



Article

Functional and Proteomic Analyses of a Putative Carbamoyl Phosphate Synthase Large Subunit in Relation to Virulence, Arginine and Pyrimidine Biosynthesis, and Siderophore Production in *Erwinia amylovora*

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Abstract: The apple is a significant global fruit cultivated extensively worldwide. Fire blight, caused by *Erwinia amylovora* (Ea), poses a significant threat to global apple production. To control this disease, characterizing the virulence mechanisms/factors is imperative. Carbamoyl phosphate synthase is an essential enzyme in the biosynthesis of arginine and pyrimidine. However, the functions of this protein in Ea remains poorly understood. This study aimed to investigate the functions of the carbamoyl phosphate synthase large subunit in Ea (CarBEa). In a virulence assay using fruitlets, an Ea strain lacking CarBEa exhibited significantly reduced virulence on fruitlets. In the auxotrophy assay, this mutant failed to grow in minimal media lacking both arginine and pyrimidine, but growth was restored when both compounds were supplemented. The comparative proteomic analysis suggests that CarBEa is involved in diverse biological processes, including amino acid and nucleotide metabolism, and inorganic ion transport. Finally, we demonstrated that CarBEa is related to siderophore secretion/production by the chrome azurol S agar plate assay. This report provides valuable insights into the functions of carbamoyl phosphate synthase large subunit, which serves as a potential target for developing efficient anti-virulence substances to control fire blight.

Keywords: fire blight; CPS; carbamoyl phosphate synthase; *Erwinia amylovora*



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1. Introduction

Apple trees (*Malus domestica*) produce a significant agricultural fruit that is distributed worldwide. Global apple production reached approximately 95.8 million tons in 2022 [1]. However, various plant diseases, including fire blight, threaten apple production globally [2]. *Erwinia amylovora* (Ea) causing fire blight disease on apples is a Gram-negative, motile, rod-shaped bacterium with peritrichous flagella [3]. It is the most economically significant and destructive bacterial disease affecting apples and pears [4]. Disease symptoms manifest in various tissues and organs, including twigs, fruits, flowers, and stems of apple trees [5]. Despite being recognized as a significant problem for over 200 years, fire blight continues to cause major sporadic losses [6,7].

Fire blight remains a perennial issue for apple growers in regions where the disease is endemic [8,9]. The continued exchange of trees or budwood between countries facilitates the disease's spread worldwide [9]. In Korea, fire blight was first reported on pears in 2015 [10]. Since its first identification, the disease has consistently spread, with the number of infected apple and pear orchards continuously increasing [8]. Despite significant

efforts by scientists to develop methods to manage the disease in Korea, fire blight continues to cause serious problems and other effective methods have to be developed [11–13]. Therefore, to effectively control the disease, it is essential to elucidate the virulence factors and mechanisms in *Ea* to develop anti-virulence reagents that can interfere with the pathogen's virulence.

Carbamoyl-phosphate synthase (CPS) is an enzyme composed of small (CPSI) and large (CPSII) subunits, encoded by the *carA* and *carB* genes, respectively [14,15]. CPS catalyzes the reaction of bicarbonate, ATP, and glutamine to produce carbamoyl-phosphate, a precursor for the synthesis of arginine and pyrimidine [16]. In bacteria, including *Escherichia coli*, the synthesis of arginine and pyrimidine is primarily catalyzed by CPSs [17]. In *Streptococcus thermophilus*, which is a Gram-positive and used in the dairy industry, inactivation of the *carB* gene induces auxotrophy for both arginine and uracil and reduced growth in milk [18]. Furthermore, a *carB*-knockout mutant of *Toxoplasma gondii*, a ubiquitous protozoan parasite, is avirulent and induces long-term immunity in its host [19]. In plant-pathogenic bacteria, it was reported that CarB contributes to pathogenicity [20]. Moreover, CarB is involved in the regulation of swimming motility and biofilm formation in *Xanthomonas citri* subsp. *citri*, the causal agent of citrus canker [21]. It was also reported that the *carB* gene in the American strain HKN06P1 of *Ea* is essential for virulence and the *carB*-deficient mutant is an auxotroph [22]. However, the functions of the *carB* gene in the Korean strain of *Ea* are poorly understood and the proteomic analysis for CarB has not been carried out.

To address this gap in the literature, this study aimed to investigate the functions of the carbamoyl-phosphate synthase large subunit in *Ea* strain TS3128. Screening the *Ea* Tn5 insertional library yielded one mutant that was not virulent on immature apples. In this mutant, the gene encoding a putative carbamoyl-phosphate synthase large subunit (*carBEa*) was disrupted by Tn5. A label-free shotgun comparative proteomic analysis was conducted to predict the biological mechanisms related to CarBEa. Additionally, various phenotypic assays were performed to demonstrate the functions of CarBEa.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

Erwinia amylovora (*Ea*) strain TS3128, whose whole genome sequence has been determined [23], was used as the wild-type strain. *Ea* strains were grown in Luria-Bertani (LB) broth (Tryptone 10 g, NaCl 10 g, Yeast extract 5 g in 1 L) as the rich medium. An *hrp*-inducing medium (Hmm) ((NH₄)₂SO₄ 1 g, MgCl₂·6H₂O 0.246 g, NaCl 0.1 g, K₂HPO₄ 8.708 g, KH₂PO₄ 6.804 g in 1 L), or an amylovoran-inducing medium (MBMA) (KH₂PO₄ 3 g, K₂HPO₄ 7 g, (NH₄)₂SO₄ 1 g, glycerol 2 mL, Citric acid 0.5 g, MgSO₄ 0.03 g, Sorbitol 10 g in 1 L) were used for minimal conditions [24]. *Escherichia coli* DH5α was used for gene cloning and plasmid construction. For selection, appropriate antibiotics were added to each medium at the following final concentrations: kanamycin 50 µg/mL and gentamicin 10 µg/mL.

2.2. Selection of *carBEa*:Tn5 and Generation of the Complemented Strain

The characteristics of bacterial strains and plasmids used in this study are described in Table S1. A random mutant pool library was generated using EZ-Tn5™ <R6Kγori/KAN-2> Tnp Transposome kit (Epicentre, Madison, WI, USA) with *Ea* strain TS3128. Briefly, transposome (1 µL) was introduced into the wild-type strain by electroporation was performed using a MicroPulser Electroporator (Bio-Rad, Contra Costa, CA, USA) at 1.8 kV and 5.1 ms, generating the mutant library. An avirulent mutant (*carBEa*:Tn5) was selected by screening the library for pathogenicity using immature apple fruits. The insertion site of the transposon in *carBEa*:Tn5 was determined through a thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR). TAIL-PCR was performed using previously reported primers [25] and arbitrary degenerate primer 1 (AD1: 5'-NGT CGA SWG ANA WGA A-3') according to the previously published protocol [26]. Genomic DNA was extracted using

Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Plasmid DNA was extracted using the LaboPass™ Plasmid Mini Kit (Cosmogenetech, Seoul, Republic of Korea). To generate the complemented strain, the 209 bp upstream region containing the putative ribosomal binding site and the open reading frame of *carBEa* was amplified using a primer set (*carBEa_cp_1-F*: 5'-AGC AAT CTT CCG GCC AAT CT-3', *carBEa_cp_1-R*: 5'-GCG TAC AGG AAG AGA CCT CG-3'). The amplicon (3875 bp) was cloned into the pGem-T easy vector (Promega), generating pGem-*carBEa*, and the sequence of the insert was confirmed by Sanger sequencing. The generated plasmid was digested with *ApaI* and *SpeI*, and the cloned DNA fragment was transferred into the pBBR1-MCS5 vector containing the *LacZ* promoter for expression, creating pMCS5-*CarBEa*. The plasmid was introduced into *carBEa:Tn5* by electroporation, generating *carBEa:Tn5*(*CarBEa*). The expression of the *carBEa* gene in *carBEa:Tn5*(*CarBEa*) was verified by RT-PCR using a primer set (*carBEa_cf_primer-F*: 5'-GGC GCA ACG GTA ATT GAG TC-3', *carBEa_cf_primer-R*: 5'-CCT ACA TCG AGC CGA TCC AC-3') (Figure S1). To avoid potential side effects from pBBR1-MCS5, an empty vector was also introduced into the wild-type and the mutant strains, creating *Ea*(EV) and *carBEa:Tn5*(EV), respectively.

2.3. Pathogenicity Test

The pathogenicity of *Ea* strains was evaluated using immature apple fruits (*Malus domestica* var. Hongro). Apples measuring less than 20 mm × 30 mm were collected, and a hole with a diameter of 4 mm was created using a cork borer. Bacterial cells were suspended in sterile distilled water (SDW) and adjusted to an optical density at 600 nm (OD_{600nm}) of 0.2, corresponding to approximately 1×10^8 CFU/mL. Following this, 20 µL of the bacterial suspension was inoculated into the hole in the immature apple fruits. The inoculated immature apple fruits were then placed on the dish (over 80% humidity) and stored at 28 °C in an incubator for 10 d. Fifteen biological replicates of each strain were used and at least three independent experiments were carried out.

2.4. Growth and Auxotroph Assay

Ea strains were grown in LB medium with appropriate antibiotics. After washing with sterilized distilled water (SDW), each strain was resuspended and adjusted to an OD_{600nm} of 0.3. The bacterial suspension was diluted (10^{-3}) in LB medium and incubated at 28 °C in a shaking incubator, with measurements taken every 12 h for 96 h. Each strain was tested in three biological replicates. For the arginine and pyrimidine auxotroph assays, *Ea* strains were grown under eight different conditions with appropriate antibiotics: MBMA, MBMA with 10 mM uracil or 50 mM arginine, MBMA with 10 mM uracil and 50 mM arginine, HMM, HMM with 10 mM uracil or 50 mM arginine, and HMM with 10 mM uracil and 50 mM arginine. Bacterial strains were resuspended in SDW and adjusted to an OD_{600nm} of 0.5. Then, 2 mL of each suspension was diluted into 18 mL of the media. Growth was measured every 24 h for 10 d at 28 °C in a shaking incubator (220 rpm). Each strain was tested in three biological replicates, and at least three independent experiments were conducted.

2.5. Proteomic Analysis

For the comparative proteomic analysis, a previously established protocol was employed with minor modifications [27]. Total soluble proteins were extracted from *Ea* and *carBEa:Tn5* strains incubated in LB medium. Three biological replicates of each strain were utilized, resulting in a total of six samples. *Ea* strains incubated in LB medium were harvested at an OD_{600nm} of 0.3, washed twice with 50 mM Tris-HCl (pH 7.8), and resuspended in lysis buffer (6 M guanidine HCl, 10 mM dithiothreitol, 50 mM Tris-HCl pH 7.8). Following sonication of the bacterial suspension, the concentration of total soluble proteins was measured using a BCA kit (Thermo Fisher Scientific, Rockford, IL, USA). Peptides were generated by trypsin digestion, and the tryptic-digested proteins were purified using a Sep-Pak Vac 1cc tC18 cartridge (Waters, Milford, MA, USA). Liquid chromatography

followed by tandem mass spectrometry analysis (LC–MS/MS) was performed according to a recently reported procedure [28]. A 1 µg sample of the tryptic-digested peptides was analyzed using split-free nano-LC (EASY-nLC II; Thermo Fisher Scientific, Bremen, Germany) coupled with an LTQ Velos Pro instrument (Thermo Fisher Scientific). The data obtained from the LC–MS/MS analysis were deposited in the ProteomeXchange Consortium via the PRIDE [29] partner repository with the dataset identifier PXD053326.

To identify and quantify proteins and peptides from the LC–MS/MS analysis, a previously reported method was employed with slight modifications [30]. The raw data obtained through LC–MS/MS analysis were analyzed using Thermo proteome discoverer (ver. 1.3.0.399) with the SEQUEST protocol algorithm. Proteins and peptides were identified using genome information of the *Ea* strain TS3128 (Accession No. CP056034). To enhance reliability, the target-decoy strategy was employed [31]. The identified proteins were imported into Scaffold4 (Proteome Software, Portland, OR, USA) for reanalysis. Peptide spectrum matches (PSMs) were utilized for the comparative analysis [32]. PSMs from individual proteins were normalized to the total PSM values in each sample. The mean value of PSMs from three biological replicates per strain was used to identify differentially abundant proteins (greater than 2-fold) between *Ea* and *carBEa:Tn5*. Statistical analysis was performed using the Student's *t*-test ($p < 0.05$). The identified differentially abundant proteins were categorized using a cluster of orthologous groups (COG) analysis [33].

2.6. Siderophore Production Assay

Siderophore production was investigated using Chrome azurol S (CAS) agar following a previously established protocol with slight modifications [34]. A total of 25 mL of CAS blue dye [35] and 225 mL of LB medium containing 8.04 g of PIPES were mixed to create LB-CAS plates (1.5% agar). Bacterial suspension was adjusted to an OD_{600nm} of 1.0. Five µL of the suspension was placed at the center of the plates and incubated at 28 °C for 2 d without shaking. The diameters of the colony and halo zones were measured. Three biological replicates of each strain were used, and at least three independent experiments were performed.

2.7. Comparison of Amino Acid Sequences and In Silico 3D Modeling

The deduced amino acid sequences of CarBEa and its homologs from other bacterial species were obtained from NCBI. The sequences were compared using the Clustal Omega program (EMBL-EBI, Hinxton, UK). For 3D structure modeling, PDB files were generated via the I-TASSER server [36] and visualized using the PyMOL program (Molecular Graphics System, San Carlos, CA, USA).

2.8. Statistical Analysis

The statistical significance of the data was analyzed using one-way analysis of variance (ANOVA) with Tukey's HSD_{ab} test using SPSS 12.0K (Chicago, IL, USA). A *p*-value (<0.05) showed a statistically significant difference.

3. Results and Discussion

3.1. Identification of the *carBEa:Tn5* Strain and Amino Acid Sequence Analysis of CarBEa

A Tn5-insertional mutant library of the *Ea* strain TS3128, consisting of 5242 random mutants, was screened using immature apples, yielding one mutant (*carBEa:T5*) that failed to cause disease on immature apples. Additionally, it was noted that a gene annotated as carbamoyl-phosphate synthase large subunit (CarBEa, Accession No. QKZ10356; Locus tag, HU055_13835) was disrupted by the transposon in the mutant. The deduced amino acid sequence of CarBEa shows high homology with carbamoyl-phosphate synthase large subunits from other Gram-negative bacteria (Figure 1). Specifically, the protein exhibits 95%, 94%, and 81% amino acid sequence similarity with the carbamoyl phosphate synthase large subunits UYM68838 in *Escherichia coli*, ECK9372303 in *Salmonella enterica*, and MBD4171480 in *Xanthomonas citri* pv. *citri*, respectively (Figure 1A). The high level of

amino acid sequence identity and similarity found between CarBEa and UYM68838 in *E. coli* (Figure 1A) is reflected in their nearly identical in silico-rendered three-dimensional structures (Figure 1B,C). This suggests that carbamoyl-phosphate synthase proteins are conserved and are likely to perform similar roles in Gram-negative bacteria.

A

MD04171480.1	MPRTOLETLILGAGVIVGGAGFQSYGAGQALREGEVYLVNSPATINTPM	60	MD04171480.1	TAYVSYTEDECEALPT-DQDKIMILGGPNRIGGGIFDYCVHMAALDQOGETM	599
QK210356.1	MPRHTKISLILGAGVIVGGAGFQSYGAGQALREGEVYLVNSPATINTPM	60	QK210356.1	TAMVSYTEEECAHQHGDIMILWLGPNRIGGGIFDYCVHMAALDQOGETM	599
UHM68838.1	MPRHTKISLILGAGVIVGGAGFQSYGAGQALREGEVYLVNSPATINTPM	60	UHM68838.1	TAMVSYTEEECAHQHGDIMILWLGPNRIGGGIFDYCVHMAALDQOGETM	599
ECK9372303.1	MPRHTKISLILGAGVIVGGAGFQSYGAGQALREGEVYLVNSPATINTPM	60	ECK9372303.1	*****	599
MD04171480.1	ADAVYIEPIHNIQVYKIVIEIPVAPALLTNGGATNGCALADKHLEIVNVELGAVR	120	MD04171480.1	NHNPETVSTDYTSQVLYPELTLEDLEIVLEVRPIQVIVGGGPTPLLAALAAAG	659
QK210356.1	ADATYIEPIHNEIVKIVIEIEIPVAPALLTNGGATNGCALADKHLEIVNVELGATA	120	QK210356.1	NHNPETVSTDYTSQVLYPELTLEDLEIVLEVRPIQVIVGGGPTPLLAALAAAG	659
UHM68838.1	ADATYIEPIHNEIVKIVIEIEIPVAPALLTNGGATNGCALADKHLEIVNVELGATA	120	UHM68838.1	NHNPETVSTDYTSQVLYPELTLEDLEIVLEVRPIQVIVGGGPTPLLAALAAAG	659
ECK9372303.1	ADATYIEPIHNEIVKIVIEIEIPVAPALLTNGGATNGCALADKHLEIVNVELGATA	120	ECK9372303.1	NHNPETVSTDYTSQVLYPELTLEDLEIVLEVRPIQVIVGGGPTPLLAALAAAG	659
MD04171480.1	EAFIMDEHLEFRVMAWELQDOTASIAHTHEELALELQITVWPIVTFPSGTGGSGG	180	MD04171480.1	PVIGTSPDIADEHVEFQGVLDVILGPNRIANNEEVLVAHEIGVLPVPSVY	719
QK210356.1	DAIDAEHQVFFANMILQDOTASIAHTHEELAAVAGFCPIVTFPSGTGGTGG	180	QK210356.1	PVIGTSPDIADEHVEFQGVLDVILGPNRIANNEEVLVAHEIGVLPVPSVY	719
UHM68838.1	DAIDAEHQVFFANMILQDOTASIAHTHEELAAVAGFCPIVTFPSGTGGTGG	180	UHM68838.1	PVIGTSPDIADEHVEFQGVLDVILGPNRIANNEEVLVAHEIGVLPVPSVY	719
ECK9372303.1	DAIDAEHQVFFANMILQDOTASIAHTHEELAAVAGFCPIVTFPSGTGGTGG	180	ECK9372303.1	PVIGTSPDIADEHVEFQGVLDVILGPNRIANNEEVLVAHEIGVLPVPSVY	719
MD04171480.1	IAYNVEELIVGQGLSPTTELVESGLQHEFEMVYVDQADKIVYALBQDM	240	MD04171480.1	LGGAHVEIVGQGLSARYVDAWISQSPVLLDFPLDAVEVDV1ADQVNLGGV	779
QK210356.1	IAYNVEEFEEICQGLSPTNELLDESILQHEFEMVYVDQADKIVYSENPDM	240	QK210356.1	LGGAHVEIVGQGLSARYVDAWISQSPVLLDFPLDAVEVDV1ADQVNLGGV	779
UHM68838.1	IAYNVEEFEEICQGLSPTNELLDESILQHEFEMVYVDQADKIVYSENPDM	240	UHM68838.1	LGGAHVEIVGQGLSARYVDAWISQSPVLLDFPLDAVEVDV1ADQVNLGGV	779
ECK9372303.1	IAYNVEEFEEICQGLSPTNELLDESILQHEFEMVYVDQADKIVYSENPDM	240	ECK9372303.1	LGGAHVEIVGQGLSARYVDAWISQSPVLLDFPLDAVEVDV1ADQVNLGGV	779
MD04171480.1	GHTGDSIVAPAGTLTOEYVIMNSAMVLEIVGTTGSGVGFANPGLVLI	300	MD04171480.1	MEHIEAGHSGSSSSPPYLSPTQDAEIVQVLMIAEGLVLMQVAFVINEAG	839
QK210356.1	GHTGDSIVAPAGTLTOEYVIMNSAMVLEIVGTTGSGVGFANPGLVLI	300	QK210356.1	MEHIEAGHSGSSSSPATLTSAEIDQVMDQVLAFLQVLMQVAFVINEAG	834
UHM68838.1	GHTGDSIVAPAGTLTOEYVIMNSAMVLEIVGTTGSGVGFANPGLVLI	300	UHM68838.1	MEHIEAGHSGSSSSPATLTSAEIDQVMDQVLAFLQVLMQVAFVINEAG	834
ECK9372303.1	GHTGDSIVAPAGTLTOEYVIMNSAMVLEIVGTTGSGVGFANPGLVLI	300	ECK9372303.1	MEHIEAGHSGSSSSPATLTSOEIDQVMDQVLAFLQVLMQVAFVINEAG	834
MD04171480.1	NPVYSSSALASATGPIAAVAVLAVGTLDLNEITGATPSPEFSIYVYVITP	360	MD04171480.1	DOIVYLEVNPVIAATPVYSVIAIGRAIIVAHQMGVLAEGQATIEIIPVYSVIEA	899
QK210356.1	NPVYSSSALASATGPIAAVAVLAVGTLDLNEITGATPSPEFSIYVYVITP	360	QK210356.1	NEVLYLEVNPVIAATPVYSVIAIGRAIIVAHQMGVLAEGQATIEIIPVYSVIEA	893
UHM68838.1	NPVYSSSALASATGPIAAVAVLAVGTLDLNEITGATPSPEFSIYVYVITP	360	UHM68838.1	NEVLYLEVNPVIAATPVYSVIAIGRAIIVAHQMGVLAEGQATIEIIPVYSVIEA	893
ECK9372303.1	NPVYSSSALASATGPIAAVAVLAVGTLDLNEITGATPSPEFSIYVYVITP	360	ECK9372303.1	NEVLYLEVNPVIAATPVYSVIAIGRAIIVAHQMGVLAEGQATIEIIPVYSVIEA	893
MD04171480.1	FFAFIEFRQAVILTQISVGEAMAGITFSLSQALQLEGTILQDPTGLDSSSE	420	MD04171480.1	IFPFAIFQGVDFPLQPESTMEWGIVGTFSAFAAGAGQAGIAPPAQVAFVYNQD	959
QK210356.1	FFAFIEFRQAVILTQISVGEAMAGITFSLSQALQLEGTILQDPTGLDSSSE	420	QK210356.1	VLPNPFQGVDFPLQPESTMEWGIVGTFSAFAAGAGQAGIAPPAQVAFVYNQD	953
UHM68838.1	FFAFIEFRQAVILTQISVGEAMAGITFSLSQALQLEGTILQDPTGLDSSSE	420	UHM68838.1	VLPNPFQGVDFPLQPESTMEWGIVGTFSAFAAGAGQAGIAPPAQVAFVYNQD	953
ECK9372303.1	FFAFIEFRQAVILTQISVGEAMAGITFSLSQALQLEGTILQDPTGLDSSSE	420	ECK9372303.1	VLPNPFQGVDFPLQPESTMEWGIVGTFSAFAAGAGQAGIAPPAQVAFVYNQD	953
MD04171480.1	DIATLHLEAGPVEFLYADAFAGATVAYVLSIFQPNFLDQIEVIEHSGEADQ	480	MD04171480.1	IKVIVLELAAGVETGVELATDQAGVHQLQQLSDEIVNVEVHGPVDSIHNGEYTV	1019
QK210356.1	ADATLHLEAGSDFHRYADAFAGATVAYVLSIFQPNFLDQIEVIEHSGEADQ	479	QK210356.1	IKVIVLELAAGVETGVELATDQAGVHQLQQLSDEIVNVEVHGPVDSIHNGEYTV	1013
ECK9372303.1	ALTHVLELDAGQVHYADAFAGATVAYVLSIFQPNFLDQIEVIEHSGEADQ	479	ECK9372303.1	IKVIVLELAAGVETGVELATDQAGVHQLQQLSDEIVNVEVHGPVDSIHNGEYTV	1013
MD04171480.1	GMALDAPRLMLKAFSGDAQMQLGSIEHVRPLRLVIRVYVYVSDCAEFATS	540	MD04171480.1	INTTAGQAEEDSLIRRSALQVYHDTTGLQAFATMALHDAEIVSYQVEIHQIT	1079
QK210356.1	GVSLATQVLTLPVRFAGDALSVLAGHVEIRHQEDHPLVYVYVYVGTCAEFST	539	QK210356.1	INTTAGQAEEDSLIRRSALQVYHDTTGLQAFATMALHDAEIVSYQVEIHQIT	1073
UHM68838.1	GIMOLDAPRLMLKAFSGDAQMQLGSIEHVRPLRLVIRVYVYVYVGTCAEFST	539	UHM68838.1	INTTAGQAEEDSLIRRSALQVYHDTTGLQAFATMALHDAEIVSYQVEIHQIT	1073
ECK9372303.1	GIMOLDAPRLMLKAFSGDAQMQLGSIEHVRPLRLVIRVYVYVYVGTCAEFST	539	ECK9372303.1	INTTAGQAEEDSLIRRSALQVYHDTTGLQAFATMALHDAEIVSYQVEIHQIT	1073
MD04171480.1	A-1080		MD04171480.1	A-1080	
	Q-1074			Q-1074	
	UHM68838.1			UHM68838.1	
	ECK9372303.1			ECK9372303.1	
	KS1078			KS1078	

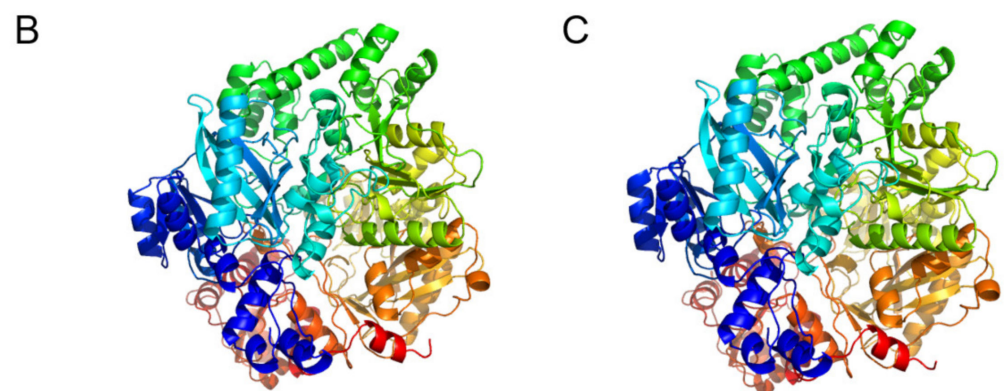


Figure 1. Amino acid sequence comparison and 3D structure of CarBEa. **(A)** Amino acid sequence alignment of QKZ10356 (CarBEa), UYM68838 (carbamoyl-phosphate synthase large subunit in *Escherichia coli*), ECK9372303 (carbamoyl-phosphate synthase large subunit in *Salmonella enterica*), and MBD4171480 (carbamoyl-phosphate synthase large subunit in *Xanthomonas citri* pv. *citri*) performed using Clustal Omega program. The ‘*’, ‘:’, and ‘.’ represent identical, conserved, and semi-conserved residues, respectively. Putative 3D structure of **(B)** CarBEa and **(C)** UYM68838 generated by I-TASSER and visualized using the PyMOL program.

3.2. CarBEa Is Required for Virulence in *Ea*

A virulence test was performed using immature apple fruits to investigate the role of CarBEa in the virulence of *Ea*. As shown in Figure 2A, the immature apple fruits inoculated with *Ea*(EV) displayed distinct typical fire blight symptoms, including bacterial oozes, whereas *carBEa:Tn5*(EV) did not cause disease on the immature apple fruits. The virulence of *carBEa:Tn5*(CarBEa) was restored, showing symptoms similar to *Ea*(EV), indicating the absence of positional or polar effects from the transposon insertion in the mutant. The disease index of the *Ea*(EV) strain increased significantly from 7 days after inoculation (DAI) and reached a value of 3 at 10 DAI (Figure 2B). In contrast, the disease index for *carBEa:Tn5*(EV) remained at zero. Furthermore, the disease index patterns of the complemented strains were similar to those of *Ea*(EV). These results demonstrate that CarBEa is indispensable for the virulence of *Ea*. Consistent with our observations, previous studies have reported a loss of pathogenicity in *carB* mutants in *E. coli*, *S. enterica*, *X. citri* pv. *citri*, and the American strain of *Ea* [20,22,37,38]. This indicates that the functions of CarBEa regarding virulence are conserved across both plant and animal pathogenic bacteria including *Ea*.

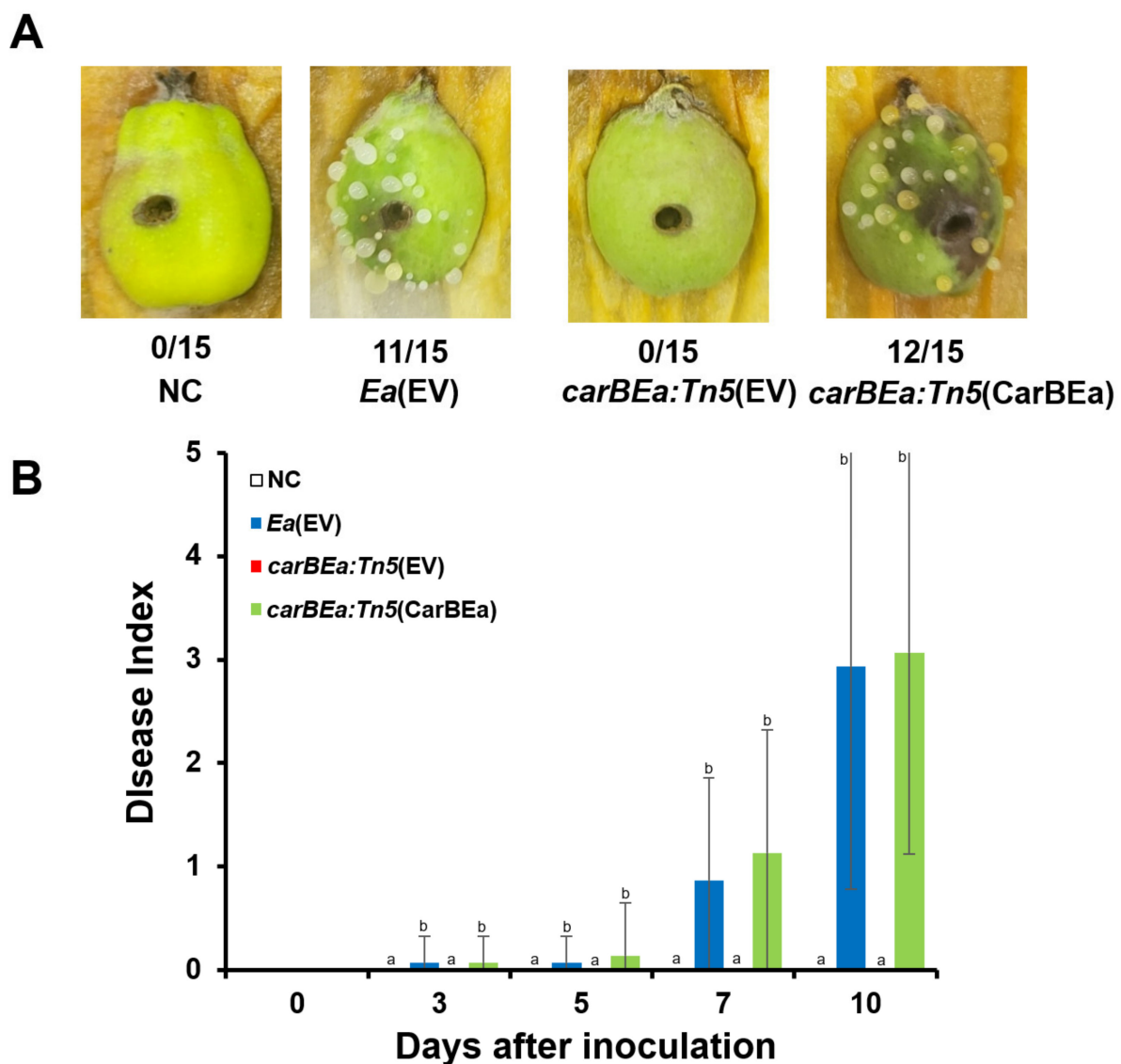


Figure 2. Fruitlet virulence assay for *Ea*(EV), *carBEa:Tn5*(EV), and *carBEa:Tn5*(CarBEa). (A) Symptoms caused by *Ea*(EV), *carBEa:Tn5*(EV), and *carBEa:Tn5*(CarBEa) on immature apples. Sterile distilled

water was used as a negative control (NC). The numbers below panel (A) indicate the number of diseased apples out of 15 apples. (B) Disease development on immature apple fruits over time. Disease severity was scored using a disease index scale from 0 to 5: 0 = no symptoms, 1 = water soaking and browning covering 1–10% of the fruitlets, 2 = water soaking and browning covering 11–25% and 11–35% of the fruitlets, respectively, 3 = water soaking and browning covering 26–50% and 36–55% of the fruitlets, respectively, 4 = water soaking and browning covering 51–75% and 56–80% of the fruitlets, respectively, 5 = water soaking and browning covering 76–100% and 81–100% of the fruitlets, respectively. Different letters on the bars indicate that the mean values differ significantly according to a one-way ANOVA test with Tukey's post hoc test at $p < 0.05$. Error bars represent the standard deviation ($n = 15$).

3.3. CarBEa Is Crucial for the Biosynthesis of Arginine and Uracil

Growth curves of *Ea*(EV), *carBEa:Tn5*(EV), and *carBEa:Tn5*(CarBEa) strains on rich media (LB) and two minimal media (MBMA and HMM) were performed to determine any effect of CarB alleles on bacterial growth. From 24 to 60 h after inoculation, *carBEa:Tn5*(EV) exhibited a slightly higher OD value; however, this difference was not statistically significant in the LB medium (Figure 3A). This indicates that CarBEa is not involved in the reproduction of *Ea*. Klee et al. reported that the *Ea* strain lacking the functional CarB did not grow well compared to the wild-type strain in the apple fruitlet medium which is relatively nutrient-limited [22]. In addition, CarBEa encodes a putative carbamoyl phosphate synthase large subunit, which is known to be responsible for the biosynthesis of arginine and pyrimidine [21]. Thus, it can be postulated that *carBEa:Tn5*(EV) is an auxotroph for both arginine and pyrimidine, resulting in the reduction of virulence in the fruitlet assay. To confirm this, the growth of *Ea* strains was evaluated in HMM and MBMA in the presence or absence of arginine and uracil (Figure 3). In MBMA, *carBEa:Tn5*(EV) did not grow, whereas the growth of the complemented strains was restored to that of *Ea*(EV) (Figure 3B), indicating that *carBEa:Tn5*(EV) is an auxotroph. To determine whether the mutant is an auxotroph for arginine, uracil, or both, the growth of the mutant was examined in MBMA supplemented with 50 mM arginine and/or 10 mM uracil. The addition of arginine or uracil alone in MBMA could not restore the growth of *carBEa:Tn5*(EV) (Figure 3C,D). Notably, the mutant recovered growth and even showed higher OD values compared to *Ea*(EV) and *carBEa:Tn5*(EV) in the presence of both arginine and uracil (Figure 3E). The growth patterns of the three strains in HMM were similar to those observed in MBMA (Figure 3F–I). *carBEa:Tn5*(EV) successfully restored growth ability in HMM when supplemented with both arginine and uracil (Figure 3I). These results demonstrate that *carBEa:Tn5*(EV) is an auxotroph for both arginine and uracil. Consistent with a previous study [21], CarBEa is also required for the biosynthesis of arginine and uracil. In other words, CarBEa is involved in both amino acid and pyrimidine metabolism, which may contribute to virulence in *Ea*. The functions of CPSII, which is primarily involved in pyrimidine and arginine biosynthesis, are conserved across all domains of life, including prokaryotic and eukaryotic organisms, but their predicted structures in plants and bacteria are different [17,39,40]. This structural difference can be exploited for the development and discovery of new agents that inhibit bacterial growth to control plant diseases, including fire blight. For example, chorismate mutase, which is required for phenylalanine and tyrosine biosynthesis in *Mycobacterium tuberculosis*, the causative agent of tuberculosis in humans, was also considered a potential target for developing anti-tubercular agents [41].

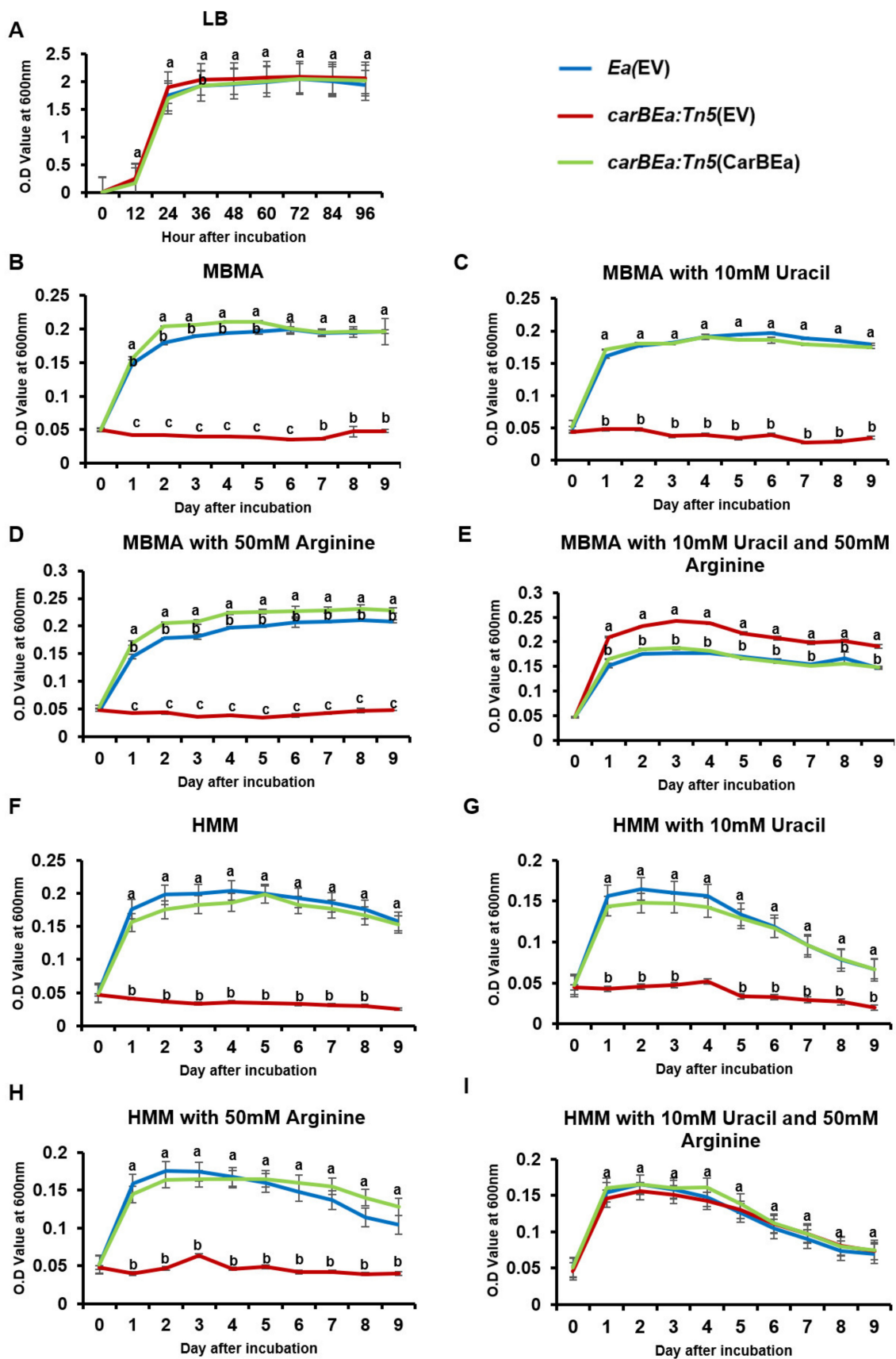


Figure 3. Growth assay in LB, MBMA, and HMM media. Growth patterns of *Ea*(EV), *carBEa:Tn5*(EV), and *carBEa:Tn5*(*carBEa*) in (A) LB, (B) MBMA (an amylovoran-inducing medium), MBMA with

(C) 10 mM uracil, (D) 50 mM arginine, (E) 10 mM uracil and 50 mM arginine, (F) HMM (an *hrp*-inducing medium), HMM with (G) 10 mM uracil, (H) 50 mM arginine, and (I) 10 mM uracil and 50 mM arginine. The OD values at 600_{nm} were measured for 4 and 9 d in LB and MBMA, respectively. Error bars represent the standard deviation. Different letters on error bars in the graph indicate statistically significant differences determined by ANOVA ($p < 0.05$) with Tukey's HSD_{ab} test.

3.4. Comparative Proteomic Analysis

We demonstrated that CarBEa is required for the biosynthesis of pyrimidine and arginine (Figure 3). However, it is possible that this protein is involved in other biological and cellular mechanisms in *Ea*. To predict biological mechanisms related to CarBEa, label-free shot-gun comparative proteomic analyses were conducted using *Ea* and *carBEa:Tn5* strains. Among the three different media, the growth of the mutant was comparable to that of the wild-type strain in LB but not in HMM and MBMA (Figure 3). This indicates that the mutant can utilize pyrimidine and arginine for survival without functional CarBEa. By identifying and categorizing proteins with altered abundance in a comparative proteomic analysis of *Ea* and *carBEa:Tn5* strains incubated in LB, we can postulate other mechanisms associated with CarBEa in addition to its role in the biosynthesis of pyrimidine and arginine. Therefore, the strains used for the comparative proteomics were cultured in an LB medium. Through LC–MS/MS analysis, 661 and 631 proteins were commonly identified in the three biological replicates of *Ea* or *carBEa:Tn5*, respectively (Table S2). These proteins were subjected to comparative analysis. Thirty-one proteins were uniquely found in *Ea* and twenty-four proteins were uniquely found in *carBEa:Tn5*. Additionally, 17 proteins were more abundant in *Ea*, whereas seven proteins were more abundant in *carBEa:Tn5* (Figure 4A). CarBEa was specifically identified in the wild-type but not in the mutant (Tables S3 and S4), confirming that the mutant lacks functional CarBEa and validating the effectiveness of the comparative proteomic analysis.

The differentially abundant proteins identified from the comparative proteomic analyses were categorized using clusters of orthologous groups (Figure 4B; Tables S3 and S4). The numbers of differentially abundant proteins classified in categories E (amino acid metabolism and transport), L (replication and repair), M (cell wall/membrane/envelope biogenesis), and O (post-translational modification, protein turnover, chaperone functions) were relatively high. Furthermore, proteins belonging to category P (inorganic ion transport and metabolism) were detected. Notably, proteins QKZ08832 and QKZ10965, related to bacterial virulence, were uniquely identified in *Ea*. Specifically, cystathionine gamma-synthase (QKZ10798) was more abundant in the wild-type strain. This enzyme catalyzes the pyridoxal phosphate-dependent synthesis of cystathionine from O-succinyl-L-homoserine and L-cysteine [42]. A recent study reported that a YggS family pyridoxal phosphate-dependent enzyme (QKZ10965) is required for virulence and other biological mechanisms in *Acidovorax citrulli*, the causal agent of bacterial fruit blotch in watermelon [27,43]. This suggests that CarBEa may be involved in a pyridoxal phosphate-dependent mechanism, which could contribute to virulence in *Ea*. Agmatinase (QKZ08436), which hydrolyzes agmatine into urea and putrescine [44], is more abundant in *Ea*. Agmatine is known as the decarboxylation product of arginine [45]. The differential abundance of agmatinase is likely attributable to the lack of CarBEa, which is required for arginine production, in the mutant.

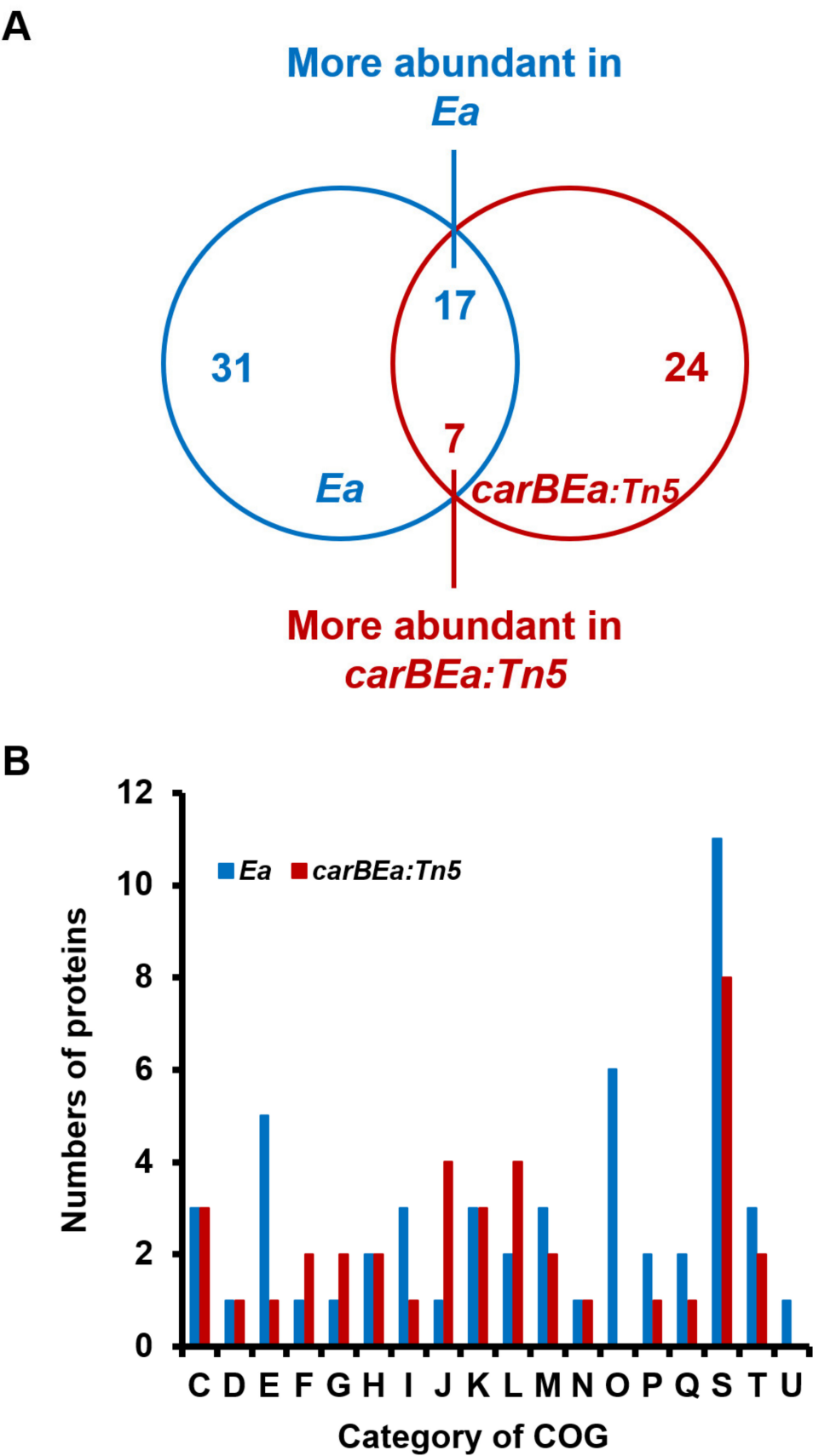


Figure 4. Label-free shotgun comparative proteomic analysis for *Ea* and *carBEa:Tn5* strains cultured in LB medium. (A) The Venn diagram displays the numbers of differentially (>2-fold) abundant

proteins identified in the proteomics analysis. (B) Classification of the differentially abundant proteins was performed using clusters of orthologous groups. The classifications are as follows: C, Energy production and conversion; D, Cell cycle control and mitosis; E, Amino acid metabolism and transport; F, Nucleotide metabolism and transport; G, Carbohydrate metabolism and transport; H, Coenzyme metabolism; I, Lipid metabolism; J, Translation; K, Transcription; L, Replication and repair; M, Cell wall/membrane/envelope biogenesis; N, Cell motility; O, Post-translational modification, protein turnover, chaperone functions; P, Inorganic ion transport and metabolism; Q, Secondary structure; R, General functional prediction only; S, Function unknown; T, Signal transduction; U, Intracellular trafficking and secretion.

3.5. *CarBEa* Is Involved in Siderophore Production

Various proteins related to inorganic ion transport and metabolism were identified through a comparative proteomic analysis. Ferric ions are known to play crucial roles in the bacterial life cycle, and several bacterial species utilize siderophores to import ferric ions from their environment [46]. To date, no written reports exist regarding carbamoyl-phosphate synthase large subunit and siderophore production. Therefore, siderophore production in *Ea* strains was investigated using the chromeazuroil S (CAS) assay. For the assay, LB-based CAS plates (LB-CAS) were employed because the growth of the mutant was not statistically different from that of the wild-type strain (Figure 5A). On LB-CAS plates, all three strains exhibited similar colony sizes, indicating no difference in bacterial growth under the given conditions (Figure 5A,B). Interestingly, the halo size in *carBEa:Tn5*(EV) was significantly reduced compared to *Ea*(EV), indicating that the amount of secreted siderophores in the mutant was less than that in the wild-type. The complemented strain restored the ability of siderophore secretion toward a level comparable to that of the wild-type strain. These results demonstrate that *CarBEa* is involved in the secretion/production of siderophore. Chen et al. reported that the pathways of arginine biosynthesis and siderophore production are likely co-regulated in *Bacillus subtilis* [47]. In *Aspergillus fumigatus*, arginine biosynthesis is also known to be related to siderophore production [48]. Therefore, it is postulated that arginine auxotrophy in *carBEa:Tn5*(EV) may be responsible for the reduced production of siderophores. However, the specific mechanisms by which *CarBEa* is associated with siderophore secretion/production remain unclear. Further studies are needed to determine the molecular and biochemical mechanisms of *CarBEa*-siderophore association.

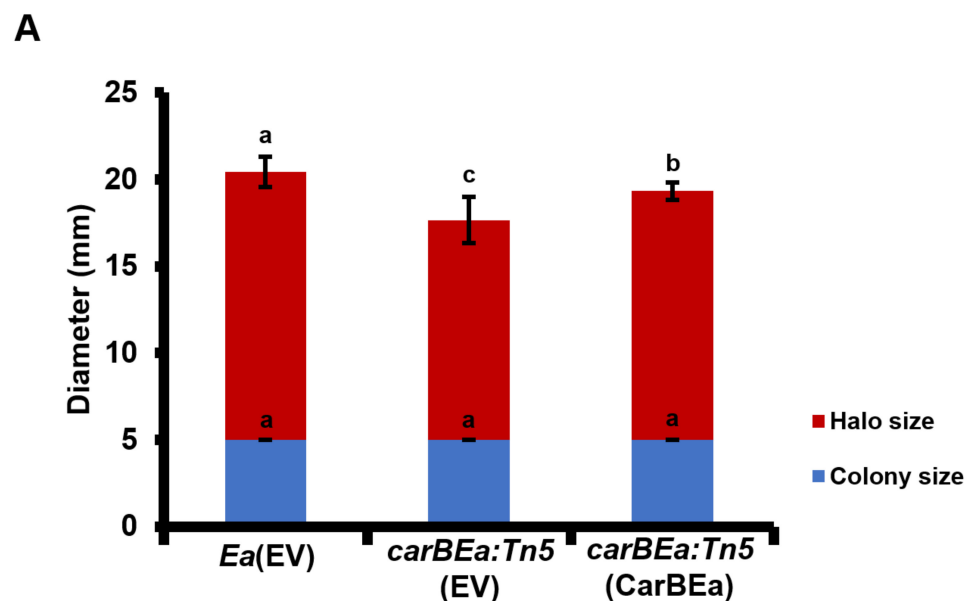


Figure 5. Cont.

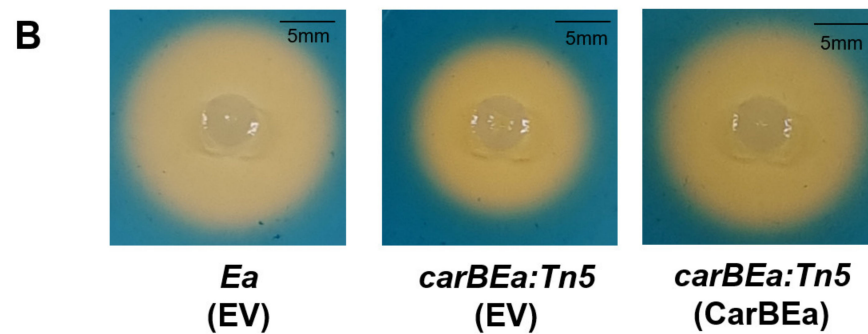


Figure 5. Siderophore assay of *Ea*(EV), *carBEa:Tn5*(EV), and *carBEa:Tn5* (*CarBEa*). Five μ L of bacterial cells suspended in LB at an OD_{600} of 1.0 was dotted on LB-CAS plates with appropriate antibiotics. (A) Measurement of colony and halo diameters. (B) Photographs of colonies and halos taken 2 d after incubation. The blue bars indicate each strain's colony diameter and the red bars indicate each strain's siderophore production halo diameter. Different letters on the error bars in the graph represent statistically significant differences determined by ANOVA ($p < 0.05$) with Tukey's HSD_{ab} test.

4. Conclusions

This study demonstrates that CarBEa, a putative carbamoyl-phosphate synthase large subunit, is essential for virulence in *Ea*, as it is crucial for the biosynthesis of arginine and uracil. Additionally, the comparative proteomic analysis results suggest that CarBEa may be involved in a range of biological and cellular mechanisms within *Ea* (Figure 6). Furthermore, this study shows an association between CarBEa and siderophore production/secretion, which is critical for iron acquisition. Enzymes involved in the synthesis of primary metabolites are increasingly recognized as promising targets for developing inhibitors to control bacterial diseases [41,49]. Thus, this study provides new insights into a previously uncharacterized virulence-related protein in *Ea*. To discover new reagents that control plant diseases by suppressing the virulence of bacterial pathogens, the characterized protein can be a potential target for screening the small molecule or chemical libraries in future research.

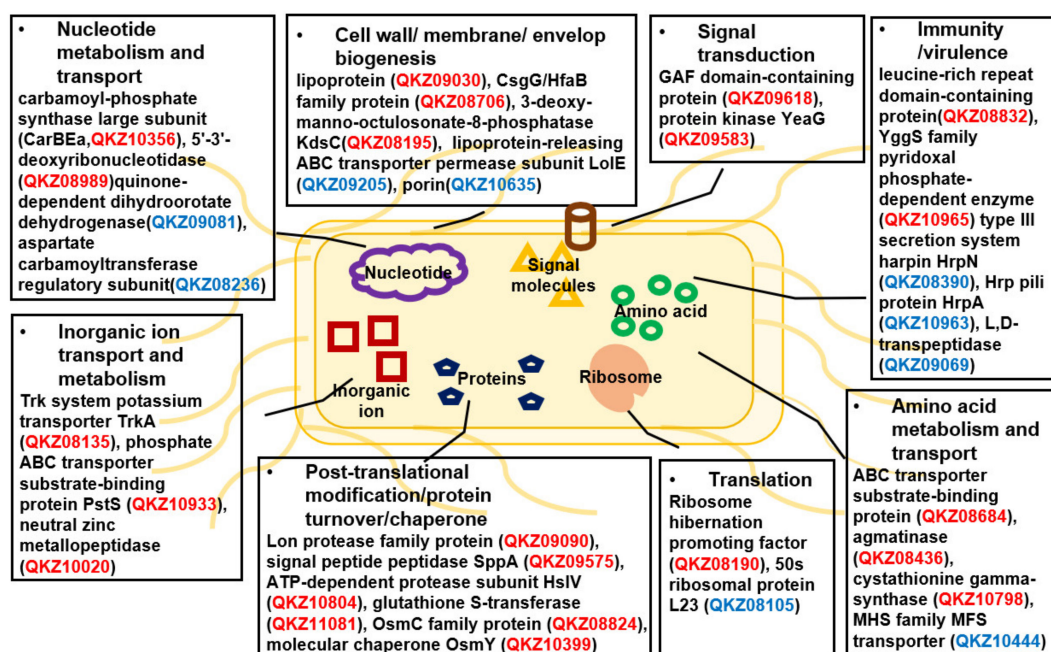


Figure 6. Diagram of putative mechanisms associated with CarBEa. Red and Blue accession numbers indicate proteins that are more abundant in *Ea* and *carBEa:Tn5*, respectively. The diagram illustrates selected proteins from the differentially abundant proteins.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10101080/s1>, Figure S1: Confirmation of expression of *carBEa* gene using RT-PCR. The predicted size of PCR product generated using *carBEa_cf_primer-F/R* set was 565 bp. cDNA generated with RNA extracted from 1, *Ea*(EV), 2, *carBEa:Tn5*, and 3, *carBEa:Tn5*(CarBEa) was used as a template; Table S1: Bacterial strains and plasmids used in this study; Table S2: Proteins and peptide spectral matches (PSM) between *Ea* and *carBEa:Tn5*; Table S3: Classification of more abundant (>2-fold change) proteins in *Ea* compared with *carBEa:Tn5* using clusters of orthologous groups; Table S4: Classification of more abundant (>2-fold change) proteins in *carBEa:Tn5* compared with *Ea* using clusters of orthologous groups. Reference [50] is cited in the supplementary materials.

Author Contributions: Conceptualization, S.-W.H.; Data curation, Y.H., S.Y.L. and S.-W.H.; Formal analysis, Y.H., S.Y.L., E.R. and S.-W.H.; Funding acquisition, Y.H. and S.-W.H.; Methodology, Y.H., S.Y.L., D.K., S.L., J.C., Y.C. and J.L.; Supervision, E.R. and S.-W.H.; Validation, Y.H. and S.Y.L.; Visualization, Y.H., S.Y.L. and S.-W.H.; Writing—original draft, Y.H., S.Y.L. and S.-W.H.; Writing—review and editing, S.-W.H. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets generated from LC-MS/MS analysis in this study can be found in the ProteomeXchange Consortium (Identifier No. PXD053326).

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Conflicts of Interest: The authors declare no conflicts of interest.

References

- Food and Agriculture Data. 2022. Available online: <https://www.fao.org/faostat> (accessed on 1 June 2024).
- Khan, A.; Svara, A.; Wang, N. Comparing apples and oranges: Advances in disease resistance breeding of woody perennial fruit crops. *Annu. Rev. Phytopathol.* **2024**, *62*, 263–287. [\[CrossRef\]](#) [\[PubMed\]](#)
- Mansfield, J.; Genin, S.; Magori, S.; Citovsky, V.; Sriariyanum, M.; Ronald, P.; Dow, M.; Verdier, V.; Beer, S.V.; Machado, M.A.; et al. Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol. Plant Pathol.* **2012**, *13*, 614–629. [\[CrossRef\]](#)
- Roberts, R.G.; Hale, C.N.; van der Zwet, T.; Miller, C.E.; Redlin, S.C. The potential for spread of *Erwinia amylovora* and fire blight via commercial apple fruit; a critical review and risk assessment. *Crop Prot.* **1998**, *17*, 19–28. [\[CrossRef\]](#)
- Eastgate, J.A. *Erwinia amylovora*: The molecular basis of fireblight disease. *Mol. Plant Pathol.* **2000**, *1*, 325–329. [\[CrossRef\]](#) [\[PubMed\]](#)
- Mendes, R.J.; Luz, J.P.; Santos, C.; Tavares, F. CRISPR genotyping as complementary tool for epidemiological surveillance of outbreaks. *PLoS ONE* **2021**, *16*, e0250280. [\[CrossRef\]](#)
- Ismailova, E.; Shemshura, O.; Sadanov, A.; Baimakhanova, G.; Turlybayeva, Z.; Kuldybayev, N.; Yelubayeva, A.; Kopzhassarov, B.; Issina, Z.; Temreshev, I.; et al. Monitoring studies of the occurrence of fire blight pathogen in Kazakhstan and identification of antagonistic microorganisms suppressing its development. *Braz. J. Biol.* **2024**, *84*, e285493. [\[CrossRef\]](#)
- Lee, H.J.; Seon, S.W.; Suh, S.-J.; Hyun, I.-H. Recent spread and potential pathways for fire blight in South Korea. *EPPO Bull.* **2022**, *52*, 135–140. [\[CrossRef\]](#)
- Pedroncelli, A.; Puopolo, G. This tree is on fire: A review on the ecology of the causal agent of fire blight disease. *J. Plant Pathol.* **2023**, *106*, 823–837. [\[CrossRef\]](#)
- Park, D.H.; Yu, J.G.; Oh, E.J.; Han, K.S.; Yea, M.C.; Lee, S.J.; Myung, I.S.; Shim, H.S.; Oh, C.S. First report of fire blight disease on Asian pear caused by in Korea. *Plant Dis.* **2016**, *100*, 1946. [\[CrossRef\]](#)
- Ahn, M.I.; Yang, H.J.; Yun, S.C. Development of K-Maryblyt for fire blight control in apple and pear trees in Korea. *Plant Pathol. J.* **2024**, *40*, 290–298. [\[CrossRef\]](#)
- Song, S.; Kim, B.; Kim, K.P.; Roh, E. Novel detection protocol for *Erwinia amylovora* in orchard soil after removal of infected trees. *Plant Pathol. J.* **2024**, *40*, 282–289. [\[CrossRef\]](#) [\[PubMed\]](#)
- Kim, B.; Lee, S.Y.; Park, J.; Song, S.; Kim, K.P.; Roh, E. Bacteriophage cocktail comprising Fifi044 and Fifi318 for biocontrol of *Erwinia amylovora*. *Plant Pathol. J.* **2024**, *40*, 160–170. [\[CrossRef\]](#) [\[PubMed\]](#)
- Nyunoya, H.; Lusty, C.J. The *carB* gene of *Escherichia coli*: A duplicated gene coding for the large subunit of carbamoyl-phosphate synthetase. *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 4629–4633. [\[CrossRef\]](#) [\[PubMed\]](#)
- Kwon, D.H.; Lu, C.D.; Walthall, D.A.; Brown, T.M.; Houghton, J.E.; Abdelal, A.T. Structure and regulation of the *carAB* operon in *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*: No untranslated region exists. *J. Bacteriol.* **1994**, *176*, 2532–2542. [\[CrossRef\]](#)

16. Raushel, F.M.; Thoden, J.B.; Holden, H.M. The amidotransferase family of enzymes: Molecular machines for the production and delivery of ammonia. *Biochemistry* **1999**, *38*, 7891–7899. [\[CrossRef\]](#)
17. Holden, H.M.; Thoden, J.B.; Raushel, F.M. Carbamoyl phosphate synthetase: An amazing biochemical odyssey from substrate to product. *Cell. Mol. Life Sci.* **1999**, *56*, 507–522. [\[CrossRef\]](#)
18. Arioli, S.; Monnet, C.; Guglielmetti, S.; Mora, D. Carbamoylphosphate synthetase activity is essential for the optimal growth of *Streptococcus thermophilus* in milk. *J. Appl. Microbiol.* **2009**, *107*, 348–354. [\[CrossRef\]](#)
19. Fox, B.A.; Bzik, D.J. Pyrimidine biosynthesis is required for virulence of *Toxoplasma gondii*. *Nature* **2002**, *415*, 926–929. [\[CrossRef\]](#)
20. Guo, J.; Song, X.; Zou, L.F.; Zou, H.S.; Chen, G.Y. The small and large subunits of carbamoyl-phosphate synthase exhibit diverse contributions to pathogenicity in *Xanthomonas citri* subsp. *citri*. *J. Integr. Agric.* **2015**, *14*, 1338–1347. [\[CrossRef\]](#)
21. Zhuo, T.; Rou, W.; Song, X.; Guo, J.; Fan, X.; Kamau, G.G.; Zou, H. Molecular study on the *carAB* operon reveals that *carB* gene is required for swimming and biofilm formation in *Xanthomonas citri* subsp. *citri*. *BMC Microbiol.* **2015**, *15*, 225. [\[CrossRef\]](#)
22. Klee, S.M.; Sinn, J.P.; Finley, M.; Allman, E.L.; Smith, P.B.; Aimufua, O.; Sittther, V.; Lehman, B.L.; Krawczyk, T.; Peter, K.A.; et al. *Erwinia amylovora* auxotrophic mutant exometabolomics and virulence on apples. *Appl. Environ. Microbiol.* **2019**, *85*, e00935-19. [\[CrossRef\]](#)
23. Kang, I.J.; Park, D.H.; Lee, Y.K.; Han, S.W.; Kwak, Y.S.; Oh, C.S. Complete genome sequence of *Erwinia amylovora* strain TS3128, a Korean strain isolated in an Asian pear orchard in 2015. *Microbiol. Resour. Announc.* **2021**, *10*, e0069421. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Lee, J.; Choi, J.; Lee, J.; Cho, Y.; Kang, I.J.; Han, S.W. Comparing protein expression in *Erwinia amylovora* strain TS3128 cultured under three sets of environmental conditions. *Plant Pathol. J.* **2022**, *38*, 410–416. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Zou, H.S.; Yuan, L.A.; Guo, W.; Li, Y.R.; Che, Y.Z.; Zou, L.F.; Chen, G.Y. Construction of a Tn5-tagged mutant library of *Xanthomonas oryzae* pv. *oryzicola* as an invaluable resource for functional genomics. *Curr. Microbiol.* **2011**, *62*, 908–916. [\[CrossRef\]](#)
26. Singer, T.; Burke, E. High-throughput TAIL-PCR as a tool to identify DNA flanking insertions. *Methods Mol. Biol.* **2003**, *236*, 241–272. [\[CrossRef\]](#)
27. Heo, L.; Cho, Y.; Choi, J.; Lee, J.; Han, Y.; Han, S.W. Proteomic and phenotypic analyses of a putative YggS family pyridoxal phosphate-dependent enzyme in *Acidovorax citrulli*. *Plant Pathol. J.* **2023**, *39*, 235–244. [\[CrossRef\]](#)
28. Heo, L.; Han, Y.; Cho, Y.; Choi, J.; Lee, J.; Han, S.W. A putative glucose 6-phosphate isomerase has pleiotropic functions on virulence and other mechanisms in *Acidovorax citrulli*. *Front. Plant Sci.* **2023**, *14*, 1275438. [\[CrossRef\]](#)
29. Perez-Riverol, Y.; Bai, J.; Bandla, C.; Garcia-Seisdedos, D.; Hewapathirana, S.; Kamatchinathan, S.; Kundu, D.J.; Prakash, A.; Frericks-Zipper, A.; Eisenacher, M.; et al. The PRIDE database resources in 2022: A hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Res.* **2022**, *50*, D543–D552. [\[CrossRef\]](#)
30. Lee, J.; Lee, J.; Cho, Y.; Choi, J.; Han, S.W. A putative 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase is involved in the virulence, carbohydrate metabolism, biofilm formation, twitching halo, and osmotic tolerance in. *Front. Plant Sci.* **2022**, *13*, 1039420. [\[CrossRef\]](#)
31. Elias, J.E.; Gygi, S.P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* **2007**, *4*, 207–214. [\[CrossRef\]](#)
32. Choi, H.; Fermin, D.; Nesvizhskii, A.I. Significance analysis of spectral count data in label-free shotgun proteomics. *Mol. Cell. Proteom.* **2008**, *7*, 2373–2385. [\[CrossRef\]](#)
33. Tatusov, R.L.; Galperin, M.Y.; Natale, D.A.; Koonin, E.V. The COG database: A tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Res.* **2000**, *28*, 33–36. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Park, H.; Do, E.; Kim, M.; Park, H.J.; Lee, J.; Han, S.W. A lysR-type transcriptional regulator LcrX is involved in virulence, biofilm formation, swimming motility, siderophore secretion, and growth in sugar sources in *Xanthomonas axonopodis* pv. *glycines*. *Front. Plant Sci.* **2020**, *10*, 1657. [\[CrossRef\]](#)
35. Loudon, B.C.; Haarmann, D.; Lynne, A.M. Use of blue agar CAS assay for siderophore detection. *J. Microbiol. Biol. Educ.* **2011**, *12*, 51–53. [\[CrossRef\]](#)
36. Roy, A.; Kucukural, A.; Zhang, Y. I-TASSER: A unified platform for automated protein structure and function prediction. *Nat. Protoc.* **2010**, *5*, 725–738. [\[CrossRef\]](#)
37. Kwaga, J.; Allan, B.J.; Van der Hurk, J.; Seida, H.; Potter, A.A. A *carAB* mutant of avian pathogenic *Escherichia coli* serogroup O₂ is attenuated and effective as a live oral vaccine against colibacillosis in turkeys. *Infect. Immun.* **1994**, *62*, 3766–3772. [\[CrossRef\]](#)
38. Mahan, M.J.; Schlauch, J.M.; Mekalanos, J.J. Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **1993**, *259*, 686–688. [\[CrossRef\]](#)
39. Shi, D.S.; Caldovic, L.; Tuchman, M. Sources and fates of carbamyl phosphate: A labile energy-rich molecule with multiple facets. *Biology* **2018**, *7*, 34. [\[CrossRef\]](#)
40. Liu, X.Y.; Cai, Y.C.; Zhang, X.; Zhang, H.F.; Zheng, X.B.; Zhang, Z.G. Carbamoyl phosphate synthetase subunit MoCpa2 affects development and pathogenicity by modulating arginine biosynthesis in *Magnaporthe oryzae*. *Front. Microbiol.* **2016**, *7*, 2023. [\[CrossRef\]](#)
41. Khanapur, M.; Alvala, M.; Prabhakar, M.; Kumar, K.S.; Edwin, R.K.; Saranya, P.S.V.K.S.; Patel, R.K.; Bulusu, G.; Misra, P.; Pal, M. *Mycobacterium tuberculosis* chorismate mutase: A potential target for TB. *Bioorg. Med. Chem.* **2017**, *25*, 1725–1736. [\[CrossRef\]](#)
42. Brzovic, P.; Holbrook, E.L.; Greene, R.C.; Dunn, M.F. Reaction mechanism of *Escherichia coli* cystathionine gamma-synthase: Direct evidence for a pyridoxamine derivative of vinylglyoxylate as a key intermediate in pyridoxal phosphate dependent gamma-elimination and gamma-replacement reactions. *Biochemistry* **1990**, *29*, 442–451. [\[CrossRef\]](#) [\[PubMed\]](#)

43. Wang, Y.; Zhao, Y.; Xia, L.; Chen, L.; Liao, Y.; Chen, B.; Liu, Y.; Gong, W.; Tian, Y.; Hu, B. yggS encoding pyridoxal 5'-phosphate binding protein is required for *Acidovorax citrulli* virulence. *Front. Microbiol.* **2021**, *12*, 783862. [[CrossRef](#)] [[PubMed](#)]
44. Maturana, P.; Orellana, M.S.; Herrera, S.M.; Martinez, I.; Figueroa, M.; Martinez-Oyanedel, J.; Castro-Fernandez, V.; Uribe, E. Crystal structure of *Escherichia coli* agmatinase: Catalytic mechanism and residues relevant for substrate specificity. *Int. J. Mol. Sci.* **2021**, *22*, 4769. [[CrossRef](#)] [[PubMed](#)]
45. Laube, G.; Bernstein, H.G. Agmatine: Multifunctional arginine metabolite and magic bullet in clinical neuroscience? *Biochem. J.* **2017**, *474*, 2619–2640. [[CrossRef](#)]
46. Frawley, E.R.; Fang, F.C. The ins and outs of bacterial iron metabolism. *Mol. Microbiol.* **2014**, *93*, 609–616. [[CrossRef](#)]
47. Chen, R.; Liu, N.; Ren, Y.; Cui, T. Transcriptomic and biochemical analysis of metabolic remodeling in *Bacillus subtilis* MSC4 under Benzo[a]pyrene stress. *Chemosphere* **2024**, *353*, 141637. [[CrossRef](#)]
48. Dietl, A.M.; Binder, U.; Bauer, I.; Shadkchan, Y.; Oshero, N.; Haas, H. Arginine auxotrophy affects siderophore biosynthesis and attenuates virulence of *Aspergillus fumigatus*. *Genes* **2020**, *11*, 423. [[CrossRef](#)]
49. Monti, S.M.; De Simone, G.; D'Ambrosio, K. L-histidinol dehydrogenase as a new target for old diseases. *Curr. Top. Med. Chem.* **2016**, *16*, 2369–2378. [[CrossRef](#)]
50. Kovach, M.E.; Elzer, P.H.; Hill, D.S.; Robertson, G.T.; Farris, M.A.; Roop, R.M.; Peterson, K.M. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **1995**, *166*, 175–176. [[CrossRef](#)]

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