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Functional Prediction and Assignment of *Clostridium botulinum* Type A1 Operome: A Quest for Prioritizing Drug Targets

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Abstract

Clostridium botulinum strain Hall is capable of producing a potent *botulinum* neurotoxin type A1 that causes food-borne, infant, and wound botulism in humans. Antibiotics and *botulinum* antitoxin are viable options for controlling growth and preventing *botulinum* intoxication in humans. The limited information on its proteins with unknown functions (operome or hypothetical proteins) hinders the discovery of new drug targets. Therefore, we aimed to apply a combined bioinformatics approach with literature support for the functional prediction, assignment, and validation of its operome. Our functional annotation scheme was based on sequence motifs, conserved domains, structures, protein folds, and evolutionary relationships of its operome. The precise functions of the 521 HPs (293 known) were annotated of which 293 HPs were previously annotated and 228 HPs not annotated from its genome. The newly identified operome contributed to the diverse cellular and metabolic processes of this organism. The function of its operome was involved in amino acid metabolism and *botulinum* neurotoxin biosynthesis. As a result of this study, 13 new virulence proteins from its operome were identified and characterized for their structure function relationships. The functions of new metabolic and virulence proteins allow this organism to colonize and interact with the human gastrointestinal tract. This study provides a quest for new drugs and targets for the treatment of *C. botulinum* underlying diseases in humans.

Keywords: *Clostridium botulinum* • Protein function • Molecular machinery • Bioinformatics • Drug target • Food spoiling • Virulence

Abbreviations: AD: Aldehyde Degradation; AAB: Amino Acids Biosynthesis; AAD: Amino Acids Degradation; ATC: Aminoacyl-tRNA Charging; CB: Carbohydrates Biosynthesis; CD: Carbohydrates Degradation; CSB: Cell Structures Biosynthesis; CPEB: Cofactors, Prosthetic Groups, Electron Carriers Biosynthesis; DRS: DNA Reactions; DR: DNA Repair; FLB: Fatty Acid and Lipid Biosynthesis; HD: Hormones Degradation; INM: Inorganic Nutrients Metabolism; MD: Mercury Detoxification; MMS: Miscellaneous; NNB: Nucleosides and Nucleotides Biosynthesis; NND: Nucleosides and Nucleotides Degradation; PDT: Phosphenolpyruvate (PEP) Dependent Transport; PS: Photosynthesis; PMR: Protein-Modification Reactions; PRS: Protein-Reactions; RRS: RNA-Reactions; SMB: Secondary Metabolites Biosynthesis; SMD: Secondary Metabolites Degradation; SMR: Small Molecule Reactions; TC: TCA Cycle; TRR: tRNA-Reactions

Highlights

- Organism-specific therapeutics is imperative for treating human botulism.
- The precise functions of the 521 hypothetical proteins annotated in this study.
- Majority of its operome mainly involved in amino acid metabolism.
- We predicted functions of 13 new virulence proteins from its operome.
- This study provides a quest for new drugs and targets.

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Introduction

Clostridium botulinum is a food-borne spore-forming rod-shaped anaerobic bacterium. This organism produces eight distinct forms of *botulinum* neurotoxin (BoNT/A-H) [1]. Human botulism is a life-threatening neuroparalytic syndrome characterized by acute febrile symmetric descending flaccid paralysis [2]. Botulism is a public health emergency with a high fatality rate (5%-10% of cases) in cases of suspected ingestion of homemade, packed, and canned foods [3]. Food-borne, infant, and wound botulism are clinical cases frequently reported in humans [4]. According to the centers for disease control and prevention, *C. botulinum* type A accounted for 42% of infant botulism and 79% of wound botulism cases. Contamination of home-prepared or home-preserved foods with type A or B strains seemingly causes 90% of food-borne botulism. It is estimated that consumption of 30–100 ng of BoNT/A might cause food-borne botulism [5]. It is also categorized as a class I bioweapon [6]. The economic and medical costs of the treatment of botulism caused by type a strains are extremely high.

Food-borne botulism is not a result of infection but is a direct functional link between metabolism and virulence. *C. botulinum* vegetative cells produce BoNT/A to kill the host rapidly for subsequent saprophytic utilization. In addition to bont/A, this organism consists of two adherence genes (*fbp* and *groEL*) and three toxin-coding genes: *cloSI, hlyA, and colA* [7]. This organism also produces some unknown virulence factors required for full virulence in the hosts. Understanding its pathophysiological mechanisms is vital to control the intoxication process. Hence, genome-scale studies on its virulence and metabolic crosstalk are of great concern in recent systems biology research [8].

C. botulinum strain hall (CBOA) was isolated in February 2015 and completely sequenced [9]. It has a circular chromosome of 3.89 Mbp in size. This genome consists of 3563 coding genes, of which 1052 (28.5%) have unknown biological functions. Automated genome annotation tools have been used for the functional assignment of completed genome sequences of prokaryotes [10]. The term operome refers to proteins with unknown biological information (hypothetical proteins or HPs) in a genome. The sequence, structure, and literature of related homologs allow us for precise functional annotation of operome. A combined bioinformatics approach has been used to functionally annotate, characterize, and categorize operome from prokaryotes [11]. Hence, our study aimed to predict and characterize the comprehensive functional contexts of the CBOA operome to assist a precise function with relevant literature. Newly annotated functions can generate high quality genome-scale metabolic networks through a metabolic gap-filling process [12]. The annotated functions of operome allow us to identify new virulence factors to discover novel drugs or vaccines against various food-borne infections.

Materials and Methods

Dataset

CBOA genome (Accession NC_009495) consisted of 1052 HPs that were included in our dataset. All HPs were listed by searching text terms (“hypothetical proteins, unknown, uncharacterized, and putative”) against the Kyoto encyclopedia of genes and genomes v103 (KEGG) [13].

Functional annotation of operome

Conserved motifs in all HPs were identified using the KEGG-Motif tool. A protein sequence containing a domain with an unknown function and an e-value below 10^{-5} was filtered from the dataset. HPs with significant motifs were only selected for the functional prediction process. The conserved domain and architecture were detected using the NCBI-CDD v3.16 tool and SMART v7.0, respectively. Functional sites in the sequences were analyzed using ScanProsite [14]. The evolutionary relationships between HPs and their protein families were predicted using the SIFTER software. The primary (instability index, aliphatic index, and grand average of hydropathicity) and secondary (helix, sheets, extended coil, loops) structural characteristics were predicted using expasy’s ProtParam server and SOPMA, respectively. The structural homologs were identified from the sequences using the Swiss model. The structural and protein fold classes of all HPs were searched using the CATH database [15].

Prediction of subcellular localization

The subcellular localization of HPs was predicted using the PSORTb v3.0.2 [16]. The propensity of a protein to become a membrane protein was predicted using the SOSUI v2.0. The transmembrane helix and topology of each protein were detected by TMHMM v2.0 Krogh et al. and HMMTOP [17]. The signal peptide and location of the cleavage site in the peptide chain were predicted using SingnalP v4.0.

Functional categorization

Sequence motifs, conserved domains, structures, protein folds, and evolutionary relationships were collectively considered in our functional annotation scheme (Supplementary file). Knowledge based discovery is a great endeavor that provides strong evidence for assigning the precise function of HPs [18]. The molecular contribution of HPs was manually analyzed and assigned to precise functions in metabolic subsystems, cellular processes, and pathogenesis. The predicted function of each protein was evaluated in agreement with the available literature in NCBI PubMed. The maximum confidence score was set to 12, in which a 50% score was assigned from overall prediction approaches, and the remaining was assisted by manual annotation and the strength of the literature validation. The predicted function of a protein with a low confidence score (<3) was not considered in this study.

Results

Functional classification and categorization

The functions of all the HPs were predicted based on the sequence and structural characteristics and then categorized for corresponding molecular functions and metabolic subsystems. Approximately 14.62% of the operome (28%) showed significant sequence similarity to known proteins by database searching. Our combined bioinformatics approach predicted the function of 6.65% of the operome, of which 121 HPs showed exclusivity to the CBOA genome. Approximately 26% of the operome harbors the Rossmann fold and 43% of the operome consists of miscellaneous folds (Figure 1). The Arc repressor mutant fold and α-β plaits occupied 4% of the operome. Immunoglobulins, jelly rolls, and the TIM barrel were also detected in the 3% operome. The annotated functions of the operome were categorized based on metabolic subsystems. Several predicted functions contributed to small-molecule reactions in diverse metabolic pathways. Most operome functions were involved in the amino acid metabolism, defense, and virulence of this organism. Some annotated protein functions were responsible to mediate the protein modifications and electron transfer systems. A high proportion of operome was predicted to be transporters (>85) and transcriptional regulators (>45). Significant operome coverage (>60) was observed for hydrolase and transferase activities. Binding proteins (DNA, RNA, and metals) covered the operome moderately. The predicted functions of HPs that were previously available in the genome annotation data are represented in Tables 1-4. The predicted functions of HPs not yet included in its genome data are only described herein. The predicted functions of the operome and its molecular contributions to transcriptional regulation, metabolic subsystems, virulence, host defense, and cell wall architecture are described below with relevant literature evidence for CBOA.

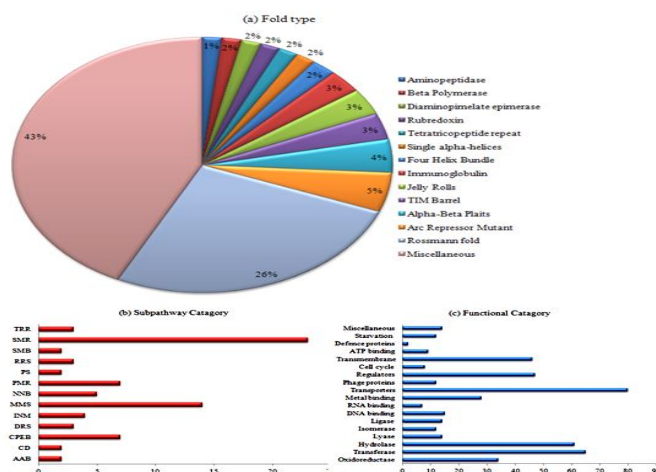

Figure 1. Functional classification of operome from *C. botulinum* type A1 based on the protein fold.

Cellular process

We predicted the functions of monopolin and tubulin from the operome of CBOA (Table 1). The listed proteins are mainly involved in chromosome segregation and cell skeleton architecture in this organism, similar to that in yeast. The identified pirin-like protein is a highly conserved nuclear protein that may function as a transcriptional regulator in cell death [19]. The NGG1p interacting factor 3 proteins (NIF3-like protein 1) are commonly found in animals. This protein shares sequence similarity with Helicobacter pylori GTP cyclohydrolase 1 type 2, which converts GTP to dihydroneopterin triphosphate. The tRNA C32 thiolase enzyme is required to modify nucleoside 2-thiocytidine in this organism, similar to archaea and bacteria. Aalanyl-tRNA synthetase predicted from this organism catalyzes the attachment of an amino acid to its cognate tRNA molecule [20].

Locus tag	Assigned function	Gene
CBO0860	Pirin-like protein	*YhhW*
CBO2935	NGG1p interacting factor 3 protein	*niF3*
CBO0165	tRNA C32 thiolase	*TtcA*
CBO1509	Aalanyl-tRNA synthetase	*alaS*

Table 1. Functional annotation of operome of *C. botulinum* type A1 involved in cellular process.

Metabolic subsystems

We assigned precise functions to the HPs involved in electron transfer, carbohydrate and lipid metabolism, and phosphate and sulfate assimilation (Table 2). NAD (P) H-binding flavin reductase from the CBOA can produce reduced flavin for bacterial bioluminescence and other biological processes. Ferredoxin reductase is a member of the flavoprotein pyridine nucleotide cytochrome reductase family, which is involved in electron transfer reactions.

The bi-functional coenzyme Pyrrolo Quinolone Quinone (PQQ) synthesis protein identified in CBOA is required for the synthesis of PQQ, but its function remains unclear. Quinoprotein forms a class of dehydrogenases distinct from NAD (P)-and flavin-dependent enzymes. The presence of this protein in CBOA can perform the oxidation of various compounds in electron transfer reactions. Phospho-L-lactate guanylyltransferase of the CBOA is involved in the initial activation of 2-phospho-L-lactate *via* pyrophosphate linkage to GMP for the biosynthesis of coenzyme F420 .

Locus tag	**Assigned function**	**Gene**	
Electron transfer			
CBO0286	NAD(P)H-binding flavin reductase	1.5.1.30	*cysJ/fre*
CBO1829	Ferredoxin	1.18.1.2	*fpR*
CBO2230	Bifunctional coenzyme PQQ synthesis protein C/D	1.3.3.11	*pqqCD*
CBO2633	Quinoprotein	1.1.5.2	*gcD*
CBO2613	2-Phospho-L-lactate guanylyltransferase	2.7.7.68	*cofC*
Carbohydrate			
CBO1145	Phosphoenolpyruvate carboxykinase	4.1.1.49	*Pck*
CBO1241	Lichenan-specific phosphotransferase	2.7.1.69	*LicC*
CBO2322	Subtilisin-like serine protease	3.4.21.62	*sdD1*
Lipid			
CBO0364	Phosphatidic acid phosphatase type 2		*plpP4*
CBO0388	Phosphoglycolate phosphatase	3.1.3.18	*Gph*
Phosphate			
CBO0464	Acid phosphatase/ Phosphotransferase	3.1.3.2	*AphA*
Sulfate			
CBO0199	Cystathionine beta-synthase (CBS domain)	4.2.1.22	*cbS*

Table 2. Functional annotation of operome of *C. botulinum* type A1 involved in metabolic subsystems.

The presence of phosphoenolpyruvate carboxykinase and lichenan-specific phosphotransferase is involved in carbohydrate metabolism. Subtilisin-like serine protease has an alpha/beta fold containing a 7-stranded parallel beta-sheet, which contributes to protein degradation in this organism. Phosphatidic acid phosphatase type 2 has a core structure consisting of a 5-helical bundle, where the beginning of the third helix binds to a co-factor. It dephosphorylates phosphatidate to diacylglycerol and inorganic phosphate in this organism. Phosphoglycolate phosphatase from this organism is highly similar to phosphoglycolate phosphatases, which catalyze the dephosphorylation of 2-phosphoglycolate. Acid phosphatase/phosphotransferase is one of several unrelated acid phosphatase families found in this organism, similar to that found in humans and other mammals. The CBS domain is located in cysteine synthase, which is responsible for the formation of cysteine from O-acetyl-serine and H2S with the concomitant release of acetic acid.

Host defense responses

We predicted the function of HPs involved in host defense responses in this organism (Table 3). The predicted functions were categorized into cell wall biogenesis, biofilm formation, starvation response, and metal detoxification. The presence of N-acetylglucosaminyltransferase II catalyzes an essential step in the cell-wall biosynthesis of this organism. Swim zinc finger domain protein has been identified in its operome, but its molecular function has not yet been characterized. The VEG protein from this organism is highly conserved among gram-positive bacteria. It stimulates biofilm formation by inducing the transcription of the tapA-sipW-tasA operon. The S-Adenosyl-l-Methionine (SAM)-dependent methyltransferases are a large class of enzymes with Rossmann-like folds. It utilizes SAM as a cofactor to methylate proteins, small molecules, lipids, and nucleic acids. This enzyme contributes to quorum sensing dependent metabolic homeostasis of the activated methyl cycle in the CBOA, similar to that in *Burkholderia glumae*.

Locus tag	**Assigned function**	**EC**	**Gene**
Cell wall			
CBO0127	N-Acetylglucosaminyltransferase II	2.4.1.141	*alG13/alG14*
CBO0014	Swim zinc finger domain protein	---	*znF*

Biofilm			
CBO0116	Veg protein	---	*veg*
CBO3144	SAM-dependent methyltransferase	2.1.1.176	*rsmB*
Starvation			
CBO0027	Phasin	---	*phaP*
CBO2926| CBO0731	Cupin domain protein	---	*RmlC/ oxdD*
CBO3367	Nitrogen metabolite repression protein		*nmrA*
CBO2373	Nucleoside triphosphate pyrophosphohydrolase	3.6.1.8	*mazG*
CBO2563	Enterocin A	---	*entA*
CBO1233	LURP1-related protein domain	---	*lurP1*
Metal			
CBO0058	Mercuric reductase	1.16.1.1	*merA*
CBO3253	Alkylmercurylyase	4.99.1.2	*merB*
CBO0617	ArsR-type HTH domain	---	*arsR*

Table 3. Functional annotation of operome of *C. botulinum* type A1 involved in host defence systems.

Phasins are granule-associated proteins detected in the CBOA, which store carbon and energy and confer stress resistance. The cupin domain protein represents the conserved barrel domain of the 'cupin' superfamily with a small barrel. It is a major nitrogen source for the survival of this organism, such as plants. The nitrogen metabolite repression protein identified from the CBOA operome is part of a system that controls nitrogen metabolite repression analogous to fungi. Nucleoside triphosphate pyrophosphohydrolase regulates oxidative and nutritional stress responses in this organism similar to the previous investigations. Enterocin A is a soluble cytoplasmic immunity protein identified in the operome. It confers bacteriocin resistance to CBOA by disorienting and closing membrane pores. The LURP1-related protein domain is comprised of a 12-stranded beta-barrel with a central C-terminal alpha helix. It is structurally similar to the C-terminal domain of the tubby protein and plays a role in the defense against competing microorganisms.

Mercuric reductase from CBOA is a FAD-containing flavoprotein that reduces Hg (II) to Hg (0) in the presence of NADPH during the detoxification of mercury compounds. This genome also contains the alkyl mercury lyase gene (merB) responsible for the reduction **and volatilization of mercury compounds. The arsR-type HTH domain is a transcriptional regulator of the arsR/smtB family that is involved in the stress response to heavy metal ions.**

Transporter proteins

We successfully annotated 18 transporter proteins in the CBOA operome (Table 4). These proteins included type II and III secretions, 2-hydroxy carboxylate transporters, cell wall-active antibiotic response proteins, inner membrane putative ABC superfamily transporter permease, ECF transporter, sulfur transporter, apolipoprotein A-I, sulfite exporter TauE/SafE family protein, thiamine-binding periplasmic, bacterial pH domain protein, and QueT transporter protein. The majority of the predicted functions are involved in secretary, sulfur sulfate, and carbohydrate transport across the CBOA membrane.

Locus tag	Assigned function	TC Number	Gene
CBO0180	Type III secretion system substrate exporter	-	*flhB /hrpN /yscU/spaS*
CBO0363	2-Hydroxycarboxylate transporter	2.A.24	*yadS*
CBO0551	Cell wall-active antibiotics response protein	9.B.116.2.1	*liaF*

CBO0778	Inner membrane putative ABC superfamily transporter permease	3.A.1.5.11	*ybhR*
CBO0790	ECF transporter protein	2.A.88.1.1	-
CBO1577|CBO1575|CBO1581	Sulphur transporter protein	2.A.1	*dsrE*
CBO1758	Apolipoprotein A-I	5.B.2.2.4	*apoa1*
CBO1904	Type II secretory pathway, pseudopilin	3.A.1.143.1	*pulG*
CBO2862|CBO3180|CBO2460|CBO2473|CBO2467|	Sulfite exporter TauE/SafE family protein	2.A.52	*tauE/safE*
CBO2910	Thiamine-binding periplasmic protein	2.A.102.4.5	*thiB*
CBO2937	Bacterial PH domain protein	3.A.1.19.1	-
CBO3177	QueT transporter protein	-	*queT*

Table 4. Functional annotation of operome of *C. botulinum* type A1 involved in transporter systems.

Discovery of new virulence proteins

Our bioinformatics approach predicted new virulence proteins in the CBOA operome (Table 5). The results of this study predicted DNA-binding and winged helix-turn-helix domains in transcription regulators of the crp-fnr family. It is involved in the regulation of virulence factors and nitrogen metabolism in CBOA similar to several pathogens. Bacteriocin is a peptide antibiotic secreted by many gram-positive bacteria that inhibit their colonization by other bacterial species.

Bacteriocin-processing endopeptidase identified from CBOA cleaves an N-terminal leader peptide in bacteriocin via the cleavage of a GlyGly bond. Consequently, CBOA can confer resistance to bacteriocin produced by probiotic bacteria in the human intestinal tract. Calcineurin is an important regulator of intracellular calcium homeostasis in the CBOA similar to several fungi and *C. difficile* and activates T cells of the human immune system. Prolyl oligopeptidase is a virulence factor commonly identified in human parasites. The presence of this protein in CBOA can cleave short peptides at the C-side of an internal proline, similar to parasites.

Locus	Score	Gene	Assigned function
CBO0747	0.785111	-	Bacteriocin-processing endopeptidase
CBO1758	0.900486	*Gp66*	Calcineurin
CBO1781	1.837087	*pop*	Prolyl oligopeptidase
CBO1828	0.810445	*ybiY*	YbjY-like metal-binding protein
CBO2138	1.655908	*pepN*	Alanyl aminopeptidase
CBO2578	1.016458	*khpB*	RNA-binding protein
CBO2603	0.486305	*wrbA*	Multimeric flavodoxin
CBO2633	0.794207	*gcd*	Quinoprotein glucose dehydrogenase
CBO2935	0.1128	*nif3*	NGG1p interacting factor 3 protein
CBO3022	0.710851	-	F5/8 type C domain protein
CBO3144	0.476182	*camA*	DNA adenine methyltransferase
CBO3353	2.351369	*fleA*	Fucose-specific lectin
CBO3430	0.54771	*ybbR*	YbbR-like protein

Table 5. Functional annotation of prioritized virulence proteins from operome of *C. botulinum* type A1.

YbeY is a highly conserved Ni2+-dependent protein that is found in bacteria. It plays a role in stress and virulence regulation in the CBOA, similar to other bacteria. The β-alanyl aminopeptidase is a biomarker for Pseudomonas aeruginosa in cystic fibrosis patients Thompson, et al. and a virulence factor (host tissue damage) in *C. chauvoei*. Similarly, we identified β-alanyl aminopeptidase as a virulence protein in the CBOA. Quinoprotein glucose dehydrogenase identified from CBOA catalyzes the oxidation of glucose to gluconic acid. This protein is involved in inorganic phosphorus-dissolving metabolism, virulence, and prodigiosin antibiotic biosynthesis, similar to proteobacteria. F5/8 type C domain-containing protein from this organism has shown high antigenicity indices, as described for *C. perfringens* type A and C strains. DNA methylation regulates virulence gene expression in *C. difficile*. The presence of DNA adenine methyltransferases in the CBOA suggests its role in controlling spore formation and colonization in response to virulence functions. Fucose-specific lectins may support host pathogen interactions *via* protein glycosylation. The presence of this protein may enhance the attachment of spores to human cell membranes and contribute to the pathogenicity of CBOA. The YbbR domain of CBOA is an important activator of non-ribosomal peptidic virulence factor biosynthesis. As shown by our analyses, we suggest additional importance for targeting these virulence proteins in drug and vaccine discovery.

Discussion

The role of the operome in bacterial genomes remains unknown due to the presence of proteins with unknown biological functions. These proteins may contribute to crucial cellular and metabolic activities. The cellular and molecular mechanisms of the operome can be elucidated by filling key knowledge gaps regarding their unknown functions. The present study used various predictive measures to identify, characterize, and validate the functioning of HPs from the CBOA according to previous investigations. Our combined knowledge with literature provides a clue to understanding its growth physiology and full virulence in the human gastrointestinal tract. The results of our study emphasize the demonstration of metabolic subsystems, virulence mechanisms, and identification of therapeutic targets based on the assigned function of the CBOA operome. Hence, biological knowledge bases determine a precise prediction of the function of bacterial operome.

Functional predictions of the operome in various pathogenic bacteria were performed using bioinformatics approaches. *C. botulinum* is a clinically prevalent bacterium in the gut microbiota, which causes botulism in humans and animals. The spores of this organism are heat-resistant and easily transmissible to hosts *via* the human-food web-animal interface. This organism has a wide genetic diversity with metabolic discrepancy and an evolutionary lineage from multiple and independent genetic rearrangements. Therefore, we investigated the missing functions from its operome to understand the metabolic subsystems using a combined bioinformatics approach. The predicted operome functions were classified and categorized according to their functional significance. Most operome contribute to amino acid metabolism, defense, and virulence. It showed the coverage of hydrolase and transferase activities. The predicted functions of its operome exhibited additional roles in protein modifications, electron transfer systems, transcriptional regulators, and transporters. The predicted functions of CBOA operome support the host defense responses, adaptability to the host environment, metal detoxification, and pathogenesis. The metabolic and transcriptional networks of this organism lack a few essential genes and functional gaps. The CBOA operome also includes biosynthetic pathways for some coenzymes. Bacteriocin, calcineurin, prolyl oligopeptidase, β-alanyl aminopeptidase, DNA adenine methyltransferases, YbeY, and YbbR domains were identified as new virulence factors in CBOA, which confer antibacterial resistance, colonization, and biofilm formation in the human gastrointestinal tract.

Conclusion

To understand the metabolic and molecular functions of this organism, it is essential to functionally assign its operome. The predicted functions of its operome characteristics provide access to fresh structural data as well as novel molecular functions that are crucial to the way of life. The predicted results cover all the functional equivalents required for various metabolic pathways and regulatory functions in this organism. The 96 metabolic enzymes from its operome recognize DNA, RNA, metals, and membranes for cellular and metabolic activities. Our approach assigned and categorized the functions of 74 metabolic enzymes, 80 transporter proteins, and 8 cell division proteins from this organism. Thirteen virulence proteins have been discovered from its operome, suggesting the importance of drug and vaccine discovery targeting these proteins. Functional prediction and assignment of its operome are fundamental for understanding the molecular machinery and full virulence spectrum at the system level. However, the assigned functions of some HPs should be tested using protein expression and purification, crystallization, and structural determination studies for the further therapeutics development process.

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Conflict of Interest

The authors confirm that this article has no conflicts of interest.

Ethics Approval and Consent to Participate

The need for ethical approval and individual consent was waived.

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