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Molecular Characterization of Overproducing L-asparaginase Recombinant *Bacillus* Fusants

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Abstract

This work aimed to characterize recombinant strains, with a high production level of L-asparaginase, previously obtained by protoplast fusion between *Bacillus subtilis* and *Bacillus cereus*. The genetic relationship between fusants and their parents was determined using a simple PCR-restriction fragment length polymorphism (RFLP) of 16S rDNAs -based method, and by analyzing the specific L-asparaginase genes and protein patterns. Our results indicate that all fusants have morphological characteristics and specific L-asparaginase genes similar to the *Bacillus cereus* strain whereas protein pattern and 16S rDNAs -RFLP analysis show mixed characteristics from both parents. Different genetic relationships were found between each fusant and its parental strain. These results confirm that protoplast fusion is an important technique in strain improvement. It was used to combine genes from different organisms for creating strains with desired properties.

Keywords: RFLP, Fusants, 16S rDNA, SDS – PAGE.

INTRODUCTION

L-asparaginase (EC.3.5.1.1; L-asparagine aminohydrolase) catalyzes the deamination of L-asparagine to L-aspartate and ammonia. This enzyme has been isolated from a wide range of sources: animals, plant, yeast, fungi, and bacteria cells (Bonthron and Jaskolski [5]; Shrivastava et al. [23]).The principal sources of asparaginase used extensively are from the bacteria *Escherichia coli* and *Erwinia carotovora* (Cammack et al. [6]).It is identified as an effective agent in the therapy of certain types of lymphoma and leukemia. L-asparaginase is able to induce complete remission in up to 80% of patients suffering from acute lymphoblastic leukemia. In spite of its effectiveness as a therapeutic agent, the drug causes severe immunological reactions (Dhavala et al. [8]; Raetz_ and Salzer [21]). The numerous side effects of L-asparaginase therapy in all patients remain to be elucidated (Lowas et al. [16]; Earl [9]). The search for other L-asparaginase sources can lead to an enzyme with less adverse side effects (Oza et al. [20]; Shrivastava et al. [23]; Oza et al. [19]; Agarwal et al. [3]).

In *Bacillus subtilis*, the L-asparaginase (*ansA*) gene is located in an operon with *ansB*, which encodes L-aspartase. Expression of the *ansAB* operon is repressed by AnsR, and the activity of AnsR has been proposed to be regulated by either asparagine or aspartate (Sun and Setlow [24]; Sun and Setlow [25]).

Protoplast fusion is an important tool in strain improvement. It has been used to combine genes from different organisms for creating strains with desired properties (Abdel-Salam et al. [1]; Abdel-Salam et al. [2]). This technology allows recombination to take place not only between related species but also between unrelated genera and is of great potential in breeding and improvement of industrial strains (Abdel-Salam et al. [1]).

Molecular characterization of bacterial strains using 16S rDNA study is a common method used for taxonomic purposes, largely due to the mosaic composition of phylogenetically conserved and variable regions within the gene (Gurtler and Stanisich [11]). Phylogenic studies require additional complicated restriction fragment length polymorphism (RFLP) schemes as part of the identification protocol. Relatively simple PCR-RFLP-based identification methods can be used to differentiate many species of *Arcobacter, Campylobacter, Helicobacter* (Marshall et. al. [17]) and *Agrobacterium tumefaciens* (Al-Karablieh et al. [4]) using either purified DNA or crude bacterial cell lysates.

The present investigation was undertaken with an aim to characterize the morphological, biochemical and genomic features of fusants between *B. subtilis* and *B. cereus* in comparison with their parental strains. For this purpose three methods of identification were used including analysis of the *B. subtilis* specific *L-asparaginase* gene, 16S rDNA-restriction fragment length polymorphism (RFLP) and analysis of protein patterns.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains used in the present experiments, their characteristics and sources are listed in Table 1. All strains were maintained at 37^{0}C on LB agar ((Davis et al. [7]).

Bioinformatics

For identification of PCR primers to amplify the L-asparaginase gene, the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) was used. The 16S rDNA sequences of both *Bacillus subtilis* and *Bacillus cereus* were obtained from the available data in the EMBL Nucleotide Sequence Database (http://www.ebi.ac.uk/embl/). These sequences were analyzed for their restriction endonuclease cutting using the Webcutter 2.0 software (http://rna.lundberg.gu.se/cutter2/).

DNA Manipulation

Restriction Fragment Length Polymorphism (RFLP)

Bacterial strains were cultivated on LB plates at 37°C for 18 hours. Crude lysates of cells (Ostuki et al. [18]) (fresh preparation) from these strains were obtained and subjected to PCR amplification using the 16S rDNA forward primer (5′-AGTTTGATCATGGTCAG-3′) and the 16S rDNA reverse primer (5′-GGTTACCTTGTTACGACT-3’). The primers were designed based on the highly conserved region of 16S rDNA from various bacteria (Weisberg et al. [28]). The negative control was done using the hot star master mix with the two primers only. Three µl of cell lysate were amplified in a 100 µl reaction mixture by using the hot star master mix from QIAGEN. The PCR was performed using a DNA thermal cycler (Perkin Elmer GeneAmp PCR System 9600). The PCR program consisted of one cycle of DNA denaturation at 95°C (5 min.), 35 cycles of 95°C (2 min), 48°C (1 min) and 72°C (4 min), plus one additional cycle of a final chain elongation at 72°C (20 min). The PCR products were purified using QIAquick PCR purification kit (QIAGEN) (Tork et al. [27]).

Amplicons representing the 16S rDNA of 1,517 or 1,483 bp were obtained from *B. subtilis* or *B. cereus*, respectively. The concentration of PCR product was estimated using ethidium bromide staining of agarose/TBE gels. The differentiation of these amplified DNAs was carried out based on the restriction digestion pattern using two restriction enzymes, namely, *Aat* II and *Hpa* I.

For restriction endonuclease digestion, a 20-µl reaction mixture was prepared by mixing 10 µl of purified PCR product with 10 U of the restriction endonuclease *Aat* II or *Hpa* I (Sambrock, et al. [22]). The mixture was incubated overnight at 37°C. Ten microliters of each digest were analyzed electrophoretically at 25 V/Cm for 1 h with a 1.5% agarose gel in 0.5X Tris-Borate-EDTA. The obtained bands were stained with ethidium bromide and visualized using a UV transilluminator (Sambrock, et al. [22]).

Amplification of Asparaginase Coding Gene

A1100-bp DNA fragment containing the *ansA* gene was obtained by PCR amplification of *B. subtilis* chromosomal DNA with hot star master mix from QIAGEN using primers ANS1 (5′-CCCAAGGAAGTCTTTTTCCA-3’) and ANS2 (5′-AGTGAAGAGGTGCATGGTATGA -3’).

The DNA sequence of the asparaginase gene (*ansA*), including 94 bp upstream and 18 bp downstream of the gene, was obtained from the available data in the EMBL Nucleotide Sequence Database of *B. subtilis* (990 bp). Primers derived from this sequence were obtained using Primer3 software. PCR conditions consisted of one cycle of DNA denaturation at 94°C (5 min), 35 cycles of 94°C (1 min), 55°C (1 min), and 72°C (2 min), plus one additional cycle of a final chain elongation at 72°C (7 min). The PCR products were purified using QIAquick PCR purification kit (QIAGEN).

Protein Content

Protein content was detected according to Laemmli [14] on a 0.75 mm thick polyacrylamide slab gel (7·8 cm) using mini gel system Bio-Rad. Low molecular weight protein standard (Amersham Pharmacia) ranging from 14-97 KD was used. The gels were stained for about 1 hr in 0.2% Commassie blue R-250 dissolved in the fixative solution (45 ml methanol, 10 ml acetic acid and 45 ml water). Then, the gels were destained by repeated soaking in the fixative solution only.

Statistical Analysis

Statistical analysis was carried out using the SPSS 10.0 for Windows software package (Statistica).

Table 1. Bacterial strains used in the present study and their characteristics.

Bacterial strains	Characteristics	Source
Bacillus subtilis	Wild type, L-asparaginase activity 16.3 U/ml	Alazhar University, Faculty of Science, Microbiology Dept.
Bacillus cereus BcNv 29	Rif^r, L-asparaginase activity 20.2 U/ml	El-Hamshary and Khattab, (2008)
B. s. :: *B. c.* FSC2 (F1)	Fusant, L-asparaginase activity 39.2 U/ml	Hegazy and Moharam, (2010)
B. s. :: *B. c.* FSC3 (F2)	Fusant, L-asparaginase activity 31.1 U/ml	Hegazy and Moharam, (2010)
B. s. :: *B. c.* FSC1 (F3)	Fusant, L-asparaginase activity 32.0 U/ml	Hegazy and Moharam, (2010)

RESULTS AND DISCUSSION

Protoplast fusion is a powerful technique used to produce genetically engineered microbial strains harboring desirable industrial properties. It is widely used to combine genes from different organisms to create strains with desired properties. It provides a mechanism by which genetic recombination can be readily achieved and should be of great potential in empirical breeding and strain improvement.

Fusants were generated by protoplast fusion between *B. subtilis* and *B. cereus* strains (Hegazy and Moharam [13]), and three fusants with higher L-asparaginase activity were selected for detailed characterization (Table 1).

Genetics Differentiation between Parents and Fusants Bacterial Strains

Amplification of L-asparaginase gene of *B. subtilis*

The *L-asparaginase* gene of *B. subtilis* (*ansA*) was amplified by PCR using primers designed based on published *ansA* gene sequences. It consists of an open reading frame of 987 nucleotides encoding a protein of 329 amino acids with a calculated molecular mass of 36441 Da. Primers amplified a 1100 bp PCR product band (Fig. 1). The band was visualized using ethidium bromide staining of agarose/TBE gels.

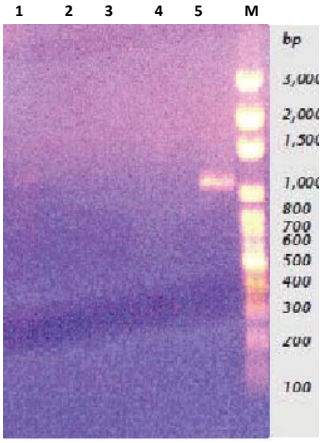

Fig.1. Agarose gel electrophoresis of PCR amplified *L- asparaginase* gene. Lanes 1, fusant F3; 2, fusant F2; 3, fusant F1; 4, B. cereus; 5, *B. subtilis*; M, AXYGEN 100 bp Ladder DNA (100 bp – 3000 bp).

An amplicon of 1,100 bp was detected in the case of the *B. subtilis* strain (Fig. 1), whereas no DNA bands were shown in the case of the *B. cereus* or the three recombinant fusants, these results indicate that the obtained recombinant fusants acquired the asparaginase gene from the *B. cereus* parent. However, the estimation of asparaginase activity revealed that the recombinant fusants had more efficient asparaginase activities i.e, 1.5 to 2.4 fold higher than their parents. Genetic modification may have occurred in the regulatory region of the *L-asparaginase* gene which results in increasing its productivity through promoter fusion as illustrated by Hartl [12].

16S rDNA RFLP analyses

Primers based on the conserved sequences of the 16S rDNA gene were used to amplify the 16S rDNA fragment for the two parents and their fusants. Restriction endonuclease digestion of the polymerase chain reaction (PCR) product enabled species differentiation (Al-Karablieh et al. [4]). Restriction enzymes were used to yield fragments that allowed identifying the similarities between parents and fusants. Variable regions between parental strains suggested earlier restriction enzyme differentiation. The choice of restriction enzymes exploited these variable regions and enabled further distinction between parents and fusants. 16S rDNA PCR amplification resulted in a 1517 base pair DNA fragment from *Bacillus subtilis* and a 1483 base pair DNA fragment from *Bacillus cereus.* The DNA sequences were obtained from the available data in the EMBL Nucleotide Sequence Database. These sequences were analyzed for their restriction endonuclease cutting using the Webcutter 2.0 software. From the obtained data two enzymes were used, i.e., *Aat* II and *Hpa* I. *Aat* II recognizes the site 5'- gacgt/c -3' and cuts the 16S rDNA of *Bacillus subtilis* into two sites at 1015 and 1184 producing three DNA fragments of lengths 1015, 333, and 169 bp, while it only cuts the DNA of *Bacillus cereus* once at 1184 producing two DNA fragments, i.e, 1184 and 229 bp. *Hpa* I recognizes the site 5'- gtt/aac -3' and does not cut the 16S rDNA of *B. subtilis* but cuts that of *B. cereus* once at 854 producing two DNA fragments sized 854 and 629 bp.

RFLP analyses of amplified 16S rDNA of *Bacillus* parental strains and their three tested fusants indicate that all fusants have a significant restriction pattern different from their parents (Table 2 and Fig. 2).

Table 2. 16S rDNA restriction patterns of bacterial parents and their fusants.

Gene name / bacterial strain	Enzyme name	NO. cuts And position	Expected fragments*	Recognition sequence
16S rDNA *B. subtilis*	*Aat*II	2 (1015,1184)	1015, 333, 169 bp	gacgt/c
16S rDNA *B. cereus*	*Aat*II	1 (1184)	1184, 299 bp	gacgt/c
Fusant 1	*Aat*II	1	1184, 299 bp	gacgt/c
Fusant 2	*Aat*II	1	1184, 299 bp	gacgt/c
Fusant 3	*Aat*II	1	1184, 299 bp	gacgt/c
16S rDNA *B. subtilis*	*Hpa*I	0	1517, 000 bp	gtt/aac
16S rDNA *B. cereus*	*Hpa*I	1 (854)	854, 629 bp	gtt/aac
Fusant 1	*Hpa*I	1	854, 629 bp	gtt/aac
Fusant 2	*Hpa*I	1	854, 629 bp	gtt/aac
Fusant 3	*Hpa*I	1	854, 629 bp	gtt/aac

* As calculated from the Webcutter 2.0 software.

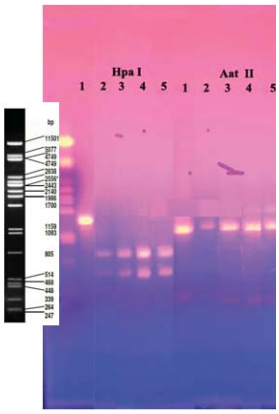

Fig.2. 16S rDNA restriction patterns of bacterial parent strains and their fusants. Lane M, Marker lambda DNA- *Pst* I, lane 1, *B. subtilis;* lane 2, *B. cereus*; lane 3, Fusant 1; lane 4, Fusant 2; lane 5, Fusant 3.

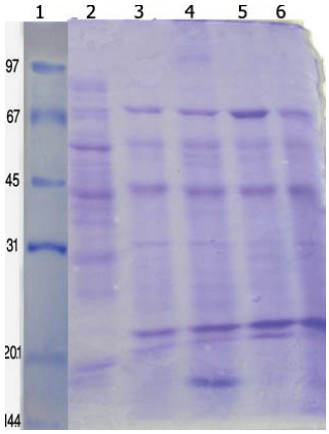

Fig.3. Protein profile of bacterial parents and their fusants. Lane1, Molecular weight markers; Lane2, *Bacillus subtilis;* Lane3, *Bacillus cereus;* Lane4, Fusant F1; Lane5, Fusant F2; Lane6, Fusant F3.

To determine whether fusants contain the genomic DNA of both the parental strains or only a small part of one of the parental chromosomes integrated into the genome of the other strain, the nucleotide sequences of the 16S rRNA gene of fusants were analyzed. The most variable region of the 16S rRNA gene was amplified from the genomic DNA of the three fusant strains using PCR. The PCR products were digested using the two restrictions enzymes *Aat* II and *Hpa*I. The data obtained demonstrated that the recombinant strains have a significant exchange of taxonomic characteristics that are displayed between the two species, although the majority apear similar to the *Bacillus cereus* parent in their morphological appearance. The three fusants have the restriction pattern of *Bacillus cereus* with *Hpa* I yielding 854 bp and 629 bp. The restriction patterns of fusants resemble *Bacillus subtilis* with *Aat* II (Table 2 and Figure 2). This observation indicates the occurrence of DNA recombination in this site. These results correlate with the results obtained by (Teeradakorn et al. [26]), which found that the *xlnB* gene of fusant D3 was similar to that of *S. cyaneus* 190-1, but that the *xlnA* gene of fusant D3 was similar to that of *S. griseoruber* 42-9.

Protein Profile

Protein profiles of both parental and their fusant strains were studied according to Laemmli [14].

SDS/PAGE analysis reveals that 20 protein bands are detected among the tested strains (Table 3 and Figure3). Twelve protein bands are unique, ten of them are detected in the *B. subtilis* parental strain and two are in the fusant F1. The latter two bands are about 41 and 16.5 kDa. These two new protein bands indicate the occurrence of DNA recombination that results in production of protein fusion and/or a new regulation system affecting transcriptional processing in this fusant as illustrated by Hartl [12].

Protein analyses reveal also the occurrence of two monomorphic bands with 71.3 and 16.6 kDa, and six polymorphic bands.

Table 3. Protein profile and polymorphism of parental and fusants strains.

Band no	MW KDa	Parental strains		Fusant strains			Polymorphism
		B.S	B.C	F1	F2	F3	
1	84.877	+	-	-	-	-	Unique
2	80.373	+	-	-	-	-	Unique
3	71.318	+	+	+	+	+	Monomorphic
4	70.575	+	-	-	-	-	Unique
5	66.830	+	-	-	-	-	Unique
6	60.557	-	+	+	+	+	Polymorphic
7	57.948	+	-	-	-	-	Unique
8	46.594	-	+	+	+	+	Polymorphic
9	45.531	+	-	-	-	-	Unique
10	42.220	+	-	-	-	-	Unique
11	41.344	-	-	+	-	-	Unique
12	39.563	+	-	-	-	-	Unique
13	35.476	-	+	+	+	+	Polymorphic
14	32.827	+	-	-	-	-	Unique
15	31.413	+	-	-	+	+	Polymorphic
16	26.954	+	-	+	-	-	Polymorphic
17	22.649	-	+	+	+	+	Polymorphic
18	20.523	+	+	+	+	+	Monomorphic
19	16.675	+	-	-	-	-	Unique
20	16.501	-	-	+	-	-	Unique

Data obtained from the EMBL Nucleotide Sequence Database reveal that the L-asparaginase gene of *B. cereus* forms of an open reading frame of 976 nucleotides encoding a protein with a molecular mass of 35327 Da. The protein band numbered thirteen (Table 3) which exists in *B. cereus* and all the three fusants probably represents the L-asparaginase of *B. cereus.* These results confirm our previous results that demonstrate that the three fusants have PCR fragments of the asparaginase gene similar to that obtained with *B. cereus.*

The relationship and dendrogram analyses (Figure 4) of fusants and parental strains based on data recorded from protein patterns (Table 3) demonstrate that F1, with the highest l-asparaginase activity, i.e., 39.2 U/ml (Hegazy and Moharm [13]), shows two additional polypeptide bands not found in both parents In addition, all three hybrids (F1, F2, F3) harbor protein bands from both parents. This observation supports the occurrence of genetic recombination between the two parental genomes. It was also found that fusants 2 and 3 are closely related to each other whereas fusant 1 is less related to each of *B. subtilis* or *B. cereus* than the two other fusants (Table 4). On the other hand, the protein pattern of the parent *B. subtilis* was clearly different from those obtained from the parent strain *B. cereus* and the three recombinant fusants. These results correspond with Law et al. [15] who obtained a number of hybrids by fusion of protoplasts of complementary auxotrophic strains of *Candida albicans.* SDS-PAGE analysis of the cell wall proteins indicated that the hybrid cell walls contained many more of the proteins associated with one parent than the other.

In conclusion, molecular characterization of higher producer L-asparaginase fusants confirms the existence of DNA recombination between the two parental strains which results in obtaining different fusants harboring different genetic backgrounds and different relatedness between each fusant and the parental strains. Specific L-asparaginase gene and protein analyses indicate that L-asparaginase produced by the recombinant fusants is closely related to the *B. cereus* parental strain. Two protein bands are detected in one of the fusants indicating the occurrence of protein fusion and/or a new regulatory system affecting the transcription process. These results confirm the importance of the protoplast fusion technique in *Bacillus* for combining genes from different organisms to create strains with desired properties.

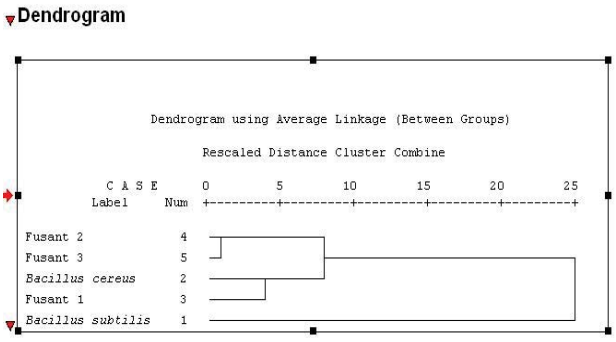

Fig.4. Dendrogram demonstrating the relationship among the three fusants and parent strains based on data recorded from protein patterns.

Table 4. Fusant protein profile relatedness to their Bacillus parental strains.

Parental strains	Fusant strains		
	F1	F2	F3
Bacillus subtilis	1/18 5.6%	3/18 16.7%	3/18 16.7%
Bacillus cereus	15/18 83.3%	17/18 94.4%	17/18 94.4%

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