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EVIDENCE OF NUTRIENT INDUCED SPORE GERMINATION IN BACTERIA FROM STRESS ENVIRONMENT

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Abstract

During unfavorable conditions many bacteria ensure their survival through spore formation, which germinate on restoration of suitable conditions. Defects in sporulation may be associated with inability of spores to germinate. Here are reported results of four *Bacillus* strains Gd64, Gd90, Gd213 and Gd208, isolated from saline environment. The spores of these strains appeared to be germination defective. To probe the defect analysis, both ALA and AGFK systems were executed. Different germinants such as L-Glutamine, L-Valine, L-Isoleucine, DL-Phenylalanine, and DL-Proline were substituted for L-Alanine and L-Asparagines in ALA and AGFK systems respectively. Results reveal that germination defective spores showed better response in AGFK system than ALA system, while germinants L-Glutamine, DL-Proline and DL-Phenylalanine exhibited better percentage germination than L-alanine and L-Asparagines. So there may be more than one mutation involved in germination defect.

Introduction

The capacity to form endospores endows spore formers with the ability to survive in adverse environmental conditions. However, the success of this survival strategy depends on the presence of an efficient mechanism for returning to the vegetative state under favourable conditions through a process called germination. Extreme environmental conditions affect the ability of spore germination. Sabri & Hasnain (1996) reported that extreme environmental conditions can cause abnormalities in spore formation, therefore, we studied impact of stress environment on morphogensis of bacteria.

Spore germination is triggered by specific molecules called germinant to act by binding to and stimulating spore receptors. A wide range of chemical and physical effectors can trigger germination of spores. In *B. subtilis*, the two best-known germination pathways are those induced by the chemical effectors like Alanine (Ala) and the combination of Asparagine (Asn), Glucose (Glu), Fructose (Fruc), and potassium ions (AGFK). This has been demonstrated by the isolation of specific mutants of *B. subtilis* defective in the Ala response (*gerA*), in the AGFK response (*gerB and gerK*), and in both responses (*gerD*). It was subsequently hypothesized that the *gerA, gerB*, and *gerK* gene products are receptors of the germinants Ala, Asn, and Glu, respectively.

The present work deals with the germination defective spore forming strains isolated from saline environment. Different germinants (L-Isoleucine, L-Valine L-Glutamine, DL-Proline, DL-Phenylalanine) in addition to L-Alanine (in ALAsystem) and L-Asparagine (in AGFK system) were used to check the evidence of nutrient induced growth in germination defective spores.

Materials and Methods

Isolation: Samples were plated on metal stressed (100 $\mu g.mL^{-1}$ $CoCi_2$, $ZnSo_4$ and K_2CrO_4 individually) and salt stressed (0.5M, 1M NaCl) plates containing Schaeffer's medium (Schaeffer *et al.*, 1965).

Characterization: Spore formation was confirmed by using the spore staining method (Schaeffer & Fulton, 1993) while spore germination ability by the tetrazolium test (Anagnostopolous & Spizizen, 1961). The bacterial strains which exhibited spore formation but being negative for tetrazolium test were designated as germination defective. These germination defective spore-forming strains were checked for evidence of nutrient induced germination. Selected colonies were purified and characterized morphologically as well as biochemically (Gerhardt *et al.*, 1994).

Spore germination: Germination of spores was induced at 37°C in two systems as follows (Vankatasubramanian & Johnston, 1989):

1) ALA system in which the spores were germinated for 60 minutes in 50 mM potassium phosphate buffer, pH 7.4 by the addition of 1mM germinant (L-Alanine, L-Asparagine, L-Isoleucine, L-Valine L-Glutamine, DL-Proline, DL-Phenylalanine) and a control without any germinant.
2) AGFK system in which the spores were germinated for 80 minutes in 50 mM Tris HCl buffer, pH 7.5 by the addition of 5.6 mM fructose, 5.6 mM glucose, 67 mM KCl and 30mM germinant (L-Alanine, L-Asparagine, L- Isoleucine, L-Valine L-Glutamine, DL- Proline, DL-Phenylalanine) and a control without any germinant.

Results

Isolation of bacterial strain: A total of 146 bacterial strains were isolated from plant, soil and water samples from a saline area. Isolation was performed at Schaeffer's media with $CoCl_2$, $ZnSo_4$, K_2CrO_4 (100 $\mu g.mL^{-1}$) individually and with NaCl (0.5 M, 1M) stress. One hundred strains were isolated at metal stress and 46 strains at salt stress.

Germination defective, spore forming bacteria: On Schaeffer's medium, 52 bacterial strains (31 with metal resistance, 21 with salt stress resistance) formed brown and light brown-coloured colonies. After spore staining, 28 strains were confirmed spore former. These strains when checked with tetrazolium overlay test for spore germination showed 6 with positive and 22 negative germination response. The 22 non-germinating strains were selected for further study. Results of 4 strains (Gd64, Gd90, Gd208 and Gd213) from those 22 strains are being reported here. These were isolated from phillоplane (64), histoplane (90), rhizosphere (208) and rhizoplane (213) of *Oryza sativa.*

Characterization of bacterial strains: Gd64, Gd90, Gd208 and Gd213 are Gram positive, capsulated, spore forming, catalase producing, facultative, anaerobic, rod shaped bacteria which can degrade glucose in the form of acetoin. Gd64 and Gd90 can reduce nitrate to nitrite (Table 1). All the strains showed affinity with family *Bacillaceae* and genus *Bacillus* that is a group of spore forming bacteria (Holt, 1994).

Table 1. Characteristics of Bacteria Gd64, Gd90, Gd208 and Gd213.

Bacterial Characteristics	Gd 64	Gd 90	Gd 208	Gd 213
Gram staining	+	+	+	+
Capsule staining	+	+	+	+
Spore staining	+	+	+	+
Catalase test	+	+	+	+
Motility	-	-	-	-
King's A	-	-	-	-
King's B	-	-	-	-
EMB	-	-	-	-
Brilliant green	-	-	-	-
Schaeffer's Media	+	+	+	+
Tetrazolium overlay test	-	-	-	-
Arginin Hydrolysis	-	-	-	-
Starch Hydrolysis	-	-	-	-
Malonate	-	-	-	-
Methyle red	-	-	-	-
Voges proskaur	+	+	+	+
Nitrate Reduction	+	+	-	-
Denitrification	-	-	-	-
Urease	-	-	-	-
H_2S production	-	-	-	-
O.F test	F.an	F.an	F.an	F.an

Effect of nutrients on growth of germination defective spores: Effect of nutrients (L-Alanine, L-Asparagines, L-Isoleucine, L-Valine L-Glutamine, DL- Proline, and DL-Phenylalanine) in ALA and AGFK system was studied on germination defective *Bacillus* strains. Germination with these germinants were compared with the results of L-Alanine in ALA system, L-Asparagines in AGFK system and without germinant buffer in both ALA and AGFK systems.

ALA system: In ALA system, Phosphate buffer was used with only one germinant, L-Alanine. No Bacillus spore showed germination in ALA system lacking L-Alanine. When 1 mM L-Alanine was added to buffer, Gd64, Gd90 and Gd208 showed rise in percent germination than without germinant buffer, while Gd213 gave no response. When the sole germinant of AGFK system i.e., L-Asparagines, replaced with L-Alanine in ALA system, spores of Gd64 (7.1 %) and Gd208 (47.5 %) exhibited decreased germination than with L-Alanine in ALA system.

Replacement of other germinants (L-Isoleucine, L-Valine L-Glutamine, DL- Proline, and DL-Phenylalanine) with L-Alanine in ALA system, spores of Gd64 showed increase with L-Isoleucine and L-Valine as compared to without germinant buffer, as compared to L-Alanine with buffer there was a 5.4 % increase with L-Isoleucine and 82.8 % decrease with L-Valine, with no germination response with L-Glutamine, DL- Proline, and DL-Phenylalanine. Spores of Gd90 exhibited an increase (182.3 %) with DL-Phenylalanine and no response with L-Asparagines, L-Valine L-Glutamine and DL- Proline relative to without germinant and L-Alanine containing buffer, whereas, a very low response with

L-Isoleucine (90.6 %) was exhibited. Gd208 showed increased germination with DL-Proline and DL-Phenylalanine than non-germinant buffer and decreases were observed (82 and 55 % respectively) compared to L-Alanine containing buffer. Spores of Gd213 did not produce any response in ALA system (Table 2).

Table 2. Percentage growth of germination defective bacterial strains in the presence of different germinants in ALA and AGFK system. (mean of three replicates).

Bacterial strains	Germinants	ALA-system	AGFK-system
Gd-64	L- Alanine	7.3±0.5	25.95±0.821429
	L- Asparagine	6.78±0.2	28.25±0.107143
	L-Isoleucine	7.7±0	28.2±0.357143
	L-Valine	1.25±0.893	26.2±1.071429
	L-Glutamine	0±0	45.895±2.567857
	DL-Proline	0±0	40.15±0.464286
	DL-Phenylalanine	0±0	33.9±0.928571
	no germinant	0±0	6.95±0.178571
Gd-90	L- Alanine	4.285±0.868	17.92±1.914286
	L- Asparagine	0±0	27.4±1.285714
	L-Isoleucine	0.4±0.286	13.35±0.75
	L-Valine	0±0	23.5±1.714286
	L-Glutamine	0±0	22.445±1.175
	DL-Proline	0±0	20.7±0.5
	DL-Phenylalanine	12.1±0.193	29.85±0.535714
	no germinant	0±0	0±0
Gd-208	L- Alanine	12.2±0.071	16.45±0.035714
	L- Asparagine	6.35±0.107	35.85±2.321429
	L-Isoleucine	0±0	29.6±3.5
	L-Valine	0±0	22.05±1.178571
	L-Glutamine	0±0	26.765±0.975
	DL-Proline	2.15±0.464	18.35±1.464286
	DL-Phenylalanine	5.45±0.321	41.8±1.428571
	no germinant	0±0	0±0
Gd-213	L- Alanine	0±0	22.775±0.196429
	L- Asparagine	0±0	44.75±0.75
	L-Isoleucine	0±0	54.55±1.178571
	L-Valine	0±0	36.75±1.678571
	L-Glutamine	0±0	39.05±1.107143
	DL-Proline	0±0	94.75±1.035714
	DL-Phenylalanine	0±0	74.535±0.332143
	no germinant	0±0	32.15±0.035714

AGFK system: In AGFK system, Tris buffer with glucose and fructose was used. Spores of Gd64 and Gd213 showed germination in buffer without germinant, although it was relatively less than with germinants, except Gd213 with L-Alanine, where lower germination was observed than without germinant buffer.

L-Asparagine (30 mM) was used as the sole germinant in AGFK system. In Gd64 (306.1 %), Gd213 (39.1 %), Gd90 and Gd 208 increase in germination over without germinant buffer was recorded. When L-Asparagine was replaced with L-Alanine spores of Gd64 (76 %), Gd90 and Gd208 showed increased germination response while Gd213

showed decreased (29 %) germination than with non-germinant buffer. Compared with L-Asparagines containing buffer, L-Alanine showed 8.14 % (Gd64), 34.5 % (Gd90), 54.1 % (Gd208) and 49.1 % (Gd213) decreases in different strains.

On replacing other germinants such as L-Isoleucine, L-Valine L-Glutamine, DL-Proline, and DL-Phenylalanine with L-Asparagine in AGFK system, spores of Gd64 showed 305.7-560.3 % increase over spores without germinant buffer. In L-Asparagines containing buffer Gd64 showed decrease with L-Alanine (8.14 %), L-Isoleucine (0.17 %) and L-Valine (7.2 %), while increased with DL-Proline (42.1 %), L-Glutamine (62.4 %) and DL-Phenylalanine (20 %). Gd90 and Gd208 showed increased germination with germinants relative to without germinant buffer. L-Asparagines in both strains caused decreases as compared with L-Alanine (34.5 %, 54.2 %), L-Isoleucine (51.2 %, 17.4 %), L-Glutamine (18.08 %, 25.3 %) and with DL-Proline (24.4 %, 48.8 %) respectively, while increases with DL-Phenylalanine (111.7 %, 66.5 % respectively) were observed. Gd213 showed increase in germination but there was no significant difference except for DL-Proline and DL-Phenylalanine which induced germination (194.7 % and 131.8 %) more than without germinant buffer. L-Asparagine containing buffer Gd213 showed decrease with L-Alanine (49.1 %), L-Valine (17.8 %) and L-Glutamine (12.7 %) whereas an increase with L-Isoleucine (21.8 %), DL-Proline (111.7 %) and DL-Phenylalanine (66.5 %) was recorded (Table 2).

Discussion

Bacterial spores can survive many years in dormant form. They can attain their original form by germination and outgrowth but bacterial morphogenesis is affected under stress conditions (Nicholson *et al.*, 2000; Abrioul *et al.*, 2002). The spores formed under such stress are unable to germinate normally (Sabri & Hasnain, 1996) and may undergo some kind of mutation (Paidhungat & Setlow, 1999; Behravan *et al.*, 2000) or deficiency. Sabri & Hasnain (1994) also reported the effect of mutation on sporulation property of *Bacillus subtilis*. Therefore, *Bacillus* strains do not germinate until they are triggered from outside. This triggering agent may be a chemical (Foster & Johnstone, 1990) or a physical change (Wuytach *et al.*, 1998, 2000).

The process of chemical or nutrient induced germination involves interaction of germinants with presumed specific receptors in the spore. The receptor is a multiunit complex consisting of at least the A and B proteins (Paidhungat & Setlow, 1999) associated with the cell membrane in dormant spores. Mutational analysis of *B. subtilis* identified several classes of spore germination mutants defective in their response to particular germinants, *gerA* mutant for example are unable to germinate in L-Alanine as sole germinant (Moir *et al.*, 1979; Sammons *et al.*, 1981) and *ger B* mutant (Irie *et al.*, 1996) as defective in the response to combination of amino acids glucose, fructose and sole germinant L-Asparagine.

In our results, germination defective *Bacillus* strains showed poor germination response in ALA system. Spores of Gd64 gave better germination response with L-Isoleucine than L-Alanine and L-Asparagine, although difference was not significant. Gd90 showed response with DL-Phenylalanine, Gd208 with L-Alanine and Gd 213 gave no response in ALA system. In AGFK system best result was obtained by Gd213 with DL-Proline and DL-Phenylalanine than L-Alanine and L-Asparagines. Even in buffer spores of Gd64 and Gd90 germinated (Fig. 1).

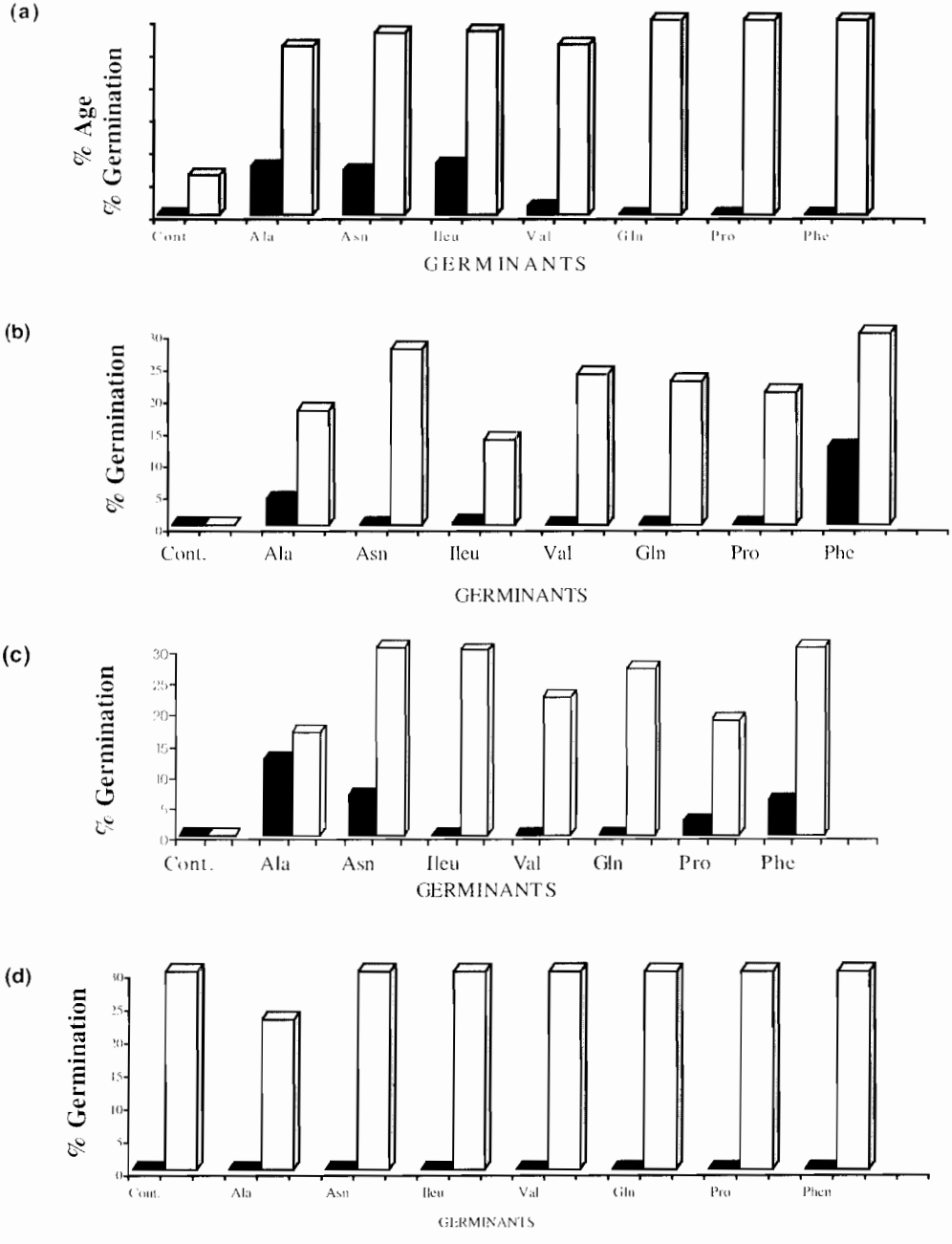

Fig. 1. Growth percentage of germination defective spores of *Bacillus* Gd64(a), Gd90(b), Gd208(c) and Gd 213(d) to different germinants L-Alanine (Ala), L-Asparagine (Asn), L- Isoleucine (Ileu), L-Valine (Val), L-Glutamine (Gln), DL- Proline (Pro), DL-Phenylalanine (Phe) in ALA (solid) and AGFK (open) system.

The results suggest that there may be more than one mutation involved in germination defect. There might be other operons than *gerA* and *gerB* involved in this process, which controlled the germination process and mutations in them caused the requirement of nutrient germinant other than L-Alanine and L-Asparagine. It has been proved that *gerB* and *gerK* operons encode homologs of *gerA* (Corfe *et al.*, 1994), while *gerI* (Clements & Moir, 1998) and *gerQ* (Barlass, 1998) operons are required for inosine germination whereas *gerI* and *gerL* operons also contribute to L-Alanine germination (Barlass, 1998).

References

Abrioul, H., M. Maqueda, A. Galvez, M. Martinez-Bueno and E. Valdivia. 2002. Inhibition of bacterial growth, enterotoxin production, and spore outgrowth in *Bacillus cereus* by bacteriocin AS-48. *Appl. & Environ. Microbiol.*, 68: 1473-1477.

Anagnostopolous, C. and J. Spizizen. 1961. Requirement for transformation in *Bacillus subtilus*. *J. Bacteriol.*, 81: 741-746

Barlass, P.J. 1998. Characterization of the L-Alanine spore germination response of *Bacillus cereus*. Ph.D. Thesis, Univ. Scheffield.

Behravan, J., H. Chirakkal, A. Masson and A. Moir. 2000. Mutation in the *gerP* locus of *Bacillus subtilis* and *Bacillus cereus* affect access of germinants to their targets in spores. *J. Bacteriol.*, 182: 1987-1994.

Clements, M.O. and A. Moir. 1998. Role of the *gerI* operon of *Bacillus cereus* 569 in the response of spores to germinants. *J. Bacteriol.*, 180: 6729-6735.

Corfe, B.M., A. Moir, D. Popham and P. Setlow. 1994. Analysis of the expression and regulation of the *ger B* spore germination operon of *Bacillus subtilis* 168. *Microbiol.*, 140: 3079-3083.

Foster, S.J. and K. Johnstone. 1990. Pulling the trigger: the mechanism of bacterial spore germination. *Mol. Microbiol.*, 4: 137-141.

Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood and N.R. Krieg. 1994. *Methods for General and Molecular Bacteriology*. Amer. Soc. Microbiol., Washington DC., U.S.A.

Holt, J.G. 1994. *Bergey's Manual of Determinative Bacteriology* (9th ed.), Williams & Wilkins Co., Baltimore, U.S.A.

Irie, R., Y. Fujita and M. Kobayashi. 1996. Nucleotide sequence and gene organization of the *ger K* spore germination locus of *Bacillus subtilis* 168. *J. Gen. Appl. Microbiol.*, 42:141-153.

Moir, A., E. Lafferty and D.A. Smith. 1979. Genetic analysis of spore germination mutants of *Bacillus subtilis* 168: the correlation of phenotype with map location. *J. Gen. Microbiol.* 111: 165-180.

Nicholson, W.L., N. Munakata, G. Horneck, H.J. Melosh and P. Setlow. 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. & Mol. Bio. Rev.*, 64: 548-572.

Paidhungat, M. and P. Setlow. 1999. Isolation and characterization of mutant D-Alanine. *J. Bacteriol.*, 181: 2513-2519.

Sammons, R.L., A. Moir and D.A. Smith. 1981. Isolation and properties of spore germination mutant of *Bacillus subtilis* 168 defective in the initiation of germination. *J. Gen. Microbiol.* 124: 229-241.

Sabri, A.N. and S. Hasnain. 1994. Amplification of *div IV*$^{+}$ gene of *Bacillus subtilis* and its effects on Spo Phenotype. *Pro. Pak. Cong. Zool.*, 14: 221-236.

Sabri, A.N. and S. Hasnain. 1996. Polypeptide products in *div IVA*$^{+}$ and div *IVA*$^{-}$ strains of *Bacillus subtilis* under varying temperature, pH and starvation condition. *Pak. J. Zool.*, 28: 303-309.

Shaeffer, A.B. and A. Fulton. 1993. A simplified method of staining endospores. *Sci.*, 77-194.

Schaeffer, P., J. Millot and J. Aubert. 1965. Catabolite repressor of bacterial sporulation. *Proc. Natl. Acad. Sci.*, USA., 54: 704-711.

Vankatasubramanian, P and K. Johnstone. 1989. Biochemical analysis of the *Bacillus subtilus* 1604 spore germination response. *J. Gen. Microbial.*, 35: 2723-2733.

Wuytach. E.Y., S. Boven and C.W. Michiels. 1998. Comparative study of pressure induced germination of *Bacillus subtilis* spores at low and high pressure. *Appl. & Environ. Microbiol.*, 64: 3220-3224.

Wuytach. E.Y., J. Soons, F. Pochet and C.W. Michiels. 2000. Comperative study of pressure and nutrient induced germination of *Bacillus subtilis* spores. *Appl. & Environ. Microbiol.*, 66: 257-26.