 at both temperatures and that of 1A317 at 37°C and 1A136, 1A318 at 45°C. Repeated Washing with water resulted in reversion of inhibitory effects of NaN₃ on 1A315, 1A316, 1A317 at 37°C and 1A318 at 45°C (Table 2). In rest of cases even washing failed to revert NaN₃ induced inhibition. While in AGFK systems NaN₃ induced spore germination in 1A317, 1A318 at 37°C; 1A292 at 45°C and 1A197 at both temperatures. Spore germination inhibition in certain *ger* mutants can be reversed with washing but even several washings with water failed to remove inhibitory effects in the strains in AGFK system. At 45°C, 1A197 and at both temperatures, 1A292 showed stimulation in spore germination response, instead of inhibition by NaN₃ in AGFK system (Table 2).

Table 2: Spore germination inhibition by NaN₃ and reversion by repeated washings with water

Percentage germination (NaN ₃)		ALA system		AGFK system	
		37°C	45°C	37°C	45°C
PY79	A	14.330	49.4400	24.2990	15.380
	B	82.180	50.8960	0.0000	52.653
1A197	A	24.680	89.5380	5.0259	39.896
	B	35.515	68.6900	0.0000	0.000
1A292	A	11.110	28.9670	81.8300	24.369
	B	68.590	91.6500	9.3100	0.000
1A314	A	9.190	21.6530	25.9330	24.067
	B	25.890	56.4810	24.8130	35.400
1A315	A	0.000	0.0000	32.4130	45.073
	B	70.832	0.0000	80.6850	72.175
1A316	A	0.000	0.0000	37.5120	37.915
	B	10.080	0.0000	72.1600	73.539
1A317	A	0.000	1.2185	19.9650	14.476
	B	71.000	76.3110	0.0000	67.500
1A318	A	0.000	0.0000	30.4910	28.335
	B	0.000	80.1760	0.0000	72.087
Percentage Inhibition (NaN ₃) (A)/Reversion (B)					
PY79	A	45.304	4.530	*	*
	B	0.000	326.800	14449.380	606.62
1A197	A	88.989	*	*	*
	B	0.000	0.000	480.970	569.09
1A292	A	*	0.000	5.002	*
	B	31.656	0.000	586.490	0.00
1A314	A	65.672	31.724	*	19.96
	B	32.844	100.420	0.000	208.76
1A315	A	33.861	47.458	100.000	0.00
	B	164.630	84.134	3027.000	0.00
1A316	A	19.362	60.624	100.000	100.00
	B	80.638	76.372	217.200	0.00
1A317	A	56.874	72.489	100.000	90.99
	B	0.000	128.290	1506.350	594.97
1A318	A	23.000	57.799	100.000	100.00
	B	0.000	107.730	0.000	266.98

* Stimulation,

A = Spore germination in the presence of NaN₃

B = Spore germination after washing with water

Inhibitory effects of HgCl₂ were also studied on wild type *B. subtilis* and its *div* mutants at both temperatures in ALA as well as in AGFK systems. PY79 spore germination was inhibited in AGFK at both temperatures but in ALA pathway PY79 remained unaffected by HgCl₂ rather exhibited improved growth at 45°C after addition of germinant (Table 3). 3 mM HgCl₂ addition in ALA system induced complete inhibition to activate spores of 1A315, 1A316 and 1A318 at 45°C. As far as spore germination inhibition was concerned it was 100% in 1A316 and 1A318 at 45°C. HgCl₂ induced inhibitory effects can be rectified by treatment with 7 mM β-mercaptoethanol. Hence this treatment reverse inhibition. Instead of inhibition, stimulatory effects of HgCl₂ were observed in 1A197, 1A317, 1A318 at both temperatures and 1A292, 1A315, 1A316 at 37 and at 45°C in 1A314. In AGFK-system HgCl₂ induced inhibition in 1A292, 1A314, 1A317 at both temperatures and 1A315, 1A318 at 45°C which could not

Table 3: Spore germination inhibition by HgCl₂ and reversion effects of β-mercaptoethanol

Percentage germination (HgCl ₂)		AGFK system		ALA system	
		37°C	45°C	37°C	45°C
PY79	A	16.440	0.000	7.486	56.937
	B	16.023	0.000	20.206	33.200
1A197	A	3.919	8.846	14.930	90.770
	B	3.622	10.190	18.506	28.214
1A292	A	21.443	55.260	20.762	32.102
	B	0.000	0.000	25.076	79.970
1A314	A	10.937	24.478	13.876	18.221
	B	0.000	0.000	27.103	47.470
1A315	A	13.149	28.833	4.287	0.000
	B	6.965	14.430	26.005	84.330
1A316	A	10.036	17.495	2.239	0.000
	B	12.029	0.000	30.705	78.456
1A317	A	16.939	18.599	4.948	8.051
	B	0.000	0.000	12.798	20.890
1A318	A	11.825	13.603	9.006	0.000
	B	6.880	0.000	19.369	59.890
Percentage Inhibition (HgCl ₂) (A)/Reversion (B)					
PY79	A	62.990	100.000	0.000	0.000
	B	36.067	0.000	356.360	395.709
1A197	A	91.410	64.298	0.000	0.000
	B	7.935	41.010	250.622	233.720
1A292	A	27.086	*	0.000	0.000
	B	0.000	0.000	214.416	0.000
1A314	A	85.522	30.558	0.000	32.650
	B	0.000	0.000	0.000	175.450
1A315	A	73.169	66.392	0.000	0.000
	B	14.212	16.820	1111.320	0.000
1A316	A	78.420	81.830	51.730	100.000
	B	25.858	0.000	661.740	23.869
1A317	A	63.410	64.650	0.000	37.230
	B	0.000	0.000	271.140	162.870
1A318	A	70.138	79.740	0.000	100.000
	B	17.374	0.000	449.600	199.430

* Stimulation

A = Spore germination in the presence of HgCl₂

B = Spore germination after washing with β-mercaptoethanol

be reversed by the addition of germinant L asparagines. Percentage inhibition of spore germination in 1A292-37°C and 1A314, 1A317 at both temperatures could not be reverted even by mercaptoethanol. In AGFK system reversal and stimulation by NaN₃ at both temperatures were also manifested by different *div* mutant strains (Table 3). The Ala and AGFK germination pathways were > 99% inhibited by 3 and 1 mM HgCl₂, respectively^[16]. The presence of 10 mM NaN₃ had no effect on ALA pathway, it inhibited the AGFK pathway by 92%^[16]. But in *gerA* mutants both AGFK and ALA systems are inhibited (85-95%) with NaN₃^[4]. HgCl₂ also had inhibitory effects on spore germination in *gerA* and *gerB* mutants both in ALA and AGFK-systems^[4]. Sabri and Hasnain^[26] reported that HgCl₂ (3 mM) completely inhibited germination in both ALA and AGFK systems. These effects are reversed by addition of β-mercaptoethanol. Inhibitory effects of NaN₃ are more evident in AGFK system^[26]. On the basis of

inhibitory effects and reversal of inhibitory effects of NaN_3 and HgCl_2 , Sabri and Hasnain^[26] aligned mutation *divIVA* to *gerB* mutation.

Rectification of D-alanine inhibition by different nutrients:

Germination response of 1A292 in AGFK was defective at 37°C while no (zero) OD₆₀₀ loss in ALA and AGFK system was recorded at elevated temperature. More over *divI* mutants (1A317 and 1A318) experienced 100% spore germination inhibition by NaN_3 at both temperatures in ALA pathway and were more sensitive to NaN_3 in AGFK system at 37°C as well as 45°C. Spore germination of *divI* mutant was arrested largely by HgCl_2 in AGFK system at both temperatures and in ALA pathway only at elevated temperature. Such peculiar behaviour need more investigation regarding their response to inhibitors and nutrients triggering reversion. Therefore, impact of Glc (glucose), Fru (fructose) on spore germination response of mutant strains was probed. 1 mg mL⁻¹ of Glc and Fru with alanine alone in case of 1A292 and 1A317, However in case of 1A318 only Glc could enhance germination rate at 37°C.

Addition of 10 mM D-alanine completely inhibited spore germination of these *div* mutants at 37°C and only in 1A318 at 45°C. Glc, fru and Glc+fru successfully rectified inhibition of 1A292 spores while Glc remained ineffective. In case of 1A317 these nutrients were successful rectifiers in combination but proved utter failure in solitary conditions. D-alanine inhibition was reverted successfully by fru and Glc at both temperatures. However their combination failed at elevated temperature to recover 1A318 spore inhibition.

Spore resistance to toluene: Spores ability to germinate in the presence of toluene was studied and their possible resistance was probed by plating toluene treated samples as well as their dilutions on nutrient agar plates following their incubations at 37 and 45°C. Resistant colonies so obtained were counted in both sets of plates (direct and dilution plates) and resistance of wild type as well as *div* mutant strains was measured. PY79 spores remained sensitive at 37°C (when plated directly) but exhibited small resistance at 45°C. 1A413 and 1A315 mutants failed to germinate at 37°C. but very little response was recorded when compared to that of wild type at 45°C. Rest of *div* mutants (1A197, 1A292, 1A316 and 1A318) responded at both temperatures, which was more than PY79 at 37°C. while less at 45°C. In case of 1A317 no germination was observed at 45°C where as significantly more than wild type germination was recorded at 37°C. Upto 1/1000 times dilutions of toluene treated samples were plated on

Table 4: Effect of temperature on teichoic acid content of wild type PY79 and *div* mutants after 24 and 96 h incubation

Strains	24 h		96 h	
	37°C	45°C	37°C	45°C
PY79	324±30.13	2512±79.80	516±14.80	667±3.50
1A197	1597±89.60	215±12.20	3520±17.10	3000±83.20
1A292	327±13.10	1707±14.45	1445±31.30	2462±15.00
1A314	1577±38.70	764±11.01	2733±25.10	740±13.20
1A315	2383±10.29	1023±17.96	3805±17.00	1310±12.70
1A316	1517±37.90	840±19.90	1202±15.01	4686±12.67
1A317	2272±20.07	1542±15.10	1727±17.90	1720±16.80
1A318	1437±31.70	2200±19.20	795±2.95	1291±11.50

nutrient agar plates and incubated at 37 and 45°C. Number of colonies were plated on nutrient agar plates and incubated at 37 and 45°C. Number of colonies counted in PY79 at 45°C was more than 37°C. Similar behaviour was observed in 1A292 and 1A315, but growth was more than PY79 at both temperatures in 1A292. Growth of 1A197, 1A314, 1A316 and 1A318 decreased at elevated temperatures (except A316) when compared with the wild type PY79.

Cell wall composition: The shape nature, structural properties, staining behavior, cell morphology and cell growth are greatly determined by peptidoglycan backbone and polymer (covalently) attached to it. For that reason peptidoglycan (hexosamine) teichoic acid and diaminopimelic acid components of walls of vegetative as well as spores of wild type and different *div* mutants were determined at 37 and 45°C. The walls of vegetative cells of *div*-strains contain significantly lower contents of teichoic acid as compared to that of *div*⁺ strains (PY79). While in spores the amount of teichoic acid was significantly higher in *div* mutants when compared with wild type (Table 4). Higher temperature in 1A292 and 1A314, both after 24 and 96 h and 1A317 (24 h) promoted teichoic acid synthesis (Table 4). Hasnain and Sabri^[30] reported that teichoic acid content decreased in *div* strains at higher temperatures. The temperature dependent change occur in conformation and organization teichoic acid which ultimately affect the cell shape and staining property. According to Archibald *et al.*^[31] temperature and ionic environment interact and determine the surface structure. Except PY79, amount of teichoic acid is more in sporulating cells as compared to vegetative cells (Table 4). Which might be due to presence of more ionic polymers in sporulating stage. of wall components is known to be affected by other factors than temperature. The walls of spores contain additional phosphate polymers while walls of vegetative cells contain only ribitol phosphate polymer as a major cell walls component^[32].

Table 5: Effect of temperature on diaminopimelic content of wild type PY79 and *div* mutants after 24 and 96 h incubation

Strains	24 h		96 h	
	37°C	45°C	37°C	45°C
PY79	1.36±0.02	2.12±0.06	2.59±0.130	1.64±0.01
1A197	1.52±0.04	1.65±0.01	3.18±0.050	4.40±0.06
1A292	0.77±0.30	0.46±0.05	2.07±0.040	0.53±0.03
1A314	4.97±0.40	1.32±0.08	3.01±0.280	2.27±0.03
1A315	1.66±0.54	1.31±0.21	4.51±0.080	2.06±0.01
1A316	1.85±0.03	0.48±0.01	3.45±0.070	1.17±0.01
1A317	3.36±0.16	0.52±0.05	3.96±0.009	5.19±0.08
1A318	1.52±0.02	0.00±0.01	3.67±0.040	0.87±0.09

Table 6: Effect of temperature on peptidoglycan content of wild type PY79 and *div* mutants after 24 and 96 h incubation

Strains	24 h		96 h	
	37°C	45°C	37°C	45°C
PY79	3.31±0.18	13.57±0.280	20.00±0.2000	16.00±0.160
1A197	6.33±0.09	27.00±0.540	3.82±0.2100	18.00±0.130
1A292	18.00±0.72	30.00±0.300	12.00±0.2300	20.00±0.200
1A314	7.20±0.07	6.00±0.180	1.87±0.0.005	2.90±0.020
1A315	9.17±0.55	4.57±0.160	16.00±0.1600	3.30±0.033
1A316	23.50±0.54	7.10±0.110	8.00±0.1600	1.57±0.047
1A317	12.00±0.75	4.10±0.082	1.30±0.1300	3.70±0.074
1A318	11.00±0.33	13.75±0.150	8.67±0.2600	4.50±0.180

The other wall component diaminopimelic acid was also analyzed in PY79 and *div* mutants. The amount of DAP after 24 h at 37°C was more in *div* mutants (except 1A292) whereas at 45°C DAP content decreased in *div* mutants. At sporulating stage (96 h) DAP increased in *div* mutants at 37° (all strains) and 45°C (except 1A292, 1A314, 1A318) (Table 5). Briehl *et al.*^[33] reported that DAP contents of temperature sensitive mutants at 47°C are 50-80% higher than in wild type, which is due to little teichoic acid in them. The phosphate and DAP, at permissive temperature is comparable to wild type but at restricted temperature a marked and specific reduction in the content of phosphate occur^[33]. Hasnain and Sabri^[27] reported that at higher temperature filament formation occur at 42°C in *div* mutants which further lead to lysis and death. The filaments obtained at 42°C show no sign of newly initiated constrictions and thus lowering wall components^[34]. The temperature cause effects on macrofibre system. In macrofibre system shape determination and maintenance appear to result from mechanical interaction among cell wall components especially PG. Favre *et al.*^[34] reported that such interactions are important in determination and maintenance of normal cell shape.

Since the rate of PG synthesis alter the cell morphology, cell division and behavior of bacterial cells, hexosamine component of PG of PY79 (wild type) *divI*, *divII*, *divIV* and *divV* mutants of *B. subtilis* was determined at 37 and 45°C, after 24 and 96 h of incubation. PG content (hexosamine) was lower at high temperature (except, 1A197 after 24 h, 1A314 and 1A316 after 96 h).

After 96 h (sporulating stage) of incubation PG content was more in *div* mutant when compared with wild type specially at 37°C (Table 6). Cell shape and cell growth are affected by PG synthesis, glycan chain geometry and orientation^[5]. In *div* mutants filaments and vegetative cell types were more perhaps that might be a reason for increased PG-content. Hasnain and Sabri^[26] reported that PG contents increased many folds in wild type after 96 h of incubation. The thickness of PG layer was 2-3 layers structure from exponential phase cells and with 4-5 layer structure from stationary phase cells^[35,36]. Generally in late phases of growth of *div* sporulating cells as well as normal cell types were less as compared to *div*⁺ strains^[27]. Hasnain and Sabri^[27] reported that although spores were present at 45°C in *div* strains but they were thick walled and defective in germination and many spores were in lysed form (with low resolution). Wall thickening is due to either altered PG metabolism or due to stoppage in protein synthesis^[37]. the wall thickening affect the penicillin-binding protein because they serve a role in linking PG to pre-existing wall and only function at no more than 1 nm. The wall thickening and wall density must decrease during transformation from septum to pole. Meador-Parton and Popham^[7] suggested that degree of peptidoglycan - cross linking could have more direct impact on the rate of spore germination and outgrowth.

From overall results it may be concluded that abnormal cell morphology and staining behavior might be due to defect in cell wall components. Altered PG content result from altered wall thickening activity. Functional homologues of *ftsA*, *ftsZ*, *ftsW*, *murD*, *murG*, *pbpB* and *div1B* genes of *E. coli* have been found in a cluster at about 133° on genetic map of *Bacillus subtilis*. Similarly *ger* mutations are located near *divII* (mp314), *divIVB* (mp247), *divV* (mp273). Very near to them are *cot*, *tag* and *his* genes which affect the cell wall composition^[1]. It appeared that mutation in division site also cause mutation in nearby genes which control spore germination, PG, TA and DAP synthesis. Hence *div* mutations are multidirectional mutations and plays an important role in morphogenetic pathway of *Bacillus subtilis*.

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