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EVIDENCE OF INVOLVEMENT OF PROTEINS IN GERMINATION OF GERMINATION DEFECTIVE SPORES OF BACILLACEAE, WITH ALANINE

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Abstract-The response of germinatin defective spores in germination pathways with and without germinant were studied on Polyacrylamide gel electrophoresis (PAGE) to explore the involvement of particular protein, which might have key role to enable the germination defective spores to germinate. The protein profile in core, cortex and coat of germinating spores in specific systems L-alanine(ALA system) and a combination of L-ala, glucose, fructose and potassium(AGFK system) along with and without germinant (alanine) was analyzed periodically. Gd160 strain use alanine as germinant in ALA system. Germinating spores of G160 showed the de novo synthesis of polypeptides with molecular weight of 53.5, 51.5, 47.8, 46 and 33.8 kDa in ALA system with alanine. While inhibition of 66, 65, 61, 31, 23 and 21 kDa was recorded. Germinating spores of Gd27 showed the synthesis of new proteins with molecular weight 61.8 (at initial stages of germination), 57.8, 56.5, 55, 53.2, 50.5, 48 and 47 kDa within 24 hours. While some proteins were inhibited such as 66, 56, 54.5, 46, 31, 15 and 12 kDa in AGFK system with alanine. In brief addition of or deletion of some proteins or even change in protein processing effects the sporulation process which directly or indirectly alter the germination responses. It exhibited a great variety, which indicate the involvement of different mechanisms in these strains.

Key words: ALA system, AGFK system, Bacillus spore, Alanine, Germination

1. INTRODUCTION

Bacillus are inhabitant of soil and they frequently face adverse environmental conditions and have gradually established advanced controlling network to respond quickly to variable changes in temperature, moisture, or nutrient availability. The developed complicated system involves the addition or removal of proteins to cope with extreme conditions. Same phenomena can be observed with Bacillus spores which add or delete some proteins in them and on the basis of that induction or reduction of proteins the morphogenetic pathways within spore germination response can be affected [1, 2, 3, 4]. Although few different results were also reported according to which proteins are not involved in triggering spore germination [5].

Whenever spores get germinant they resume germination. The avaibility of nutrients initiate spore germination through interaction of germinant with germinant receptors at the spore's inner membrane. Borsch- Pederson et al. 2016 [6] reported that spores of B. licheniformis germinate efficiently in response to a range of different single amino acid germinants. Mutational analyses revealed that the GerA and Ynd germination receptors function together in initiating an effective germination response with germinants. Neither GerA nor Ynd could function alone in stimulating spore germination. Different receptors are involved individually or cooperatively for different nutrients, which ultimately triggered the germination by synthesizing or inhibiting various proteins.

In this regard to explore the involvement of different proteins, protein profile of germinating spores of germination defective spore forming bacteria in response to both systems (ALA, AGFK) after different intervals was studied. In this study we tried to know the kind of proteins introduced or hindered to trigger germination on basis of molecular weight.

2. MATERIAL AND METHODS

2.1. Strains Used

Spore former Isolates 22, 23, 25, 27, 28, 29, 31, 32, 33, 37, 38, 39 126, 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 (Gd22, Gd27, Gd29, Gd31, Gd32, Gd33, Gd37, Gd160, Gd161, Gd126) which were germination defective spore forming bacteria were used for this study.

2.2. Characterization

The isolates were characterized according to Gerhardt et al.1994 [7].

2.3. Spore Staining

To check the ability of spore formation, 0.76% malachite green is used on smear providing steam. Gram safranine applied as secondary stain.

2.4. 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 positive result. The isolates which were unable to show pink coloration were selected for further study [8].

2.5. Nutrient Induced Germination Pathways

Isolates which produce unable to germinate spores 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 Venkatasubramanian and Johnstone,1993[9].

2.6. Polyacrylamide Gel Electrophoresis (Page)

Method of Laemmli (1970) [10] is applied to detect addition or deletion of protein of different molecular sizes in germinating spores in both systems(ALA, AGFK with and without alanine) after different intervals and the proteins of core and coat of those germination defective spores.

3. RESULT

Twenty five spore forming isolates were obtained from different stress environments (Industrial and polluted) and characterized according to Gerhardt et al.1994 [7], the spore forming ability was checked by spore staining. The potential of spore germination of isolates was checked at TBBA (Tryptose blood base agar) medium. The ten Bacillus spp among those twenty five spore forming strains were unable to germinate on TBBA medium. Ten isolates were selected for further study (Table-3.1). The germination response of those ten germination defective spore forming strains to alanine as germinant in both AGFK and ALA nutrient induced germination pathways was observed. Gd27 in AGFK with alanine and Gd160 in ALA with alanine exhibited prominent germination.

Table-1 Soil Samples Used to Isolate Spore Forming Bacteria

S.No	Sample Location	Sample Condition	ISOLATES OBTAINED FROM	
			Soil	**Water**
1	**Kasur**	Industrial area	**Gd31, Gd32, Gd33, Gd37**, 38, 39,	**Gd22,** 23, 25, **Gd27**, 28, **Gd29**
2	**Zafer Ali road LHR**	Polluted area	156, 157, 158, 159, **Gd160, Gd161**	**Gd126**, 127, 128, 129, 130, 131, 132,

Prefixed with Gd and in bold are germination defective spore forming bacteria

Finally both were selected to study induction and inhibition of protein synthesis in response to alanine The samples to load on PAGE were collected after different intervals during incubation (0 min,20 min,40 min,60 min and 24 hours) of both strains in ALA and AGFK system with and without alanine as germinant. In Gd160 six polypeptides of 21, 23, 31, 61, 65, 66 kDa in spore core, and 65 and 69 kDa in spore wall were observed. Gd160 strain used alanine as germinant in ALA system. Germinating spores of Gd160 showed the de novo synthesis of polypeptides

with molecular weight of 53.5, 51.5, 47.8, 46 and 33.8 kDa in ALA system with alanine. While inhibition of 66, 65, 61, 31, 23 and 21 kDa, was recorded (Table-3.2).

Table-3.2 Polypeptide profiles (kDa) of Spores of Gd160 in ALA System

SPORE		GERMINATING SPORES IN ALA SYSTEM AFTER DIFFERENT TIME INTERVALS									
CORE	CORTEX +COAT	WITHOUT ALANINE					WITH ALANINE				
		0min	20min	40min	60min	24 hours	0min	20min	40min	60min	24 hours
66	69	61.2	61.2	61.2	61.2	61.2	61.2	61.2	61.2	61.2	61.2
65	65	51.7	57.5	57.5	57.5	55.5	57.5	57.5	57.5	57.5	57.5
61	-	43	55.5	55.5	55.5	51.7	53.5	51.5	53.5	53.5	53.5
31	-	30.5	51.7	30	44	44	51.5	47.8	51.5	51.5	51.5
23	-	26	36	28	35	35	47.8	44	47.8	44	46
21	-	-	30	26	26	26	44	36	44	35	44
-	-	-	26	-	-	-	35	35	35	28	36
-	-	-	-	-	-	-	26	28	28	26	35
-	-	-	-	-	-	-	-	26	26	-	33.8
-	-	-	-	-	-	-	-	-	-	-	28
-	-	-	-	-	-	-	-	-	-	-	26

kDa: kilo dalton, molecular weight of protein bands

In Gd27 seven proteins of 12-66 kDa were observed in spore core and four proteins of 63-69 kDa in the spore cell wall. In spore cell polypeptides of 31 (expression manyfolds) and 64kDa were expressed more. Protein pattern of germinating spores was determined in AGFK system using alanine as germinant. Germinating spores of Gd27 showed the synthesis of new proteins with molecular weight 61.8 (at initial stages of germination), 57.8, 56.5, 55, 53.2, 50.5, 48 and 47 kDa within 24 hours. While some proteins were inhibited, such as 66, 56, 54.5, 46, 31, 15 and 12 kDa, in AGFK system with alanine (Table-3.3).

Table-3.3 Polypeptide Profiles (kDa) of Spores of Gd27 in AGFK System

SPORE		GERMINATING SPORES IN AGFK SYSTEM AFTER DIFFERENT TIME INTERVALS									
CORE	CORTEX +COAT	WITHOUT ALANINE					WITH ALANINE				
		0min	20min	40min	60min	24 hours	0min	20min	40min	60min	24 hours
66	69	70	70	68	70	67.8	70	69	69	69	67.8
64	68	68	68	65	67.8	65	69	65	65	65	65
56	65	65	65	64	65	64	65	64	63	64	63
54.5	63	64	64	63	64	63	64	63	59	63	59
46	-	63	63	48.8	63	60	63	-	-	-	57.8
31	-	-	-	-	-	59	61.8	-	-	-	56.5
15	-	-	-	-	-	58	52	-	-	-	55
12	-	-	-	-	-	52	50	-	-	-	53.2
-	-	-	-	-	-	-	49	-	-	-	52
-	-	-	-	-	-	-	-	-	-	-	50.5
-	-	-	-	-	-	-	-	-	-	-	49
-	-	-	-	-	-	-	-	-	-	-	48
-	-	-	-	-	-	-	-	-	-	-	47

kDa: kilo dalton, molecular weight of protein bands

DISCUSSION

Spores of B.subtilis have three clear cut parts, the central one is known as the core; consisting on chromosomal DNA, few specific protein involved to protect nucleotide and are important to proceed germination and outgrowth of spore is assumed to exist in the core [11]. A thin cell wall layer; the cortex, exterior to core include very less proteins required for germination. The outer covering of spore known as spore coat comprised of two layers; include

a large number of proteins of various sizes, few of proteins among them were regulating processes of cell metabolism and morphogenesis [12]. The polypeptide profile of germinating spore cell part (core) and wall component (cortex + coat) of selected strains (Gd 27, Gd160) was analyzed.

To study polypeptide profile of germination defective spores of selected strains (Gd27, Gd160), spores were germinated in germinating system along with germinant with which specific strain yielded optimum germination response and proteins were detected on SDS PAGE. The protein profile of spore cell, spore cell wall and germinating spores in germination system (with and without particular germinant) were compared. Germinating spores of Gd27 and Gd160, exhibited protein profiles both with and without alanine in germination pathways which enhanced their germination responses. In both strains a variety of protein bands ranging from 12-70 kDa were observed in spore components. Nevertheless a great variability observed in protein profileof both strains. Kuwana et al. (2002) [13] via SDS PAGE resolved protein bands from 4.5 to 66kDa from dormant B. subtilis spores. The comparison of polypeptide bands of these spores (which show germination) with intact spore cell, spore cell wall and germinating spores in without germinant germination system revealed the de novo synthesis of proteins and also inhibition / reduction of some polypeptides during germination relative to with germinant germination system. De novo synthesized polypeptides were of molecular weights 61.8, 57.8, 56.5, 55, 48, 47 (in Gd27) 47.8, 46, 33.8, 51.5 (in Gd160) With new polypeptides formation, some polypeptides were reduced too such as 56, 54.5, 15, 12(in Gd27), 61, 21, 23 (Gd160). Commonly reduced polypeptides were 66, 31 (in Gd27 and Gd160). Spores do respond to chemical stimuli for germination (Fig.4.1).

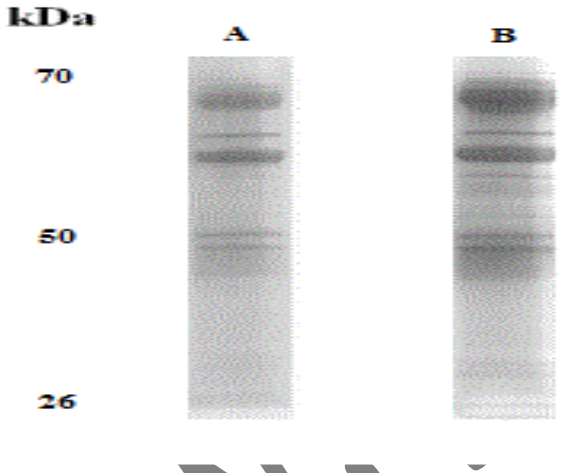

Fig. a Polypeptide Profile of Gd27(A) and Gd160 (B) after 24 hours with alanine as germinanat in ALA and AGFK system

The initial steps in spore germination include synthesis of receptor proteins compatible to GerAA, GerAB and GerAC proteins [14] and localized in the membrane [15]. Receptor for germinant, after combining trigger a chain of reactions [16]. De novo synthesis of cortex-specific hydrolytic enzymes is later step in germination. The hydrolysis of spore-cortex peptidoglycan by enzymes is an essential event during germination before commencing the outgrowth. SleB and CwlJ proteins are important enzymes for germination of B. subtilis spores. SleB has evidence to be involved in the germination by activating lytic transglycosylase activity, whereas spores obtained from sleB mutant are reluctant in completing last steps of germination. A ypeB kind of mutantional defect in gene downstream of sleB, also exhibited similar defects [17]. Both SleB and CwlJ proteins are actually cortex lytic enzymes, which are redundant in function [18]. SleB located both in the inside membrane of the spore and in outer layers and YpeB localized in the outer membrane. YpeB required for the localization and stabilization of SleB. Some proteins such as YaaH is present in large amounts in exudates of germinating spore of B. subtilis but donot have any evidence of key role in the process of germination [19]. Protein profiles of germinating spores of germination defective spores of strains being described here exhibited a great variability for some polypeptides. On the other hand some protein bands were common between different strains. The novel proteins synthesized during germination in different strains are compatible to one another or follow the system similar to SleB/YpeB has yet to be resolved.

Many genes and proteins required for coat have been purified and analysed in details and record of these have been shown, involvement of proteins with evident roles in coat structure, coat function and spore germination [19]. CotA, CotB, CotC and CotD are proteins with molecular sizes of 65, 59, 12 and 11 kDa respectively. Spores obtained from strains having mutations in either of the genes responsible to synthesize coat proteins exhibited the wild type pattern of coat polypeptides, except the mutation of cot gene whose product showed variation in potein profiles. Spores

bearing inactive alleles of cotA, cotB or cotC did not show any prominent change in phenotype. However, cotD mutant spores germinate somewhat more slowly than do non-mutant spores. Another coat protein CotE had the molecular size of 24 kDa. The bacteria which are unable to synthesize CotE due to mutation in relevant gene, formed spores with optical refractility of normal range, These spores of mutant strain were heat resistant but were unable to resist to lysozyme and are abnormal to germinate as compared to spores of wild type [20].The mutants of cotH gene produced spores which are unable to germinate normally although those spores were normally heat, lysozyme and chloroform resistant, later studies revealed that cotH gene encodes a protein molecular weight of 42.8kDa. 7.8 kDa coat protein is evolved from protein of molecular weight 10.1 kDa which is known as CotT. It consists of proline, glycine and tyrosine as major components, and overall it is made up of seven different kinds of amino acid. A trypsin kind of proteolytic activity converts inactive to active form of protein of molecular weight 7.8 kDa. Altered inner coat layers are recorded in spores from mutation or inactivation of cotT gene, which results in spores with morphologically variation, it is possible that CotT protein which is an inner coat and is cause of slow germination in spores ven then provided with a mixture of fructose, glucose and asparagines. A variet proteins with different molecular weight are observed which plays significant role in resistance and germination of spores such as CotX (18.6 kDa), CotY (17.9 kDa) and CotZ (16.5 kDa). Spores from cells bearing deletions in cotX, cotYZ or cotXYZ are heat and lysozyme resistant but readily clump and respond more rapidly to germinants than do wild type spores. These proteins are functionally involved in access of spore's interior to germinant and have effect on spore hydration activity [21]. The spoIVA gene encodes a protein of 55kDa, after the 2nd hour of sporulation. It is involved in attatchment of matrix to forespore and is located on the mother cell side of the forespore membrane. The yabG gene encode a 33kDa protein. It has no clear signal sequence or hydrophobic regions. An SDS-PAGE analysis showed that the protein sample solublized from yabG mutant spores in the presence of SDS and 2-mercaptoethanol contained increased levels of CotT (15 kDa), YeeK (18 kDa), YxeE (21kDa), CotF (23 kDa), Yrb A (31 and 45 kDa) and SpoIVA (55 kDa) which were not visible or barely visible in the preparation from wild type spores. Takamatsu et al. (2000) [22] reported that the 18 kDa polypeptide might be generated by proteolysis of the primary YeeK because the B. subtilis yeeK gene would potentially encode a 43 kDa protein. Kodama et al (2000)[23] pointed out the role of yxeE gene in B. subtilis, encoding a 15 kDa protein, and the YxeE protein extracted from yabG mutant spores was estimated to be 18 kDa. Both yeeK and yxeE were functionally unknown but their preliminary results suggested that these genes were expressed during sporulation. Wang et al. (2015) [24] relates the triggering of germination within defective spores with memory of SpoVA proteins which stimulate the release of dipicolinic acid in response to germinants and so as a result germination begins otherwise blocked.

In brief addition or deletion of some proteins or even change in protein processing affect the sporulation process which directly or indirectly alter the germination responses. The protein profiles of strains described here exhibited a great variety, which indicating the involvement of different mechanisms in these strains.

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