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# Molecular characterization of *Bacillus subtilis* OKR isolated from wheat rhizosphere reveals key outer membrane proteins associated with plant growth promotion: A step towards climate-smart agriculture

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**Abstract**

*Bacillus subtilis*, is reported to be an efficient bacterium and a universal cell factory for industry, medicine and biomaterials. It also possesses plant growth promotional activities. In the current study, we aimed to identify and characterize wheat rhizosphere *B. subtilis* at molecular level such as by 16S RNA gene, *in vitro* screening, and outer membrane proteins using host mimic simulation by two-dimensional gel electrophoresis. Based on 16S RNA gene results, the isolate OKR was identified as *B. subtilis* and clustered with *Bacillus subtilis* strain QD517 and *B. subtilis* strain LQ20. The isolate assigned the name as *B. subtilis* strain OKR under accession no. KP201140.1. *In vitro* screening of OKR for growth proportional and biocontrol activities has revealed it as an effective type of plant growth rhizobacterium. Furthermore, the outer membrane protein analysis not only substantiated the attributes of *B. subtilis* strain OKR as PGPR, but also revealed that leaf extract (LE) media could be used as an alternative *in vivo* model for plants to characterize plant promotional activities at molecular level. Each condition was found to correspond to a unique protein expression profile due to the significant protein's expression diversity observed between the control and the simulated host condition. Two-component response regulators and heat shock proteins are key proteins identified under host-mimicking conditions that appear to be indispensable for plant growth-promoting activities. Furthermore, the identification of common proteins such as $Na^+/H^+$ antiporters and ABC transporter-like proteins holds a promise for survival and metabolic activity in each niche. We believe that our findings will contribute to the development of environment-friendly, sustainable, and climate-smart agricultural technology strategies that can succeed under limited environmental conditions.

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## Introduction

Rapid urbanization has dramatically affected agricultural land use, resulting in less arable

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land and less space to grow crops. Under such conditions, increasing crop production is crucial for addressing global food security challenges. However, achieving this goal is not always straightforward, as crop production can be negatively impacted by a range of biotic and abiotic stressors (Godfray et al., 2010; Masmoudi et al., 2023). The plant growth promoting rhizobacteria (PGPR) are being effectively used in agricultural systems for maximizing growth of crops along with achieving enhanced plant defense against a variety of pathogens (Zhang et al., 2024). The use of PGPR in agriculture can lead to a reduction in the use of synthetic fertilizers and pesticides, which can have negative environmental impacts. Additionally, PGPR can help improve soil health and increase plant resilience to biotic and abiotic stressors (Tripathi et al., 2024).

PGPR can affect plant growth directly or indirectly. For example, the direct mechanism involves gains of resources such as nitrogen fixation, phytohormone synthesis, important minerals such as nitrogen and phosphorus or modulation of plant hormone levels, whereas the indirect mechanism refers to the resistance of abiotic and biotic stresses and may also include biocontrol agents (Kang et al., 2015; Jeyanthi and Kanimozhi, 2018; Bouremani et al., 2023). *Bacillus* spp. have been extensively studied for their beneficial effects on plant growth, and have been shown to improve seed germination, root development, nutrient uptake, and provide protection against a range of plant pathogens. As such, they are commonly used as biofertilizers and biocontrol agents in agriculture (Leser et al., 2008; Gouda et al., 2018; Zhang et al., 2023).

Along with the existing several molecular biology techniques, next generation transcriptome sequencing, microarrays, and proteomics studies have not only widely explored the host strategies, but also the microbes (Shoresh and Harman, 2008; Shi et al., 2024) using host-microbe interaction. Leaf extracts have been used as a host-mimicking carrier in several studies aimed at understanding the molecular basis, mechanisms, and adaptations of microbial host niches. This approach involves extracting soluble compounds from plant leaves, which can then be used as a growth medium to culture and study microbial communities (Vaou et al., 2021). The use of leaf extracts as a growth medium can also help overcome some of the challenges associated with studying microbial communities *in vivo*. For example, it can be difficult to isolate and culture specific microorganisms from complex environmental samples, such as soil or plant tissues. By using leaf extracts as a growth medium, researchers can more easily manipulate the microbial community and study the effects of specific environmental factors on microbial growth and function.

In the present study, we identified the *B. subtilis* resourced from a wheat field, tested efficacy of *B. subtilis* by various biochemical tests, and developed the culture media to simulate the plant hosts and LB media as a control following extraction of outer membrane proteins and LC-MS/MS analysis. Overall, the identification of a *Bacillus subtilis* strain from the wheat field and the development of a culture medium to simulate the plant host environment can be important steps in understanding the beneficial effects of this microbe on plant growth and health. It can also provide insights into the ecology of plant-microbe interactions in agricultural ecosystems and the potential for developing sustainable approaches to agriculture based on these interactions.

## Materials and Methods

### Sample collection, culture conditions and biochemical tests

Twenty rhizobacterial isolates were collected from the wheat rhizosphere, transferred to Microbial Genomics Laboratory at COMSATS University Islamabad, Sahiwal Campus and stored at 4 $^{o}$C. Five grams of rhizosphere soil were added to a 250 mL conical flask which was filled with 100 mL sterile distilled water. The suspension was sieved to remove any heavy materials from it. The flask was shaken on a rotary shaker at 180 rpm for 10 minutes.

The culture was streaked and incubated for 24 h at 37 $^{o}$C to observe bacterial colonies. Following day, the colonies showing different morphology appeared on the medium and they were chosen for further purification. A single colony was selected after repeated re-streaking on new LB agar plates and kept warm for the process of incubation. This procedure was performed three times to get pure cultures. The selected four isolates were diluted in the LB media and optical density was measured by a spectrophotometer at each interval of 30 minutes in order to obtain a suitable optical density (OD) for the inoculation experiments. The log phase culture stage was chosen for biochemical tests.

Various biochemical tests were conducted to evaluate and screen 20 isolates named as Okara 1-Okara 20 for their various potential biochemical plant growth promotional activities. The key tests considered for characterizations were production of indole acetic acid (IAA) and organic acids, hydrogen cyanide (HCN) production and protease activity as described by Tsegaye et al. (2019).

## DNA extraction

Of 20, only two isolates based on their biochemical characteristics were selected to proceed 16S RNA gene amplification and sequencing. Total genomics DNA was extracted from an overnight grown bacterial culture at 37 °C using Fermentas Genomic DNA purification kit (Thermo Fisher). Briefly, the bacterial culture was centrifuged at 14,000 × *g* for 10 minutes, the supernatant removed, the pellet resuspended in 800 µL of S1-Lysis buffer, vortexed ups and down, and transferred each to a Bead Tube. The S2-Lysis enhancer (100 µL) was added, incubated for 10 minutes at 65 °C following homogenization for 10 minutes by bead beating on the vortex mixture by a maximum speed and centrifuged for 2 minutes at 14,000 × *g*. The supernatant was taken and transferred to a fresh tube and an aliquot of 900 µL of S4-Binding buffer was added, briefly vortexed, and 700 µL of the sample mixture were loaded onto a spin column-tube assembly. The sample was centrifuged for 1 minute at 14,000 × *g*. The spin column was placed in a clean collection tube with the addition of 500 µL of S5-Wash buffer following centrifugation for 1 minute at 14,000 × *g* for 1 minute. The step was repeated two times and finally 100 µL of S6-Elution buffer were added, incubated at room temperature for 1 minute, centrifuged for 1 minute at 14,000 × *g.* Finally, the purified DNA was stored at 4 °C for further use.

## PCR amplification and sequencing of 16S rRNA

For amplification, universal 16S rRNA gene primers were used, and the amplified product was sequenced for the downstream sequence analysis. The PCR was conducted with following PCR reaction conditions: each reaction was 25 µL having 13.8 µL Taq polymerase 2X Master mix, 0.5 µL of forward primer, 0.5 µL of reverse primer, 15.3 µL of water and 2 µL template DNA. PCR was done in 25 µL reaction volumes. The primes used were 27f and 1525r (Forward 5’AGAGTTTGATCMTGGCTCAG, Reverse AAGGAGGTGWTCCARCC). The PCR initial denaturation was set for 5 min at 95 °C which was followed by denaturation for 30 s at 94 °C of 35 cycles. Annealing was performed for 30 s at 62 °C. Then, extension was performed for 1 min at 72 °C. Another final step of extension was followed for 5 min at 72 °C. The quantity and size of PCR product was assessed using gel electrophoresis. An aliquot of 0.8 µL of agarose was mixed with 100 mL TBE buffer and both reagents were heated for 2 min approximately. Ethidium bromide 3-4 µL was added to the Eppendorf tube. An aliquot of 5 µL of DNA sample was mixed with the 2 µL loading dye electrophoresed at 100 V for approximately 30 min, visualized, and photographed with a UV transilluminator (WEALTEC) by fluorescence at 488 nm. The PCR product was sent to Korea for 16S RNA gene sequencing (Macrogen, Korea).

## Sequencing, accession number and evolutionary analysis

The raw data of 16S RNA sequence was obtained, trimmed and converted into FASTA format. The nucleotide blast (blastN) of the Fasta sequence was performed with default parameters (expect threshold 0.05, word size 28 and gap cost linear) and confirmed sequences were submitted to the GenBank for assigning of accession number. Following BLAST analysis, the sequence with >80% similarity with maximum query coverage was selected for evolutionary analysis. The maximum likelihood method was adopted to drive phylogeny using Molecular Evolutionary Genetics Analysis software (MEGA) v.10.

## Preparation of outer membrane proteins

The OMPs of *B. subtilis* strain Okara were prepared by the sucrose gradient method as described by Jagannadham and Chowdhury (2012) with little modifications. In brief, lysozyme 60 µg/mL in the membrane buffer (1 mM PMSF, pH 8.0, 2 mM EDTA, 0.75 M sucrose and 10 mM Tris) was used to treat the cells. The cells were broken by sonification and centrifugation at 4 °C for 10 minutes using a centrifugation of 8000 rpm. The supernatant was taken and proceeded for centrifugation at 4 °C with 30,000 rpm for 2 h. The pellets were suspended in the membrane buffer having 2% Triton X-100 and incubated for 30 min at room temperature. Thereafter, the samples were centrifugated at 4 °C for 2 h at 30,000 rpm to separate the proteins associated with inner membrane while the proteins associated with OM remained in the pellets. The pellets enriched with OM proteins were dissolved in the lysis buffer comprising 2% CHAPS, 1.98 M thiourea and 8.5 M urea. The extracted OM protein was treated with EDTA and RNase to remove ribosomal contamination as described by Schell et al. (2011) and the proteins were separated by the 2-D gel electrophoresis.

## In-gel digestion and extraction of peptides

In order to proceed for the In-gel digestion, the de-staining of 2-D gel electrophoresis was carried out by removing the selected protein gel bands and exposing to in-gel trypsin digestion as described by Ibrahim et al. (2012). Briefly, the gel pieces were preserved with acetonitrile (ACN, 1:1) and 50% of 25 mM ammonium bicarbonate. Moreover, the drying of the solvent was done with the help of a SpeedVac concentrator. Keeping in view the size of the gel band, additional 10-20 µL of trypsin (10 µg/mL in

ammonium bicarbonate) was added to the gel pieces and incubated at 37 °C for 16-18 h. Then resulting peptides were extracted with 5% trifluoroacetic acid in 50% ACN. The solutions comprising peptides were merged and dried up in a speed-vac device until proper volumes were approached (25-50 µL).

### LC-MS/MS of trypsin-digested proteins

The preparation of peptides from purified outer membrane were done in two biological replicates following analysis by LC-MS/MS as described by Ibrahim et al. (2012). Briefly, the gel bands were excised, shifted to 0.5-mL Eppendorf tubes and 1:1 solution of 100 mM sodium thiosulfate. Potassium ferricyanide (30 mM) was used for de-staining. Then the digestion was performed using 2% formic acid (v/v) and 50% acetonitrile (v/v) solution using a Multiprobe II Plus Ex robotic liquid handling system (Perkin Elmer Waltham, MA, USA). The peptides (10 µL) so produced were proceeded for separation using an UltiMate 3000 Nano LC system (Thermo Scientific Dionex, MA, USA). The peptides profile obtained from the LC-MS/MS were managed to detect the proteins against *B. subtilis* by the MASCOT database which is an automated database (Perkins et al., 1999). By using SEQUEST, cross-correlation scores (X corr) were calculated and placed for protein detection (Eng et al., 2008). The X corr of charged peptides either singly, doubly or triply were more than 1.8, 2.5, and 3.5, respectively. In order to increase the overall sequence coverage, peptides having values less than the specified thresholds (with a cutoff value of 50) were also taken into consideration. A final list of potential OM proteins was prepared by combining all obtained putative proteins from LC-MS/MS runs, and their manual verification was done.

### *In silico* analysis of LC-MS/MS released peptides

A subcellular localization of proteins from whole proteome obtained from the LC-MS/MS was predicted for *B. subtilis* strain Okara in depth *in silico* studies by using Protparam and PSORTb (Yu et al., 2010) at a significant score > 7.5. The peptides obtained by the LC-MS/MS from the *B. subtilis* strain 168 were employed as bait proteome for studying the similarities using the local BLAST. While complete sequences of the proteins were extracted from the proteome of *B. subtilis* strain 168; the grand average of hydropathicity (GRAVY) score of peptides and signal peptides estimated by SignalP 3.0 server were also assessed to examine the nature of those proteins either hydrophobic or hydrophilic. The functional annotation of each protein sequence was done by Gene Ontology, UniProt, Pfam, PDB, etc. A list of OM-associated proteins of *B. subtilis* strain Okara correlated with other PGPRs were also manually assembled by applying keywords such as OM, porin, secretin, receptors, pili, flagella, quorum sensing, lipoprotein, flagella, pili, secretin, porin, receptor, and surface.

## Results and Discussion

### Isolation and biochemical tests

Overall, twenty pure bacterial samples were selected for biochemical tests for testing plant growth promoting activities such as organic acid and IAA production (**Table 1**).

**Table 1. Evaluation of *Bacillus* strains for their potential to produce different metabolites**

| Isolate | IAA production | Organic acid production | HCN production | Antifungal activity |
|---|---|---|---|---|
| Okara 1 | - | + | + | - |
| Okara 2 | +++ | +++ | +++ | +++ |
| Okara 3 | - | - | - | - |
| Okara 4 | + | ++ | + | + |
| Okara 5 | - | - | - | - |
| Okara 6 | - | + | - | + |
| Okara 7 | - | - | - | - |
| Okara 8 | - | - | - | - |
| Okara 9 | + | - | - | ++ |
| Okara 10 | - | - | - | - |
| Okara 11 | ++ | + | ++ | + |
| Okara 12 | + | ++ | + | + |
| Okara 13 | +++ | +++ | +++ | +++ |
| Okara 14 | ++ | - | + | + |
| Okara 15 | - | - | - | - |
| Okara 16 | - | - | - | - |
| Okara 17 | - | - | - | - |
| Okara 18 | + | ++ | ++ | + |
| Okara 19 | - | - | - | - |
| Okara 20 | - | - | - | - |

- no response; ++ moderate response; + little response; +++ high response

Out of 20 only two isolates Okara 2 and Okara 13 produced promising amount of IAA and organic acids. Similar results were obtained when these isolates were evaluated for biocontrol activities such as HCN production and antifungal activities (**Table 1**). The useful plant-microbe association in the rhizosphere is considered as essential contributing factor of soil fertility and plant health (Solomon et al., 2023). PGPRs correspond to a wide variety of soil associated bacteria which interact with the plant host and promote growth. These microbes used a variety of actions to improve plants by using plant's secreted metabolites. The production of IAA, as well as organic acids substantiated the characteristics of these isolates as PGPRs. Earlier published studies have reported similar observations for IAA production (Selvakumar et al., 2008; Khianngam et al., 2023). Similarly, both siderophores and HCN are known as potential fungal inhibitors, i.e., they show antifungal activity and protect the plants from fungal diseases (Flaishman et al., 1996; Martinez-Arias et al., 2020).

## 16S RNA sequencing and evolutionary analysis

Of 20, only two isolates were processed for DNA extraction and PCR amplification based on their PGPR impact. The universal primer of 16S rRNA gene was used for amplification using the extracted DNA as a template. The obtained product size of the 16S rRNA gene was about 1400 bp (1.4 kb) long as shown in **Figure 1**. The lane 1 represents the positive control, lanes 2 and 3 negative controls, while 4 and 5 represent the isolates Okara 2 and Okara 13 (**Figure 1**). The isolate Okara 13 was successfully sequenced and considered for future studies. The nucleotide sequences of 16S rRNA genes were subjected to BLAST analysis with the NCBI database. The sequences showing high similarity scores with 16S rRNA genes were downloaded based on homology analysis, i.e., maximum score, maximum identity and with lowest E-value. The results showed that isolate Okara 13 had a close identity with *B. subtilis* strain LQ20 and *B. subtilis* strain QD517 as the sequence similarity of these strains was > 99% (**Figure 2**). Moreover, the strain also existed in the cluster which encodes *B. subtilis* and *B. amyloliquefaciens* species. It is also worth to mention that the cut-off value of sequence identity considered in the phylogenetic tree was 50. The isolate was designated as strain Okara and the 16S sequence was submitted to NCBI Genbank under accession number KP201140.1 *B. subtilis* strain Okara. In the cluster, closely related species such as *Bacillus amyloliquefaciens* and *Bacillus subtilis* group has well renowned significance in the agriculture sector and mostly used as biopesticides (Dame et al., 2021).

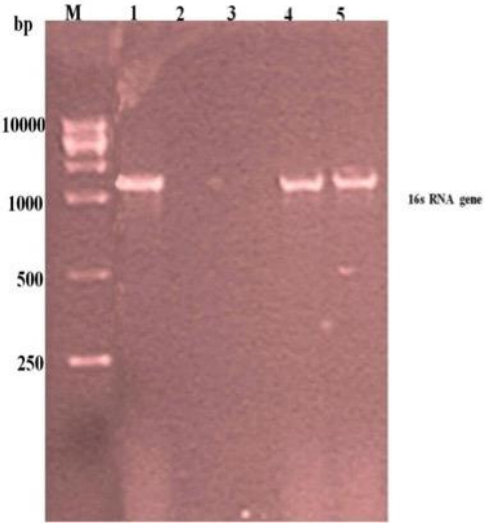


**Figure 1. Visualization of 16S rRNA gene amplification by agarose gel electrophoresis analysis amplified from 20 bacterial isolates. Lanes 4 and 5 show the amplified PCR product of 16S rRNA gene, 3 and 4 negative controls and 1 as positive control.**

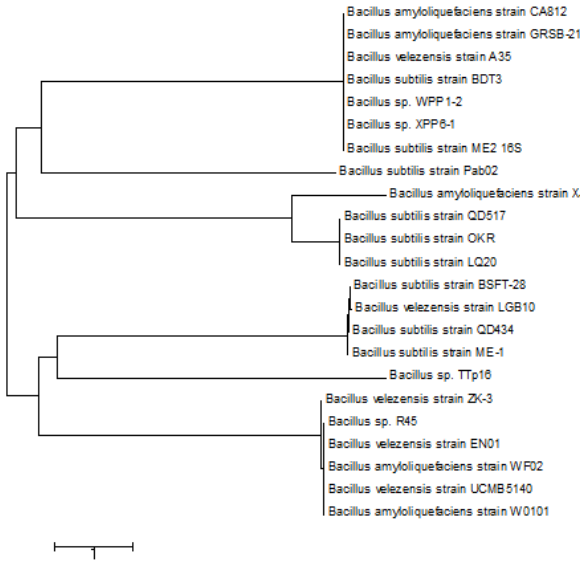


**Figure 2. Evolutionary tree of 16S rRNA gene. The tree was built with full-length 16S rRNA gene sequences employing the neighbor-joining method. The values of the most important internal nodes (Bootstrap values with 1000 replicates) are shown in the tree.**

## 2-D profile of OMPs under *in vitro* and host *mimic growth conditions*

The results of 2D gel electrophoresis revealed that the outer membrane protein profile of *B. subtilis* varied in the control and leaf extract, although there was a resemblance in some protein bands under the two growth conditions. We observed several protein spot differences between LB control media and LE media (**Figure 3**) which may have comprised a significant number of proteins. The protein bands looked in the silver

staining were cut out from the gel into small pieces and digested with trypsin. The subsequent peptides were then evaluated with the help of LC-MS/MS.

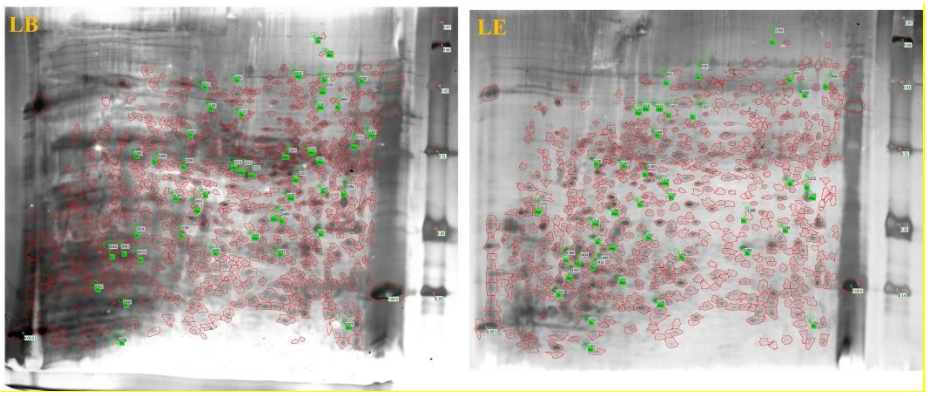


**Figure 3. 2-D gel electrophoresis stained by silver staining. Lanes 1 and 2 represent the protein bands extracted from OMPs of leaf extract media, and Lanes 3 and 4 represent the control media.**

## OMPs profiling of *B. subtilis*

Bacteria are capable of changing their expression of genes and proteins quickly and proficiently in response to signals from the environment for the purpose of survival, virulence, energy metabolism, and stress response (Malard et al., 2022). In our current study, we aimed to examine the proteome expressed under two different growth conditions based on the LC-MS/MS method. In addition, *in silico* analysis was carried out to predict the subcellular localization and the functional categorization of these LC-MS /MS identified OMPs (**Table 1** and **Table 2**).

**Table 2. LC-MS/MS identified common membrane proteins under control and leaf extract media**

| Locus Tag | Protein type |
|---|---|
| BSU00970 | Hypothetical protein |
| BSU03060 | L-lactate permease |
| BSU03080 | Transcriptional regulator, MarR family |
| BSU03090 | Transporter, LysE family |
| BSU03230 | Regulator of polyketide synthase expression |
| BSU03260 | Aromatic hydrocarbon catabolism protein |
| BSU03340 | GTP cyclohydrolase I (EC 3.5.4.16) type 2 |
| BSU03350 | ABC-type sugar transport systems |
| BSU04230 | Permease components |
| BSU04590 | Transmembrane protein, distant homology with ydbS |
| BSU05730 | Membrane protein |
| BSU07190 | Hypothetical protein |
| BSU07400 | Major facilitator family transporter |
| BSU09890 | ABC transporter, ATP-binding protein |
| BSU09900 | ABC transporter, permease protein |
| BSU12069 | Hypothetical protein |
| BSU12070 | Hypothetical protein |
| BSU29530 | Protease IV |
| BSU30060 | NAD(FAD)-utilizing dehydrogenases |
| BSU30070 | Glycine betaine transporter OpuD |
| BSU30200 | Biotin synthase (EC 2.8.1.6) |
| BSU30840 | Uncharacterized protein |
| BSU31010 | yuaG membrane protein |
| BSU31090 | K(+)-uptake protein KtrA, peripherally bound subunit |
| BSU31330 | Hypothetical protein |
| BSU31560 | Nucleoside ABC transporter, permease protein 1 |
| BSU31640 | Na(+) H(+) antiporter subunit E |
| BSU31650 | Na(+) H(+) antiporter subunit F |
| BSU33000 | Serine protease |
| BSU33690 | Choline ABC transporter, substrate-binding protein |
| BSU33710 | OpuCC |
| BSU33850 | Multiple antibiotic resistance protein marC |
| BSU33990 | LysR-family transcriptional regulator Bsu YvbU |
| BSU37410 | Hypothetical protein |
| BSU37440 | 17 kDa surface antigen |

The identified proteins were pooled, duplicates removed, and a non-redundant list of OMPs collected based on the membrane proteome of *B. subtilis* (**Table 1** and **Table 2**). Overall, the results of this pooled data showed that 55 OMPs were identified under the LB culture condition, and 43 under the leaf extract culture condition simulated to *in vivo* mimic growth condition. Furthermore, these proteins were identified in at least 2 replicates under each condition with 96% confidence level. These recognized proteins were mainly annotated based on the Cluster of Orthologues Groups (COG).

In catalog of these proteins, there were common and unique membrane proteins among the LB and LE host mimic growth conditions of *B. subtilis*. **Table 2** listed 35 most abundant proteins which were recognized in both conditions of growth of *B. subtilis*. **Table 3** presents 29 unique outer membrane proteins identified only under the LB medium, while **Table 4** and **Figure 4** present the distinctly expressed outer membrane proteins in the LE medium.

**Table 3. LC-MS/MS identified membrane proteins exclusively under LB conditions**

| Locus Tag | Protein type |
|---|---|
| BSU01140 | Hypothetical protein |
| BSU01600 | Trilactone hydrolase [bacillibactin] siderophore |
| BSU02170 | Putative transporter |
| BSU03050 | L-lactate dehydrogenase |
| BSU03070 | Drug resistance transporter, EmrB/QacA family |
| BSU03240 | DUF1980 domain-containing |
| BSU03250 | Hypothetical protein |
| BSU03359 | Hypothetical protein |
| BSU04220 | General stress protein 26 |
| BSU04360 | Manganese transport protein MntH |
| BSU04370 | Integral membrane protein |
| BSU04490 | ABC transporter, ATP-binding protein |
| BSU04500 | ABC transporter, permease protein |
| BSU04600 | Membrane-flanked domain |
| BSU04610 | Rhomboid family protein |
| BSU05540 | Probable membrane protein yetF |
| BSU05620 | D-serine/D-alanine/glycine transporter |
| BSU07200 | Hypothetical protein |
| BSU25000 | Uncharacterized protein YqgF |
| BSU25010 | Putative membrane protein |
| BSU26620 | Uncharacterized transporter YrdR |
| BSU29520 | Unknown conserved protein |
| BSU31030 | Hypothetical protein |
| BSU31540 | ABC transporter, periplasmic nucleoside-binding protein |
| BSU31550 | ABC transporter, ATP-binding protein |
| BSU31620 | Na(+) H(+) antiporter subunit C |
| BSU31630 | Na(+) H(+) antiporter subunit D |
| BSU33090 | Sensor histidine kinase LiaS |
| BSU33110 | Conserved protein LiaG in *B. subtilis* in Lia cluster |

**Table 4. LC-MS/MS identified membrane proteins exclusively under leaf extract conditions**

| Locus Tag | Protein type |
|---|---|
| BSU06020 | Heat shock protein 60 family co-chaperone GroES |
| BSU06030 | Heat shock protein 60 family chaperone GroEL |
| BSU07360 | Methyl-accepting chemotaxis protein |
| BSU08240 | Catechol-2,3-dioxygenase |
| BSU08290 | Two-component system sensor kinase |
| BSU08300 | Two-component response regulator |
| BSU10380 | Methyl-accepting chemotaxis protein |
| BSU14010 | Chemotaxis protein CheV |
| BSU19200 | DNA-binding response regulator, LuxR family |
| BSU28710 | Carbon starvation protein A |
| BSU30390 | Two-component sensor histidine kinase BceS |
| BSU30400 | Two-component response regulator BceR |
| BSU31520 | Two-component sensor histidine kinase |
| BSU32110 | Thioredoxin reductase |
| BSU32770 | VBIBacSub10457_3401 |

## Characterization of common outer membrane proteins

The topology of the common class of OMPs (**Table 2**) identified in our study was generally as follows: ABC transporter proteins that encompass the most of identified and pragmatic OMPs and localized as outer membrane by Protparam and PSORTb were identified abundantly under both growth conditions. The transporter proteins of the bacteria are well conserved for performing an indispensable role in intermediating the uptake. They take the large molecule to outer cell surface and small molecules with the environment (Ozkan et al., 2024). The transporters such as ABC transporter proteins consist of a group of heterogenous proteins which represent their various cellular and functional roles (Zeng and Charkowski, 2021). So, identification of these proteins is consistent with the ecological niche of *B. subtilis* strain Okara.

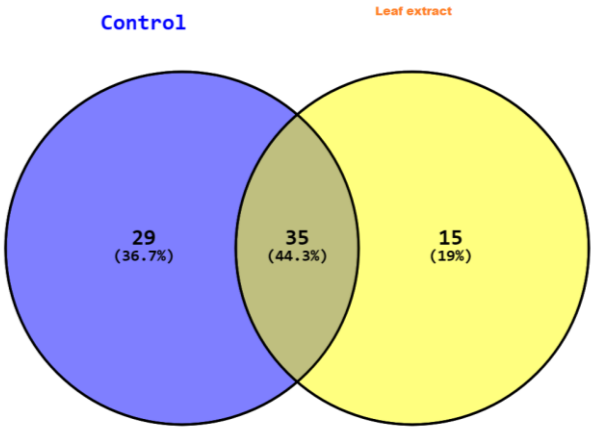


**Figure 4. Venn diagram representing the differentiation among membrane proteins identified from leaf extract and control media.**

The other group of identified proteins in every condition of growth was Na(+) H(+) antiporter subunit C. Previous genomic studies have reported numerous copies of the Na(+) H(+) antiporter genes most of the time in the form of operon, particularly in those bacteria living under challenging environments of temperature, toxic salt concentrations, extremes of pH and other environmental factors. The number of $Na^+(K^+)/H^+$ antiporters depend on versatility of the bacteria such as 5-9 copies are reported in general, however, some particularly ecologically stressed comprise higher numbers of cation/$H^+$ antiporters (Krulwich and Hicks, 2009; Mesbah et al., 2009; Wang et al., 2023). Looking into the key roles of $Na^+/H^+$ antiporters such as maintenance of stress, pH, cation homeostasis and salt we consider that *B. subtilis,* $Na^+/H^+$ antiporters seem to be indispensable for survival in each niche.

Bacterial adaptation to various environments is an interesting area of study and shows a considerable capacity for understanding the niche adaptation; it may be contingent on regulation of various combined molecular mechanisms which may help bacteria to sense the environmental changes, process the signals, and orchestrate the physiological changes accordingly (Pranavathiyani et al., 2021; Rabbi et al., 2021). To address this, the genome and the proteome of various microbes have been investigated by previous studies. However, there are few coding sequences in the genome that are predicted to code for proteins but having no evidence of expression at the translation level (Desler et al., 2009; Dall'Agnol et al., 2014). Proteins encoded by such sequences were assigned as “hypothetical” or “uncharacterized” These hypothetical proteins were found to constitute a major portion of microbial genomes. Such as in our study the list of common proteins includes the abundant number of hypothetical or uncharacterized, i.e., 10 out of 29 proteins identified by the LC-MS/MS.

Besides the afore-mentioned proteins, several other proteins in single copy were also identified such as L-lactate dehydrogenase which is an important enzyme of the anaerobic metabolic pathway (Mercer et al., 2019). Manganese transport protein MntH functions to manage manganese which is essential for several biological processes as an enzyme cofactor and serves as a protectant of oxidative stress (Kehres and Maguire, 2003; Puccio et al., 2022). The identification of these proteins seems to be indispensable for the survival of *B. subtilis* in every niche.

## OMPs expression under LE medium

Bacterial variation in the environment of plants is very broad and happens at various cellular levels due to which gene expression and metabolic activity occur. Different omics methodologies are significant to understand completely the system levels (Barnard et al., 2007; Okoye et al., 2024).

This fact is well understood that the proteome is considered to be the final resultant of gene expression. From this research, we have exposed for the first time the profiling of proteome of bacterium *B. subtilis* strain Okara 1 which is PGPR for plants under plant circumstances in a biolab by incubating the bacterium in the LE medium; this is similar to what has been reported earlier (Kall et al., 2004; Barnard et al., 2007; Khianngam et al., 2023). In this research, the results show that 17 proteins were recognized under the LE culture condition, exposing the response of plant material against the modifications in membrane proteome of bacteria (**Table 4**). By analyzing comparatively the proteome of outer

membrane, it has been found that under both LE culture conditions, the expression of 17 proteins was induced due to adding plant materials, which were recognized under the LE medium conditions (**Figure 4**), but not under the LB culture conditions (**Table 4**).

Surprisingly, the majority of these proteins were found to be related to those exposed under *in vivo* circumstances, e.g., energy producing, energy assimilation, conversion proteins, NADH dehydrogenase subunit B, etc. Additionally, some proteins associated to recognize and synchronize under the LE medium circumstances were also identified under *in vivo* circumstances, e.g., heat shock protein 60 family co-chaperone GroES, methyl-accepting chemotaxis protein, two-component system sensor kinase and chemotaxis protein CheV (**Table 4**).

A protein can undergo post-translational modification (PTM), which is a covalent alteration of an amino acid, changing its characteristics and functions. These functions include folding, ligand binding, migration to a new location, interaction with other molecules, and other specific roles (Ramazi and Zahiri, 2021). In the case of molecular chaperones, these roles could include any of the various ways they chaperone client polypeptides and their non-canonical tasks (Begum et al., 2019). In reaction to stressful situations, cells create a class of proteins known as heat shock proteins (HSPs) (Dhawi, 2020). Originally associated with heat shock, it is now recognized that they can also manifest in response to other stressors, including as exposure to cold or UV radiation, as well as during wound healing or tissue remodeling. Numerous members of this category carry out chaperone duties by stabilizing newly synthesized proteins to guarantee proper folding or by assisting in the refolding of proteins that have been damaged by cellular stress. The hypothesized genes involved in the transport and catabolism of chemicals obtained from plants as well as tolerance to various environmental stressors such as heavy metals, ROS, cold, heat, or osmotic shock are associated with the remarkable adaptation of PGPR to their environment (Chen et al., 2018). These genes, widely distributed in the PGPR proteome, lay the groundwork for rhizosphere fitness.

Bacteria primarily employ two component systems (TCS) to detect their environment and modify their behavior accordingly (Kall et al., 2004; Lazar and Tabor, 2021). They are crucial components of a variety of adaptive processes, including virulence, chemotaxis, metabolism, motility and others. Therefore, the requirement for adaptability and the capacity to react to environmental cues are fundamental for microorganisms interacting with plants. The two-component system sensor kinase found in our research has four copies.

Certain intestinal bacteria have a family of transmembrane receptors called the methyl-accepting chemotaxis proteins (MCP, commonly known as the aspartate receptor) that regulate chemotactic response (Park and Seo, 2015; Hida et al., 2020). For root colonization, chemotaxis-mediated response to root exudates, starting by sensing-specific 21 ligands through methyl-accepting chemotaxis proteins (MCPs), is crucial.

Verily, these proteins were not able to be identified under the *in vitro* LB medium (**Table 1**), which shows that these proteins might be a sound reason for the response of *B. subtilis* hosts.

## Conclusion

Identification of *B. subtilis* and its evolutionary analysis provides a useful insight about *B. subtilis* features which is known as a universal cell factory for industry, medicine and biomaterials. Several significant proteins were recognized differentially between the two niches, showing the complexity of relation between *B. subtilis* and wheat host. Identification of various molecular bases such as two-component response regulator, two-component sensor histidine kinase, and heat shock proteins under leaf extract conditions seem to be vital for respective niches; it is promising for survival and metabolic activities in each niche. The use of leaf extract medium for *in vivo* simulation is a promising area of research. Leaf extract media may contain a wide range of biologically active compounds that can simulate the complex environment of living organisms. By using leaf extract media, researchers may be able to study biological processes under conditions that more closely resemble the natural environment.

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This study does not involve human/animal subjects, and thus no ethical approval is needed.

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The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of the experiment, all materials were properly discarded to minimize/eliminate any types of bio-contamination(s).

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**Authors' consent**
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It is declared that we the authors did not use any AI tools or AI-assisted services in the preparation, analysis, or creation of this manuscript submitted for publication in the International Journal of Applied and Experimental Biology (IJAaEB).

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