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Proteolytic activity analysis in germination defective spores of Bacillus

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ABSTRACT

Proteolytic enzymes are characteristically produced by *Bacillus* species during sporulation and germination. The hydrolysis of spore proteins to free amino acids, is accomplished by proteases, in first 20 minutes of spore germination. The analysis of *Spo* mutants (strains which lack Sporulation (*Spo)* gene or have its inactive form) revealed that many of these strains did not produce extracellular proteases. Some evidence for the involvement of serine proteases in sporulation has been provided by inhibitor studies. Nevertheless variation in germination response of germination defective strains with different nutrients was recorded in ALA system, but overall responses of germination defective spores with substituted amino acids and alanine are not much different. Therefore all the amino acid proved competent germinant in place of alanine in ALA system. The overall role of germinants as a replacement in AGFK is satisfactory and they trigger germination in germination defective bacterial spores as do asparagines. In present studies all the germination defective spores showed very low proteolytic activity in comparison with proteolytic activity of normal spores of wild type *Bacillus subtilis* PY79. It appears that defective spores lack enzymes with proteolytic activity which is necessary for germination. Since all protease deficient mutants are asporogenous. Although some mutants, producing very low levels of protease are still able to sporulate, with some drawbacks.

Keywords: Bacillus, Spores. Protease, Germination, ALA system, GFK system

INTRODUCTION

Proteolytic enzymes are characteristically produced by *Bacillus* species during sporulation and germination. Site-directed mutagenesis and structural studies suggest that the germination protease, GPR, in spores of *Bacillus* species is an atypical aspartic acid protease (Carroll & Setlow, 2005)**.** Acid-active proteases are synthesized during sporulation and germination (Cavallo *et al*., 1999). Microbial proteases have been divided into four groups on the basis of the mechanism of action. These are: i) serine proteases ii) metal proteases iii) thiol proteases and iv) acid proteases. The extracellular proteases of the genus *Bacillus* are either serine or metal enzymes. The biochemical events, including exoenzyme synthesis associated with sporulation, have been divided into three physiological categories. These are i) primary sequence of dependent events specifically concerned with spore formation ii) side events that are not specifically involved in the process but are initiated by the occurrence of some of the events in i), and iii) phenotypic changes induced by the changes in cultural conditions that initiate sporulation. The analysis of *Spo* mutants revealed that many of these strains did not produce extracellular protease and that reversion to sporogeny was accompanied by the synthesis of protease. All mutants that display altered serine protease activity possess altered sporulation characteristics. Moreover, Leighton *et al.* (1972, 1973) described temperature-sensitive mutants of *B. subtilis* that produced an inactive serine protease and was asporogenous at 47^{o}C, the nonpermissive temperature. Revertants to wild-type invariably acquired both a normal serine protease and the ability to sporulate at 47^{o}C. Some evidence for the involvement of serine proteases in sporulation has been provided by inhibitor studies. Phenylmethylsulfonylfluoride (PMSF) inhibits extracellular serine protease activity and prevents

sporulation in *B. subtilis*. It is difficult to assign a particular function in sporulation to extracellular serine proteases. The metal proteases are not functional in the process of sporulation. Esterases the third extracellular proteases have not been studied in detail.

CodY, is a transcriptional regulator whose function is to respond to the amino acids, which are present intracellular. Expression of the *aprE* and *nprE* genes is also controlled by CodY, either directly or indirectly. These are the genes responsible for function of major metabolic enzymes AprE and NprE, in Bacillus subtilis which act as exoproteases. Direct control of CodY-mediated repression of Exoproteases is not clear yet, exoproteases are not produced in presence of proteins or their degraded products. In the absence of CodY activity the metabolic activity of these exoprotease is controlled by *scpC* gene indirectly to maintain their significant level of repression. (Barbieri *et al*., 2016),

In the present study the levels of proteolytic activity in germination defective spores, either in the presence or absence of any germinant replaced in ALA and AGFK systems of germination are discussed and evidence of any relation of this spore germination defect with protease catalysis has been explored.

MATERIALS AND METHODS

Strains used

Spore former Isolates 05, 4, 19, 22, 23, 25, 27, 28, 29, 31, 32, 33, 37, 38, 39,85, 87, 99, 126, 127, 128, 129, 130, 131, 132, 156, 157, 158, 159, 160 and 161were isolated from stressed environment, industrial and polluted areas. Among these isolates, Bacillus strains (Gd05, Gd4, Gd19, Gd22, Gd27, Gd85, Gd87, Gd99, Gd160) which were germination defective spore-forming bacteria were used for this study (Table-1).
Bacillus subtilis PY79 strain was used as wild-type.

Table I: Soil samples used for bacterial isolations

Sr. No	Sample location	Sample condition	Isolates obtained from	
			Soil	Water
1.	Moridke	Industrial area	Gd 04	Gd 05
2.	Kala Shah Kaku	Polluted area	Gd 19	
3.	Kasur	Industrial areas		Gd 22, Gd 27,
4.	Narang Mandi	Polluted area		Gd 85, Gd 87,
5.	Muslim town LHR	Polluted area	Gd 99	
6.	Zafer Ali Road LHR	Polluted area	Gd 160	

Characterization

The isolates were characterized according to Gerhardt *et al*., 1994.

Spore staining

To check the ability of spore formation, 0.76% malachite green is used on smear providing steam. Gram safranine applied as a secondary stain **(**Schaeffer & Fulton, 1933).

Medium to check germination potential: strains were grown on tryptose blood base agar (TBBA) to check germination potential of bacterial spores. Pink color appearance showed a positive result. The isolates which were unable to show pink coloration were selected for further study (Moir, 1981).

Response to variable nutrients

Isolates which produce spores that are unable to germinate were finally selected to check their germination response in specific systems L-alanine (ALA system) and a combination of L-ala, glucose, fructose and potassium (AGFK system) with and without alanine as germinant, according to method described by Venkatasubramanian & Johnstone,1993.

Proteases activity

Protease activity is analyzed by mixing 1ml of 0.5% azocoll suspension with 100µl of spore samples and incubating overnight at 37°C under agitation of 300rpm. Next day suspension was filtered and absorbance measured at 520nm (Chavira *et al.,* 1984).

RESULT

Germination response in ALA system

ALA system

Germination defective bacterial spores exhibit a variable response in ALA system. Spores of Gd27, showed no response in without any germinant ALA system. While spores of Gd05, Gd4, Gd19, Gd22, Gd85, Gd87, Gd99 and Gd160 exhibit restricted germination response in ALA buffer (Table-2)

Table II: Germination of germination defective bacterial spores in ALA system with different germinants (mean of three replicates)

Sr. No.	Strain	Alanine	Aspara-gine	Iso-leucine	Valine	Gluta-mine	Proline	Phenyl-alanine	Control
1.	**Gd05**	3.00	0.00	2.73	8.28	3.45	5.55	4.59	2.15
2.	**Gd4**	9.44	9.30	9.53	8.79	8.18	7.76	7.62	6.78
3.	**Gd19**	5.47	11.33	7.70	2.59	16.52	11.32	10.04	11.56
4.	**Gd22**	26.21	0.00	2.97	2.04	43.74	4.96	0.00	0.74
5.	**Gd27**	9.74	8.89	21.93	8.30	20.72	9.59	0.24	0.00
6.	**Gd85**	0.81	6.82	6.65	8.17	7.05	7.44	0.00	1.30
7.	**Gd87**	4.48	2.42	5.13	8.06	3.06	7.60	3.84	2.70
8.	**Gd99**	39.36	6.21	9.13	6.97	7.91	6.59	9.23	5.65
9.	**Gd160**	51.85	8.74	30.17	8.13	7.76	13.42	18.35	0.86

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Alanine

With alanine as sole germinant in ALA system, high germination response (>50) was observed with spores of Gd160, while relatively low (<10) germination response was recorded with Gd05, Gd4, Gd19, Gd27, Gd85 and Gd87 (Table-2).

Asparagine

Germination defective bacterial spores showed variable responses on replacement of sole germinant alanine with asparagine. Gd05, Gd22, showed no germination with asparagines, while spores of Gd4, Gd27, Gd85, Gd87, Gd99, Gd160, exhibited stimulatory response (< 10) but not to the extent of other strains (Table-2).

Isoleucine

When isoleucine used as sole germinant in ALA system, <10 germination was observed with spores of Gd05, Gd4, Gd22, Gd85, Gd87 and Gd99 (Table-2).

Valine

Germination in valine supplemented ALA system, all spores of exibited low germination response (Table-2).

Glutamine

All germination defective bacterial spores gave germination response with glutamine. Low (<10) germination rates with spores of Gd05, Gd4, Gd85, Gd87, Gd99 and Gd160 was observed (Table-2).

Proline

All germination defective bacterial spores germinate with proline in ALA system. Spores of Gd05, Gd4, Gd5, Gd22, Gd27, Gd85, Gd87 and Gd99 yielded comparatively low (<10) germination (Table-2).

Phenylalanine

All bacterial spores except Gd22, Gd85, could germinate with phenylalanine in ALA system. Low germination (<10) was observed in spores of Gd05, Gd4, Gd87 and Gd99 (Table-2).

Protease activity

Spore of Gd05 strain exhibited 1.829 $g^{-1}h^{-1}$ proteolytic unit, which was 73% less than normal spores of *Bacillus subtilus* PY79. Spores of Gd4 showed 1.078 $g^{-1}h^{-1}$ proteolytic unit, which was 84 percent less as compared to normal spores of PY79. Spores of Gd19 strain had 0.538 $g^{-1}h^{-1}$ proteolytic unit, which was 92% less as compared to wild type spores of PY79. Germination defective spores of Gd22 exhibited 0.286 $g^{-1}h^{-1}$ proteolytic unit, which was 95% less than in wild type spores of PY79. Proteolytic activity of germination defective spores of Gd27 strain was 0.095 $g^{-1}h^{-1}$ proteolytic unit, which was 98% less than normal spores of PY79. Proteolytic activity of spores of Gd85 strain was 0.179 $g^{-1}h^{-1}$ proteolytic unit, which was 97% less than wild-type spores of PY79. Germination defective spores of Gd87 strain had 5.556 $g^{-1}h^{-1}$ proteolytic unit, which was 20% less than normal spores of PY79. Germination defective spores of Gd99 had 0.240 $g^{-1}h^{-1}$ proteolytic unit, which was 96% less than normal spores of *Bacillus subtilis*. Spores of Gd160 strain had 0.303 $g^{-1}h^{-1}$ proteolytic unit which was 95% less than wild type spores of PY79.

Relatively high proteolytic activity was exhibited by spores of Gd87. Spores of strain Gd27 showed very low proteolytic activity (Table-4).

DISCUSSION

Variation in germination response with different nutrients was recorded in ALA system, but overall responses of germination defective spores with substituted amino acids and alanine are not much different. Therefore all the amino acid proved competent germinant in place of alanine in ALA system. Although spores of Gd04, with alanine; spores of Gd05, Gd22 with asparagine; spores of Gd85 with glutamine; spores of Gd04 with proline; spores of Gd04, Gd22, Gd29, Gd85 with phenylalanine showed no germination response in ALA system (Table-2). There may be some kind of defect in the triggering mechanism of germination response via ALA system.

The overall role of germinants as a replacement in AGFK is satisfactory and they trigger germination in germination defective bacterial spores as do asparagines (Table-3).

Table III: Germination of germination defective bacterial spores in AGFK system with different germinants (mean of three replicates)

Sr. No.	Strain	Aspara-gine	Alanine	Iso-leucine	Valine	Gluta-mine	Proline	Phenyl-alanine	Control
1.	**Gd05**	28.3	50.7	49.5	54.3	54.0	33.3	27.9	5.8
2.	**Gd4**	28.3	65.1	27.7	27.2	44.5	46.1	29.1	6.3
3.	**Gd19**	18.3	38.7	44.7	36.0	61.0	61.7	31.2	2.4
4.	**Gd22**	32.5	41.1	43.9	42.7	38.7	37.6	32.3	16.1
5.	**Gd27**	19.1	69.0	28.9	23.1	24.0	19.2	23.4	1.0
6.	**Gd85**	32.0	54.5	28.9	30.7	58.1	59.4	31.8	5.8
7.	**Gd87**	31.4	48.6	47.6	51.3	58.8	27.9	29.2	7.1
8.	**Gd99**	23.6	23.3	23.4	21.2	23.3	18.5	37.7	0.7
9.	**Gd160**	80.9	73.8	70.5	45.3	63.4	65.2	55.1	38.2

Table IV: Activity of proteases in spores of germination defective bacterial strains and wild type *Bacillus subtilis* PY79.

Sr. No.	Bacterial strains	Protease activity (PU $g^{-1}h^{-1}$)
1.	**Gd05**	1.829
2.	**Gd4**	1.078
3.	**Gd19**	0.538
4.	**Gd22**	0.286
5.	**Gd27**	0.095
6.	**Gd85**	0.179
7.	**Gd87**	5.556
8.	**Gd99**	0.240
9.	**Gd160**	0.303
10.	**PY79**	7.027

During germination, the proteins degraded are a group of small, acid-soluble spore proteins (SASP) which are unique to the spore stage of the life cycle. Germination protease (GPR) is also unique to the dormant and developing spore and is specific for cleavage of SASP. GPR is synthesized during sporulation as a tetrameric zymogen termed P_{46}, after the molecular mass of (46kDa). The zymogen is converted to the active enzyme (termed P_{41}; also a tetramer) ~ 2 hours later in sporulation through the removal of 15 or 16 amino terminal residues. This is an auto processing reaction of P_{46} – P_{41} which is stimulated both *in vitro* and *in vivo* by desiccation, a decrease in pH, and accumulation of dipicolinic acid (DPA), an abundant small molecule in dormant spores. The conversion of P_{46} to P_{41} also appears to be associated with at least some structural change in the protein, as the enzyme's single sulfahydryl group is reactive in P_{46} but not in P_{41}. Synthesis of GPR as an inactive zymogen is essential for the formation of spores exhibiting their full resistance to several treatments (Illades-Aguiar & Setlow, 1994b). If zymogen is unable to activate in spores then the spores will be unable to germinate. One of the protease is also responsible for the processing of the proform of germination specific lytic enzyme (GSLE) to active enzyme for starting germination. Spore cortex-lytic enzymes have been purified from spores of *Bacillus megaterium, B. cereus* (Chen *et al*., 2000 a, b) and *Clostridium perifringens* (Miyata *et al*., 1995b). Based on structural analysis of spore peptidoglycan and its dynamics during germination, Atrih *et al*. (1998, 1999) have suggested that there are at least three different types of enzyme activities involved in spore – cortex hydrolysis and modification during germination glucosaminidase, transglycosylase, and a possible non-hydrolytic epimerase. SleB and CwlJ proteins have been recognized as important enzymes in normal spore germination of *B. subtilis*. The SleB protein (Moriyama *et al*., 1996b; Atrih & Foster, 2001b; Chirakkal *et al*., 2002) has apparent lytic transglycosylase activity (Boland *et al*., 2000). The CwlJ protein is required for rapid germination. Spore cortex lytic enzymes (SCLE) which acts on intact spores, needs some activation process for expression of the activity. The mechanism of activation is crucial to an understanding of bacterial spore germination. Proteolytic cleavage of the premature junction of pro SCLE in the complex (the linkage between Val 49 and Val 150 of SleC) during germination generates active SCLE with a mass of 31kDa (Okamura *et al*., 2000). The protease involved in the conversion of pro SCLE to SCLE denoted germination-specific protease (GSP) has been detected in germinated spores (Urakami *et al*., 1999).

Exoproteases have been studied extensively, firstly due to their importance in biotechnology and secondly their role to initiate sporulation. Although AprE and NprE are metabolic enzymes but are not directly involved in growth and sporulation of *Bacillus subtilis.* The transcriptional regulators which regulate spore formation are also involved in regulating genes *aprE* and *nprE.* For instance, the *aprE* gene is directly repressed by AbrB, ScoC, and SinR and is activated by phosphorylated DegU (Ogura *et al*., 2004, Kobayashi, 2007). CodY transcriptional regulator which controls more than 200 genes in *B.subtilis* (Molle *et al*., 2003, Brinsemade *et al*., 2014). Its binding ability to DNA is enhanced with branched-chain amino acids (isoleucine, leucine, and valine [ILV]) (Silvers & Sonenshein, 2004) and GTP (Brinsmade & Sonenshein, 2011.), as ligand. Therefore the activity of CodY is more in amino acid rich media than amino acid exhausted media. It is revealed that *aprE* and *nprE* regulatory regions contain strong binding sites after global analysis of CodY-binding sites *in vitro* and *in vivo*. Null mutants of *CodY* did not exhibit any change in expression of genes. A reason may be that CodY regulates other regulator of protease genes which ultimately repress the genes of these enzymes.

aprE and *nprE* are directly repressed by ScoC, a pleiotropic transcriptional regulator, which also controls expression of a minor extracellular protease (Epr), oligopeptide permeases, and other proteins (Balitsky *et al*., 2015a, 2015b). Simultaneous inactivation of three negative regulators, AbrB, CodY, and ScoC, or the later two regulators is required to observe high *aprE* or *nprE* expression, respectively, under conditions of nutrient excess. (Barbieri et al., 2016).

In the present studies all the germination defective spores showed very low proteolytic activity in comparison with proteolytic activity of normal spores of wild type *Bacillus subtilis* PY79 (Fig-1). It appears that defective spores lack enzymes with proteolytic activity which is necessary for germination. Setlow *et al*. (2004) demonstrated specific proteins required for the hydrolysis of β-MUG, which is hydrolyzed to 4-methyumbelliferone (MU) during germination and outgrowth. The level of protein needed for this hydrolysis is low in dormant spores in *B.atrophaeus* (formerly *B.subtilis* Var. niger). Lack of many general proteins of phosphotransferase system for sugar uptake also hinders β-MUG hydrolysis during spore germination and outgrowth (Setlow *et al*., 2004).

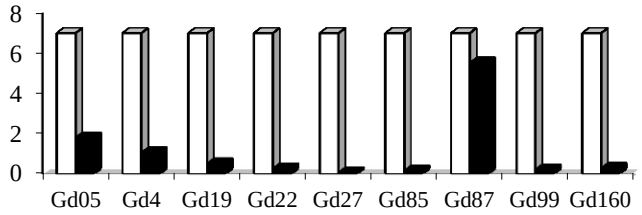

Fig. 1: Comparative representation of proteolytic activity in germination defective spores of isolated srains(shaded) and normal spores of *Bacillus subtilis* PY79(white)

CONCLUSION

All the germination impaired spores of isolated Bacillus strains exhibited very low germination response in ALA and AGFK systems with alternated germinants to alanine and asparagines respectively. Comparative analysis of proteolytic activity of germination defective spores with normal spores of wild type *Bacillus subtilis* PY79 showed very low proteolytic activity in defective spores than normal ones. This guides to vital role of proteases to trigger germination in impaired spores even variable germinants were provided.

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