

Proteomic Insights into the Antibacterial Mechanism of *Brevibacillus brevis* HK544 Producing Edeine B₁ against *Erwinia amylovora*

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Recently, we reported that *Brevibacillus brevis* HK544, which produces edeine B₁ (EB₁), exhibits promising antimicrobial activity against plant pathogens. To elucidate its antibacterial mechanisms against *Erwinia amylovora*, the causal agent of fire blight, we conducted label-free shotgun proteomic analysis of *E. amylovora* TS3128 treated with *B. brevis* culture filtrate (CF) or purified EB₁. Comparative analysis showed significant (over twofold) changes in 45 of 624 and 81 of 846 quantified proteins following CF and EB₁ treatments, respectively. Functional categorization revealed altered abundances of proteins associated with energy production, ribosome biogenesis, and cell wall/membrane/envelope biogenesis in both treatments. Notably, 42 and

75 translation-related proteins were differentially expressed in CF- and EB₁-treated samples, respectively, consistent with the proposed translation-inhibitory activity of EB₁. These findings provide insights into the cellular targets and antibacterial mode of action of *B. brevis* HK544 and EB₁, supporting their potential as biocontrol agents against fire blight.

Keywords : antibacterial mechanism, *Brevibacillus brevis*, edeine B₁, *Erwinia amylovora*, label-free shotgun proteomics

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Fire blight, caused by *Erwinia amylovora* belonging to the order *Enterobacterales*, is a devastating plant disease on Rosaceae plants, including apples, pears, and quinces, leading to severe losses in both crop quality and yield (Hauben and Swings, 2015; Pedroncelli and Puopolo, 2023). Among the various control methods, chemical treatments have been considered one of the most effective strategies for protecting plants. However, the use of chemical compounds can negatively affect the environment through phytotoxicity and the emergence of resistant phytopathogens (Sundin and Wang, 2018). To counteract these issues, biological control methods have been explored as eco-friendly and alternative strategies (Dagher et al., 2020; Lim et al., 2025). Recently, we found a soil-borne bacterium, *Brevibacillus brevis* HK544, which exhibits broad-spectrum antimicrobial activity against various plant pathogens (Kim et al., 2024a; Kim et al., 2024b). From the HK544 culture filtrate (CF), we also identified edeine B₁ (EB₁) as the major active compound responsible for its antimicrobial activity (Kim et al., 2024a; Kim et al., 2024b). EB₁ is an antimicrobial pep-

tide containing five free basic groups and one free carboxyl group, and it is known to inhibit initiation of protein synthesis by blocking the P site of the bacterial 30S ribosome (Polikanov et al., 2018; Westman et al., 2013).

Herein, we evaluated the *in vitro* antibacterial activity of HK544 CF and EB₁ against *E. amylovora* using a two-fold serial dilution assay (Espinell-Ingroff et al., 2005). The bacterial pathogen *E. amylovora* TS3128, provided by the Rural Development Administration (Wanju, Korea), was maintained on mannitol glutamate yeast extract (MGY) medium (D-mannitol 10 g, L-glutamic acid 2 g, KH₂PO₄ 0.5g, NaCl 0.2 g, MgSO₄·7H₂O 0.2 g, yeast extract 1 g per liter) at 28°C. HK544 CF was prepared from 3-day-old *B. brevis* HK544 cultures grown in tryptic soy broth medium (17 g of tryptone, 3 g of soytone, 2.5 g of glucose, 5 g of NaCl, and 2.5 g of K₂HPO₄ per liter) at 28°C. The active compound EB₁ was purified from *B. brevis* HK544 CF as previously described (Kim et al., 2024b). For the antibacterial activity assay, a bacterial suspension (2×10^5 CFU/mL in MGY) of *E. amylovora* TS3128 was dispensed into the wells of a 96-well microtiter plate, followed by the addition of HK544 CF in two-fold serial dilutions starting from 2.5%. The plates were incubated for 24 h at 28°C, and then growth inhibition (%) was calculated as follows: $[1 - (\text{OD}_{600} \text{ of treatment} / \text{OD}_{600} \text{ of non-treatment}) \times 100]$. Growth of *E. amylovora* TS3128 was completely inhibited at CF concentrations $\geq 0.63\%$ (Fig. 1A). In an *in vivo* antibacterial assay performed as described previously (Kim et al., 2022), CF treatment resulted in a disease control efficacy of 87% compared to the non-treatment control (NC), which

is comparable to that of streptomycin treatment (Fig. 1B). Considering that EB₁ is the major active compound responsible for the antimicrobial activity of *B. brevis* HK544, we further assessed the antibacterial activity of EB₁ against *E. amylovora*. The growth of *E. amylovora* TS3128 was completely inhibited at EB₁ concentrations $\geq 5 \mu\text{g/mL}$ (Fig. 1C). All the assays were performed twice with three replicates at all concentrations investigated.

To further elucidate the molecular mechanism underlying the antibacterial activity of HK544 CF and EB₁, the proteomes of *E. amylovora* TS3128 treated with HK544 CF or EB₁ were compared with those of the NC using a label-free shotgun proteomic approach (Kim et al., 2020). For protein extraction, *E. amylovora* TS3128 was cultured overnight in Luria Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) at 28°C, and then the bacterial cell suspension (1 mL) was inoculated into 100 mL of LB broth medium containing CF (0.1%) or EB₁ (1.25 $\mu\text{g/mL}$); these concentrations of CF and EB₁ correspond to IC₅₀ values determined from the results of the antibacterial activity assay in Fig. 1. When the OD₆₀₀ value of the untreated *E. amylovora* reached 0.6, all bacterial cultures including CF or EB₁ treatments were harvested and disrupted using an Ultrasonic Processor (Colo-Parmer, Vornon Hills, IL, USA). Total soluble proteins from nine samples, including the NC, were extracted, concentrated, and digested with trypsin to generate tryptic peptides, as previously described (Cho et al., 2025).

Tryptic-digested peptides (1 μg) of each sample were analyzed by liquid chromatography-tandem mass spec-

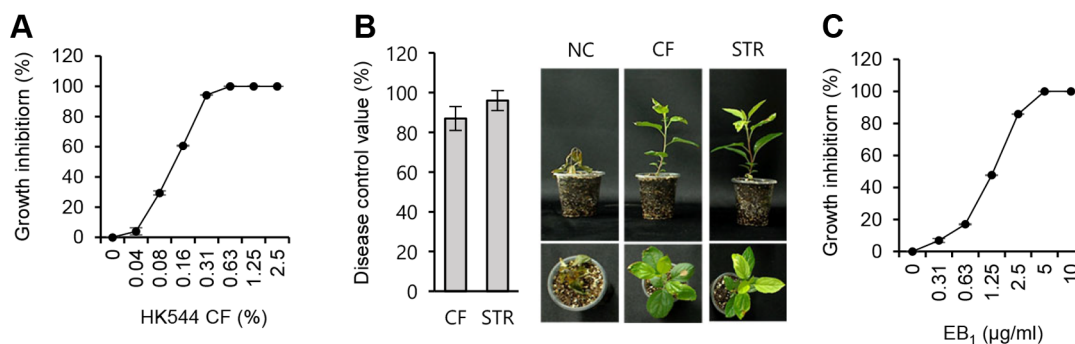


Fig. 1. *In vitro* and *in vivo* antibacterial activity of *Brevibacillus brevis* HK544 culture filtrate (CF) and its metabolite edeine B₁ (EB₁) against *Erwinia amylovora*. (A) Growth inhibition of *E. amylovora* by different concentrations of *B. brevis* HK544 CF. The growth inhibition rate was determined by measuring the OD₆₀₀ value 24 h after CF treatment, relative to the non-treatment control. (B) Disease control efficacy of *B. brevis* HK544 CF on Chinese crab-apple seedling against *E. amylovora*. Plants were inoculated with a bacterial cell suspension (4×10^8 CFU/mL) of *E. amylovora* TS3128 at 24 h after treatment. STR, streptomycin (100 $\mu\text{g/mL}$). The disease control value was calculated based on disease severity at 9 days post-inoculation, relative to the non-treatment control (NC). (C) Growth inhibition of *E. amylovora* at different concentrations of EB₁. All experiments were performed twice with three replicates, and the data are presented as the mean $\pm$ standard deviation.

trometry (LC-MS/MS) (Cho et al., 2025). The samples were injected into a split-free nano LC system (EASY-nLC II; Thermo Fisher Scientific, Bremen, Germany), which was connected to an LTQ Velos Pro instrument (Thermo Fisher Scientific) equipped with the Proteome Discoverer software (ver. 1.3.0.399; Thermo Fisher Scientific). Full MS spectra were acquired with nine data-dependent MS/MS scans and analyzed using the SEQUEST algorithm within the Proteome Discoverer software. Spectra were matched against the *E. amylovora* TS3128 database (chromosome accession no. CP056034.1 and plasmid accession no. CP056035.1, National Center for Biotechnology Information). To control the false discovery rate and enhance identification confidence, the target–decoy approach was applied. The resulting data were imported into Scaffold 4 (Proteome Software, Portland, OR, USA) for comparative analysis. Peptide spectra matches (PSMs) for each protein were normalized to the total PSMs, and the average normalized PSMs from three biological replicates were calculated to identify differentially abundant proteins (Choi et al., 2008). Functional classification of the identified proteins was performed according to clusters of orthologous groups (COG) (Galperin et al., 2025). The MS proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository under the dataset identifier PXD067784 (Perez-Riverol et al., 2019).

The protein numbers shared between each biological replicate were 507, 624, and 846 for NC, CF, and EB₁, respectively (Supplementary Table 1). The number of proteins obtained from three biological replicates corresponded to approximately 16.6–17.1%, 20.6–26.8%, and 26.3–33.7% of the total predicted protein-coding sequences (CDSs), respectively; it has been reported that *E. amylovora* TS3128 contains about a 3.8 Mb genome including 3,237 CDSs, 22 rRNA, and 77 tRNA (Kang et al., 2021). The normalized average of the PSMs in the three biological replicates was calculated and quantitatively analyzed to identify proteins with counts that were significantly different from the mean (over twofold). Statistical analyses were performed using Student's *t*-test to assess the significance of the differences ($P < 0.05$). A total of 52 and 166 proteins were uniquely detected in NC and CF, respectively. Additionally, 54 proteins in NC and 45 proteins in CF exhibited significant differences in abundance (over twofold) compared with each other. In the presence of EB₁, the expression of 497 proteins was altered, of which 26 and 345 proteins were unique to NC and EB₁, respectively, while 45 and 81 proteins were significantly more abundant (over twofold) in NC and EB₁, respectively (Fig. 2A). Proteins exhibiting differential abundance were classified through COGs analysis to eluci-

date the antibacterial mechanisms underlying the response of *E. amylovora* TS3128 to the CF or EB₁ treatment. Differentially abundant proteins identified in the NC, CF-, and EB₁-treated groups are presented in a heatmap, where both biological replicates and proteins were co-clustered using complete-linkage hierarchical clustering with Euclidean distances (Fig. 2B). The protein dendrogram segregated into two large clusters, clearly distinguishing the expression profiles of NC, CF, and EB₁. These differentially abundant proteins were further categorized by COG classification and subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (Supplementary Table 2, Fig. 2C). Between NC and CF, only COGs E (amino acid metabolism and transport) and S (function unknown) showed higher abundance in NC, whereas all other COG categories had equal or greater abundance in the CF-treated group (Supplementary Table 2). In contrast, all COGs exhibited more abundance in the EB₁-treated group. Among the COGs, group J (translation) showed the most pronounced change in response to both CF and EB₁ (Supplementary Table 2). Comparative proteomic analysis across nine biological replicates revealed 50 proteins with distinct abundance patterns, of which 20 proteins were more abundant in NC, while 30 proteins showed increased abundance in the presence of CF and EB₁.

A more detailed comparison between the NC- and CF-treated samples revealed notable differences in proteins associated with amino acid metabolism, as indicated by KEGG enrichment analysis (Fig. 2C). Among the 54 proteins that were more abundant in NC than in the CF-treated samples, 10 proteins were associated with amino acid metabolism, including aspartate aminotransferase family protein (QKZ08075), argininosuccinate lyase (QKZ10791), aminopeptidase N (QKZ09075), aspartate/tyrosine/aromatic aminotransferase (QKZ09072), ornithine carbamoyltransferase (QKZ08238), oligopeptidase A (QKZ07977), oligopeptide ABC transporter substrate-binding protein OppA (QKZ09548), aminomethyl-transferring glycine dehydrogenase (QKZ08467), ABC transporter substrate-binding protein (QKZ08684), and glutamine ABC transporter substrate-binding protein GlnH (QKZ10990) (Table S3). Notably, of the 52 proteins detected exclusively in NC, eight proteins were related to amino acid metabolism: agmatinase (QKZ08436), amino acid ABC transporter substrate-binding protein (QKZ08876), lactoylglutathione lyase (QKZ09346), carboxypeptidase M32 (QKZ09464), lysine/arginine/ornithine ABC transporter substrate-binding protein ArgT (QKZ09913), aminopeptidase PepB (QKZ10074), methylenetetrahydrofolate reductase (QKZ10796), cystathionine gamma-synthase (QKZ10798)

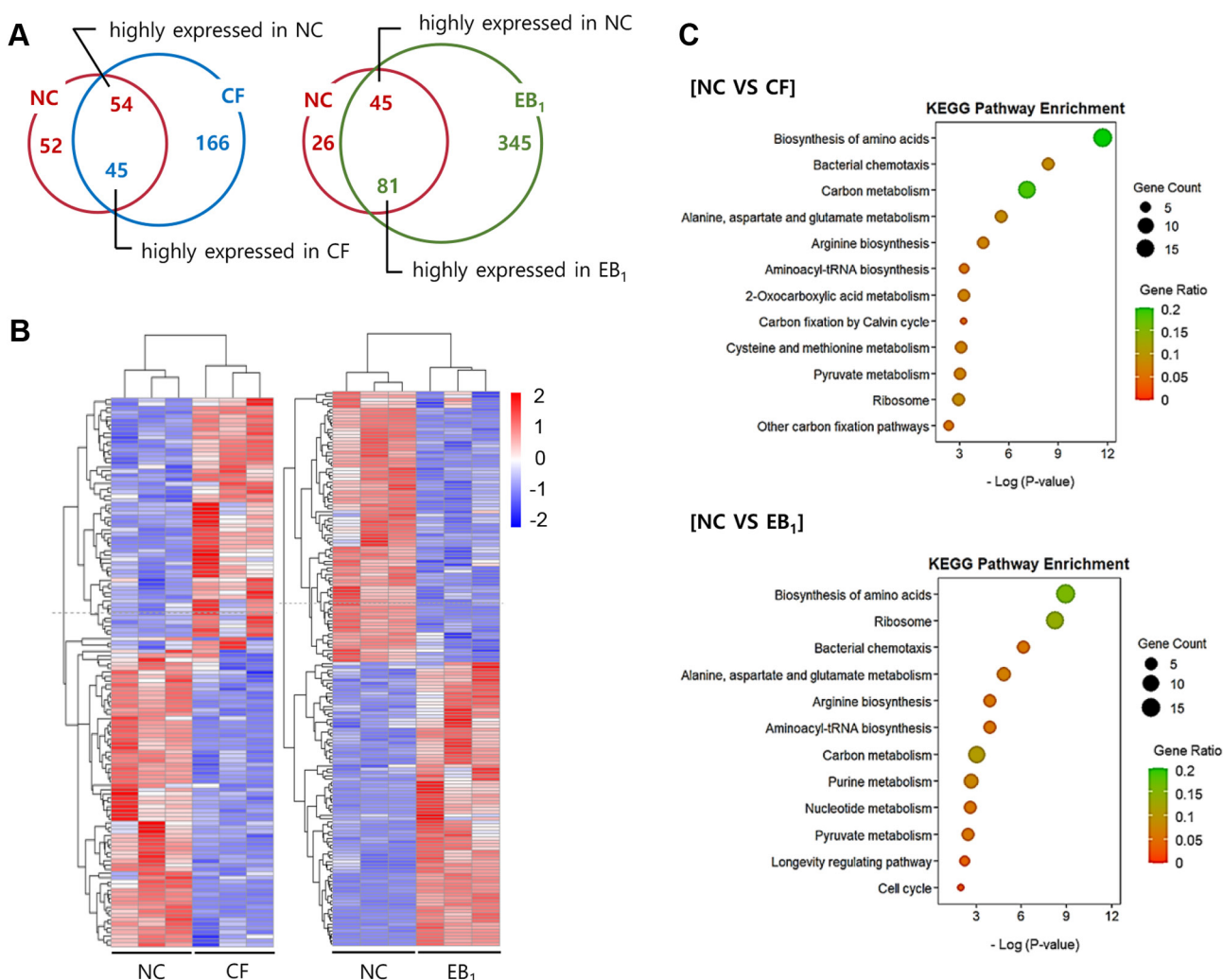


Fig. 2. Comparative proteomic analyses of *Erwinia amylovora* TS3128 treated with culture filtrate (CF) and edeine B₁ (EB₁) at IC₅₀ concentrations. (A) Venn diagrams showing the numbers of proteins with significant differences in abundance (over twofold). (B) Hierarchical clustering of differentially expressed proteins between NC and CF, and between NC and EB₁. (C) KEGG pathway enrichment analysis. The vertical axis indicates the pathway category, and the horizontal axis represents the pathway enrichment score. NC, non-treatment control; CF, treatment with *Brevibacillus brevis* HK544 culture filtrate; EB₁, treatment with edeine B₁; KEGG, Kyoto Encyclopedia of Genes and Genomes.

(Supplementary Table 3). In contrast, among the 45 proteins that were more abundant in the CF-treated samples relative to NC, three proteins were associated with amino acid metabolism: chorismate synthase (QKZ09938), aspartate ammonia-lyase (QKZ10561), and dTDP-4-amino-4,6-dideoxygalactose transaminase (QKZ10759) (Supplementary Table 4). Additionally, eight amino acid metabolism-related proteins were detected exclusively in the CF-treated samples: pyrroline-5-carboxylate reductase (QKZ08692), N-acetyl-γ-glutamyl-phosphate reductase (QKZ09231), asparaginase (QKZ09576), 4-hydroxy-tetrahydrodipicolinate reductase (QKZ10358), aspartate/tyrosine/aromatic aminotransferase (QKZ10650), lysine-

sensitive aspartokinase 3 (QKZ10681), acetylglutamate kinase (QKZ10793), and a transporter substrate-binding domain-containing protein (QKZ09025) (Supplementary Table 4). Therefore, these results suggest that CF and EB₁ treatments may induce a metabolic shift in *E. amylovora* by suppressing amino acid metabolism and selectively re-directing amino acid biosynthetic pathways, which may in turn bacterial growth, replication, cell envelope integrity, and antibiotic resistance, given the essential roles of amino acid metabolism in bacterial survival (Stokes et al., 2019).

A comparison between the NC and EB₁-treated samples revealed that translation-associated proteins were strongly affected by EB₁ treatment. Six 30S ribosomal proteins

(QKZ08109, QKZ09057, QKZ08611, QKZ08125, QKZ08126, and QKZ08117) were more abundant (over twofold) in the EB₁-treated samples compared with the NC-treated samples. In addition, translation initiation factor IF-3 (QKZ11007), histidine-tRNA ligase (QKZ10058), and seven 50S ribosomal proteins (QKZ08106, QKZ10707, QKZ08613, QKZ10708, QKZ10706, QKZ08206, and QKZ08207) were more abundant (over twofold) in the EB₁-treated samples (Supplementary Tables 5 and 6). Moreover, seven 30S ribosomal proteins (QKZ08098, QKZ08107, QKZ08112, QKZ08116, QKZ08180, QKZ08282, and QKZ10529) and six 50S ribosomal proteins (QKZ08105, QKZ08111, QKZ08119, QKZ08121, QKZ09311, QKZ09846, and QKZ10853) were detected exclusively in the EB₁-treated samples (Supplementary Tables 5 and 6). EB₁ is known to inhibit protein synthesis by binding to the P-site of the bacterial 30S ribosomal subunit, thereby preventing the formation of the 30S pre-initiation complex and blocking the association with the large subunit to form the 70S initiation complex (Pioletti et al., 2001; Polikanov et al., 2018). Disruption of the ribosomal subunits by antibiotic treatment can halt ribosome assembly and lead to the accumulation of defective ribosomal particles, such as free 30S and 50S subunits, along with unassembled ribosomal proteins (Siibak et al., 2011). Consistent with this mechanism, our proteomic results indicate that COG J (translation) was the most strongly affected functional category in *E. amylovora* by the EB₁ treatment (Supplementary Table 2). In addition to translation-associated proteins, the universal stress protein UspE (QKZ09445) and the envelope stress response regulator CpxR (QKZ10827) were detected exclusively in the EB₁-treated samples (Supplementary Table 6). Universal stress proteins (Usps) are highly conserved across bacteria, archaea, and protozoa and are known to play roles in bacterial cell motility, with UspE-deficient mutants exhibiting impaired motility (Nachin et al., 2005). The CpxR protein is a key regulator of the Cpx stress response pathway, which contributes to antibiotic resistance in *E. coli* (Raivio et al., 2013). The exclusive detection of UspE and CpxR suggests that *E. amylovora* may activate stress response pathways in response to EB₁ treatment, including antibiotic pressure, nutrient limitation, and oxidative stress (Stokes et al., 2019).

EB₁ treatment also affected the nutrient metabolism of *E. amylovora*, including pathways related to carbohydrates, amino acids, lipids, and inorganic components. The oligopeptide ABC transporter substrate-binding protein OppA (QKZ09548), glutamin ABC transporter substrate-binding protein GlnH (QKZ10990), PTS N-acetyl glucosamine

transporter subunit IIABC (QKZ08888), MetQ/NlpA family lipoprotein (QKZ10190), metal ABC transporter substrate-binding protein (QKZ09713), and iron uptake system protein EfeO (QKZ09690) were more abundant (over twofold) in NC compared with EB₁-treated samples (Supplementary Table 5). In addition, amino acid ABC transporter substrate-binding protein (QKZ08876), lysine/arginine/ornithine ABC transporter substrate-binding protein ArgT (QKZ09913), α -amylase (QKZ09702), glycoside hydrolase family 68 protein (QKZ10931), and murein tripeptide/oligopeptide ABC transporter ATP-binding protein OppF (QKZ09544) were detected exclusively in NC (Supplementary Table 5). These proteins are associated with nutrient metabolism, representing COGs E (amino acid metabolism and transport), G (carbohydrate metabolism and transport), and P (inorganic ion transport and metabolism). Bacterial cells acquire and utilize various organic and inorganic nutrients to survive in their environment, including carbon, nitrogen sources, phosphates, vitamins, and metal ions (Kirchman, 1994; Muñoz-Elías and McKinney, 2006). Antibiotic treatment can alter bacterial nutrient metabolic states, thereby contributing to lethality, stasis, or tolerance (Stokes et al., 2019). In this context, EB₁ may suppress nutrient metabolism in *E. amylovora*, as suggested by the increased abundance (over twofold) of proteins related to nutrient metabolism, particularly those in COGs E, G, and P, in the NC compared with the EB₁-treated samples.

Bacterial cell walls and membranes can contribute to antibiotic resistance through modifications of peptidoglycans, lipopolysaccharides (LPS), porins, and efflux pumps (Balibar and Grabowicz, 2016; Garneau-Tsodikova and Labby, 2016; Viveiros et al., 2007). Among 29 proteins that were more abundant in both the CF- and EB₁-treated samples, we found that the carboxy terminal-processing peptidase (QKZ09616), 3-deoxy-8-phosphooctulonate synthase (QKZ09277), LPS assembly protein LptD (QKZ10348), and outer membrane protein assembly factor BamC (QKZ10022) were more abundant (over twofold) in both the CF- and EB₁-treated samples compared with the NC-treated samples (Supplementary Tables 4 and 6). Furthermore, among 145 proteins that were exclusively detected in both the CF- and EB₁-treatment compared with the NC-treatment, we found that the OmpA family lipoprotein (QKZ07876), outer membrane protein assembly factor BamE (QKZ10111), acyl-ACP-UDP-N-acetylglucosamine O-acyltransferase (QKZ10206), and peptidoglycan glycosyltransferase FtsI (QKZ10327) were detected exclusively in the CF- and EB₁-treated samples (Supplementary Tables 4 and 6). LptD has been reported to confer resistance to antibiotics, such as rifampin, tetracycline, ciprofloxacin,

and imipenem (Balibar and Grabowicz, 2016), while Omp-related LPS assembly proteins can contribute to polymyxin B resistance by modifying outer membrane proteins through upregulation of LPS biosynthetic genes (Moffatt et al., 2019). These results suggest that *E. amylovora* may modulate cell wall- and membrane-associated proteins to counteract the antibacterial effects of CF and EB₁, a mechanism similar to those observed in other bacterial pathogens (Balibar and Grabowicz, 2016; Moffatt et al., 2019).

E. amylovora has been investigated under various culture conditions and across different strains to compare their protein expression profiles (Holtappels et al., 2015; Lee et al., 2022). Holtappels et al. (2015) demonstrated that flagellin and chemotaxis proteins were differentially regulated among virulent *E. amylovora* strains. Similarly, Lee et al. (2022) reported that virulence-related proteins, including components of the type III secretion system and amylovoran, were differentially expressed under diverse nutrient conditions. These findings suggest that *E. amylovora* exhibits distinct protein expression patterns depending on both culture conditions and strain differences. In this study, we analyzed the proteomes of *E. amylovora* TS3128 treated with CF or EB₁. A wide range of proteins in *E. amylovora* was affected by both treatments, with notable changes in COGs J (translation), M (cell wall/ membrane, envelope biogenesis), and E (amino acid metabolism). Among them, ribosome assembly proteins within COG J (translation) were significantly influenced by the CF- and EB₁-treatment. Moreover, we also found that protein expression patterns were slightly different between the CF- and EB₁-treated samples. Considering that *B. brevis* is known to produce various bioactive metabolites, including EB₁, gramicidin, and tyrocidine (Kim et al., 2024a), it is likely that HK544 CF also contains various bioactive compounds capable of modulating protein expression in *E. amylovora*. Taken together, our proteomic results provide valuable insights into the antibacterial mode of action of HK544 CF, including EB₁, and may also inform future studies on potential resistance mechanisms against antibiotics.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (<http://www.ppjonline.org/>).

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