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Genomic Insights of Pathogenicity of *Comamonas kerstersii*

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Abstract: To achieve better management of *Comamonas kerstersii* (*C. kerstersii*) infections, the aim was to understand its virulence and pathogenicity by analyzing the virulence factor genes (VFGs) in the genome of *C. kerstersii* 121606. The genome was sequenced previously, and the VFGs were predicted by BLAST searching through the VFDB database. The results showed that *C. kerstersii* contained a large number of VFGs, some of which were exemplified to correlate to the survival and growth in humans, and the pathogenicity of *C. kerstersii* in the gastrointestinal tract and lungs. Genomic analysis revealed that *C. kerstersii* might be an intracellular pathogen. The harbored VFGs were conducive to explaining its virulence and pathogenicity in gastrointestinal tract and lung infections reported in the literature or encountered in clinic. The data also provided ideal targets for novel antibiotic and vaccine development for the therapy of *C. kerstersii* infections.

Keywords: *Comamonas kerstersii*; virulence factor gene; pathogenicity; intracellular pathogen; gastrointestinal tract infection; lung infection

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Comamonas kerstersii 细菌致病性的基因组分析

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摘要: 为了更好地管理 *Comamonas kerstersii* (*C. kerstersii*) 感染, 通过分析 *C. kerstersii* 菌株 121606 基因组中的毒力因子基因(VFGs)来了解其毒力和致病性。对已经完成测序的 *C. kerstersii* 121606 基因组, 通过 BLAST 搜索 VFDB 数据库来预测其 VFGs。结果表明: *C. kerstersii* 含有大量 VFGs, 其中一些与其在人体内的生存和生长, 以及在胃肠道和肺部的致病性有关。基因组分析表明: *C. kerstersii* 可能是一种细胞内病原体。 *C. kerstersii* 所携带的 VFGs 有助于解释其在文献中所报道的胃肠道感染和临床工作中所遇到的肺部感染中的毒力和致病性, 这些数据也为新型抗生素和疫苗开发提供了理想的靶点, 以用于治疗 *C. kerstersii* 感染。

关键词: *Comamonas kerstersii*; 毒力因子基因; 致病性; 细胞内病原体; 胃肠道感染; 肺部感染

Comamonas kerstersii (*C. kerstersii*) is a Gram-negative, aerobic, motile rod bacterium^[1], which is found ubiquitously in water, soil, and plants^[1]. As summarized in our previously submitted work,

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we knew that, it primarily causes intra-abdominal infections, such as peritonitis and appendicitis, as well as extra-abdominal infections, including lung infection encountered in our clinical settings. Due to difficulties in identification using conventional phenotypic methods or even 16S rRNA gene sequencing, the actual prevalence of its infections are underestimated. While it is rarely resistant to antibiotics and is closely associated with a favorable clinical prognosis, we have also isolated a pan-drug resistant *C. kerstersii* strain 121606 from an 82-year-old male patient. Given the potential health threat posed by pan-drug resistance, we have conducted genomic studies to elucidate the mechanisms of pan-drug resistance at the genomic level (submitted). In this article, we aim to further understand the survival ability, virulence, and pathogenicity of *C. kerstersii*, particularly in the gastrointestinal tract and lungs, by analyzing the virulence factor genes (VFGs) in the genome of the exemplified *C. kerstersii* 121606 strain. This strain shares nearly identical VFGs with our two other *C. kerstersii* strains: one (strain 12322-1) isolated from the sputum of a patient with a fatal lung infection, to whom it promoted the patient's death, and another (strain 202149) isolated from peritoneal pus of a patient with acute perforated appendicitis.

1 Materials and Methods

1.1 Genome Sequencing, Assembly, Annotation for *C. kerstersii* 121606

These works had been completed as described in previously submitted article. The 16S rRNA gene sequence (1 460 bp, GenBank No. KY014106) and the genome sequences of *C. kerstersii* strain 121606 (GenBank assembly accession No. GCA_002002445.1, ASM200244v1) had been deposited into GenBank.

1.2 Prediction of VFGs in *C. kerstersii* 121606 Genome

The VFGs in *C. kerstersii* 121606 were predicted by conducting a BLAST search against the VFDB protein Set B database^[2] in collaboration with Beijing Novogene Bioinformatics Technology Co., Ltd.. The identity parameter was set at >40%. No stringent cut-off parameters were applied to further filter the initially predicted VFGs because doing so would exclude a large number of potentially unknown VFGs and result in an inability to correlate the remaining VFGs to the infectivity of *C. kerstersii* in patients.

2 Experimental Results and Analysis

The VFGs in *C. kerstersii* 121606 were listed in table 1 (discussed) and table 2 (not discussed), such as the genes involved in formation/biosynthesis/regulation of capsular polysaccharide (*rmlA*, *rmlB*, *rmlC*, *wbfV/wcvB*, *wbjD/wecB*, *wecC*, *gtaB*), LOS (*galE*, *kdsB*, *kdtA/waaA*, *lpxA*, *lpxB*, *lpxC*, *orfM*), lipopolysaccharide (*acpXL*, *fabZ*, *gtrB*, *hisF*, *kdsA*, *lpxD*, *kdtB*), motility/attachment apparatuses, fimbriae (*cupA1*, *cupA3*, *cupD2*, *cupD4*, *cupD5*), type IV pili (*pilA*, *pilB*, *pilC*, *pilD*, *pilE*, *pilF*, *pilG*, *pilH*, *pilI*, *pilJ*, *pilK*, *pilL*, *pilM*, *pilN*, *pilO*, *pilP*, *pilQ*, *pilR*, *pilS*, *pilT*, *pilU*, *pilV*, *pilW*, *pilX*, *pilY*, *pilZ*, *rpoN*), flagella (motility: *flaA/flmA*, *flmH*, *fleN*, *flgB*, *flgC*, *flgD*, *flgE*, *flgF*, *flgG*, *flgH*, *flgI*, *flgJ*, *flhA*, *flhB*, *flhC*, *flhD*, *fliA*, *fliC*, *fliE*, *fliF*, *fliG*, *fliH*, *fliI*, *fliJ*, *fliK*, *fliL*, *fliM*, *fliN*, *fliO*, *fliP*, *fliQ*, *fliR*, *fliS*, *fliT*, *fliU*, *fliV*, *fliW*, *fliX*, *fliY*, *fliZ*, *motA*, *motB*; chemotaxis: *cheA*, *cheB*, *cheC*, *cheD*, *cheE*, *cheF*, *cheG*, *cheH*, *cheI*, *cheJ*, *cheK*, *cheL*, *cheM*, *cheN*, *cheO*, *cheP*, *cheQ*, *cheR*, *cheS*, *cheT*, *cheU*, *cheV*, *cheW*, *cheX*, *cheY*, *cheZ*, *tsr*, *tar/cheM*), yersiniabactin siderophore (*fyuA*, *ybtQ*, *ybtP*, *irp2*, *ECVR50_2112*), *Haemophilus* iron transport system HitABC (*hitA*, *hitC*), molecular chaperones (*groEL*, *dnaK*), hemolysin (*hlyIII*, *hlyA*), heme uptake system (*hutC*, *hutD*, *bhuT*, *hmuV*, *phuS*), HasA-type hemophore-mediated heme uptake system (*hasD*, *hasE*), heme biosynthesis pathway (*hemB*, *hemC*, *hemE*, *hemH*, *hemL*, *hemN*), heme utilization (AB57_0992), the ATP-binding cassette (ABC)

transporter-FbpABC (*fbpC*) and other iorn uptake systems (*fatD*, *vctC*, *irgA*, *fur*).

Tab. 1 VFGs in genome of *C. kerstersii* 121606 (discussed)

表 1 *C. kerstersii* 121606 基因组中的 VFGs(讨论)

Classification of Virulence Factors	VFGs	121606	Virulence Factors
Yersiniabactin siderophore	<i>fyuA</i>		Putative pesticin receptor precursor
Yersiniabactin	<i>ybtQ</i>		Inner membrane ABC-transporter YbtQ
Yersiniabactin siderophore	<i>ybtQ</i> (now named as <i>KPHS_34630</i>)		Yersiniabactin-iron ABC transporter permease and ATP-binding protein YbtQ
Yersiniabactin	<i>ybtP</i>		Pipoprotein inner membrane ABC-transporter
Yersiniabactin	<i>irp2</i>		Putative peptide synthetase
Yersiniabactin siderophore	<i>ECVR50_2112</i>		Putative cytoplasmic transmembrane protein
Haemophilus iron transport locus	<i>hitA</i>		Extracellular solute-binding protein family 1
HitABC	<i>hitC</i>		Iron III ABC transporter, ATP-binding protein
Haemophilus iron transport locus	<i>hitC</i>		ABC transporter related
ABC transporter	<i>fbpC</i>		ABC transporter, ATP-binding protein, iron related
Petrobactin	<i>fatD</i>		Iron compound ABC transporter permease protein
Periplasmic binding protein-dependent ABC transport systems	<i>vctC</i>		Iron III ABC transporter, ATP-binding protein
Enterobactin receptors	<i>irgA</i>		Iron-regulated outer membrane virulence protein, TonB receptor family
Fur	<i>fur</i>		Transcriptional repressor of iron-responsive genes Fur family ferric uptake regulator
Hsp60	<i>groEL</i>		Molecular chaperone GroEL
Hsp70	<i>dnaK</i>		Molecular chaperone DnaK
Hemolysin III	<i>hly</i> III		Hemolysin
Hemolysin, HlyA	<i>hlyA</i>		Transporter
Heme uptake system	<i>hutC</i>		ABC-type hemin transporter, permease protein
Heme uptake system	<i>hutD</i>		ABC-type hemin transporter, ATP-binding protein
Heme uptake system	<i>bhuT</i>		Putative hemin binding protein
Heme uptake system	<i>hmuV</i>		Hypothetical protein
Heme uptake system	<i>phuS</i>		Hemin degrading factor
HasA-type hemophore-mediated heme uptake system	<i>hasD</i>		ABC transporter ATP-binding protein
HasA-type hemophore-mediated heme uptake system	<i>hasE</i>		ABC transporter periplasmic substrate-binding protein
Heme biosynthesis	<i>hemB</i>		Porphobilinogen synthase
Heme biosynthesis	<i>hemC</i>		Porphobilinogen deaminase
Heme biosynthesis	<i>hemE</i>		Uroporphyrinogen decarboxylase
Heme biosynthesis	<i>hemH</i>		Ferrochelataase
Heme biosynthesis	<i>hemL</i>		Glutamate-1-semialdehyde aminotransferase
Heme biosynthesis	<i>hemN</i>		Coproporphyrinogen III oxidase
Heme biosynthesis	<i>hemN</i>		Oxygen-independent coproporphyrinogen III oxidase
Heme utilization	<i>AB57_0992</i>		Hypothetical protein
Type IV pili	<i>pilA</i>	—	Fimbrial protein
Type IV pili	<i>pilB</i>		Pilus biogenesis protein
Type IV pili biosynthesis	<i>pilC</i>		Type IV pilus biogenesis protein PilC

Continue table
续表

Classification of Virulence Factors	VFGs	121606	Virulence Factors
Type IV pili biosynthesis	<i>pilD</i>		Prepilin peptidase
Type IV pili	<i>pilE</i>		Fimbrial protein P9-2 Pilin
Type IV pili biosynthesis	<i>pilE</i>		Tfp pilus assembly protein PilE-like protein
Type IV pili twitching motility related proteins	<i>pilG</i>		Type IV pilus response regulator PilG
Type IV pili	<i>pilH</i>		Twitching motility protein PilH
Type IV pili	<i>pilJ</i>		Twitching motility protein PilJ
Type IV pili biosynthesis	<i>pilM</i>		Type IV pilus assembly protein PilM
Type IV pili biosynthesis	<i>pilN</i>		Fimbrial assembly
Type IV pili biosynthesis	<i>pilQ</i>		Type II and III secretion system protein
Type IV pili biosynthesis	<i>pilR</i>		Putative two-component system, response regulator
Type IV pili biosynthesis	<i>pilR</i>		Two component, sigma-54 specific, transcriptional regulator, Fis family
Type IV pili biosynthesis	<i>pilT</i>		Twitching motility protein
Type IV pili	<i>pilT2</i>		Putative pilus retraction protein
Tap type IV pili	<i>pilU</i>		Tfp pilus assembly protein, ATPase PilU
Type IV pili biosynthesis	<i>pilZ</i>		Type IV pilus assembly PilZ
Type IV pili	<i>rpoN</i>		RNA polymerase factor sigma-54
Flagella	<i>flaA (flmA)</i>		Flagellin
Polar flagella	<i>flmH</i>		3-oxoacyl-ACP reductase
Flagella	<i>fleN</i>		Flagellar synthesis regulator FleN
Peritrichous flagella	<i>flgB</i>		Flagellar basal body rod protein FlgB
Flagella	<i>flgC</i>		Flagellar basal body rod protein
Flagella (cluster I)	<i>flgD</i>		Flagellar basal body rod modification protein
Flagella	<i>flgE</i>	—	Flagellar hook protein FlgE
Flagella	<i>flgF</i>		Flagellar basal body rod protein FlgF
Flagella (cluster I)	<i>flgG</i>		Flagellar basal-body rod protein FlgG
Flagella	<i>flgH</i>		Flagellar L-ring protein precursor FlgH
Flagella	<i>flgI</i>		Flagellar basal body P-ring protein
Flagella	<i>flgJ</i>		Peptidoglycan hydrolase
Flagella	<i>flhA</i>		Flagellar biosynthesis protein FlhA
Flagella	<i>flhB</i>		Flagellar biosynthetic protein FlhB
Flagella	<i>flhC</i>		Flagellar transcriptional activator FlhC
Flagella	<i>flhD</i>		Transcriptional activator FlhD
Flagella	<i>fliA</i>		Flagellar biosynthesis sigma factor
Flagella (cluster I)	<i>fliC</i>		Flagellin FliC
Flagella	<i>fliC</i>		A-type flagellin
Flagella	<i>fliE</i>		Flagellar hook-basal body complex protein FliE
Flagella	<i>fliF</i>		Flagellar MS-ring protein
Flagella	<i>fliG</i>		Flagellar motor switch protein G
Flagella	<i>fliI</i>		Flagellum-specific ATP synthase FliI
Flagella	<i>fliM</i>		Flagellar motor switch protein FliM
Flagella	<i>fliN</i>		Flagellar motor switch protein FliN
Peritrichous flagella	<i>fliQ</i>		Flagellar biosynthesis protein FliQ
Flagella	<i>fliP</i>		Flagellar biosynthesis protein FliP
Flagella	<i>fliR</i>		Flagellar biosynthetic protein FliR
Peritrichous flagella	<i>fliS</i>		Flagellar protein FliS

Continue table
续表

Classification of Virulence Factors	VFGs	121606	Virulence Factors
Flagella	<i>motA</i>		Flagellar motor protein MotA
Flagella	<i>motB</i>		Flagellar motor protein MotB
Flagella	<i>cheA</i>		Signal transduction histidine kinase CheA
Flagella	<i>cheB</i>		Chemotaxis-specific methylesterase CheB
Flagella	<i>cheD</i>		Chemoreceptor glutamine deamidase CheD
Peritrichous flagella	<i>cheD</i>		Methyl-accepting chemotaxis protein CheD
Peritrichous flagella	<i>cheR</i>		Chemotaxis methyltransferase CheR
Flagella	<i>cheR</i>		Chemotaxis protein CheR
Flagella	<i>cheW</i>		Chemotaxis protein CheW
Peritrichous flagella	<i>cheY</i>		Chemotaxis regulatory protein CheY
Flagella	<i>cheY1</i>		Chemotaxis two-component response regulator CheY1
Flagella	<i>tsr</i>		Methyl-accepting chemotaxis protein
Flagella	<i>tsr</i>		Methyl-accepting chemotaxis protein I
Flagella	<i>tsr</i>		Methyl-accepting chemotaxis sensory transducer
Peritrichous flagella	<i>tar/cheM</i>		Methyl accepting chemotaxis protein II
Urease	<i>ureA</i>		Urease alpha subunit UreA
Urease	<i>ureB</i>		Urease beta subunit UreB
Urease	<i>ureG</i>		Urease accessory protein ureG
SodB	<i>sodB</i>		Superoxide dismutase SodB
Hsp70	<i>dnaK</i>		Molecular chaperone DnaK
EF-Tu	<i>tuf</i>		Elongation factor Tu
Mip	<i>mip</i>		Macrophage infectivity potentiator
ClpC	<i>clpC</i>		Endopeptidase Clp ATP-binding chain C
ClpP	<i>clpP</i>		ATP-dependent Clp protease proteolytic subunit
Hsp70	<i>dnaK</i>		Molecular chaperone DnaK
Magnesium transport	<i>mgtC</i>		Mg ²⁺ transport P-type ATPase C MgtC
Isocitrate lyase	<i>icl</i>		Isocitrate lyase Icl

Notes: “—” indicates that VFGs absent in the genome of *C. kersstersii* 121606, but present in our another two *C. kersstersii* strains (12322-1, 202149); keeping “—” in table is convenient for later comparative analysis with *C. kersstersii* 12322-1 and 202149.

注: “—”表示 VFG 不存在于 *C. kersstersii* 121606 的基因组中,但存在于另外两个 *C. kersstersii* 菌株(12322-1, 2002149)中;在表中保留“—”便于以后与 *C. kersstersii* 12322-1 和 202149 进行比较分析。

Several conclusions can be drawn from table 1:

- 1) *C. kersstersii* possesses multiple iron acquisition systems vital for the bacterial survival and virulence.
- (a) The yersiniabactin siderophore system sequesters irons from transferrin and lactoferrin and delivers them to the cytosol and is an essential virulence factor; the yersiniabactin siderophore system also acts as chelator of Cu²⁺, Zn²⁺, Ni²⁺ and plays the corresponding important role in bacterial survival in enviroment and virulence in human;
- (b) Haemophilus Iron Transport system functions in assimilation of iron from human transferrin;
- (c) FbpABCtransporter functions in the periplasm-to-cytosol transport of iron;
- (d) Molecular chaperones are involved in iron uptake;
- (e) Hemolysins lyse erythrocyte cells and release heme into blood and act as vital virulence fac-

- tors;
- (f) Heme uptake pathways;
 - (g) Heme biosynthesis pathway.
- 2) *C. kerstersii* possesses many virulence factors crucial for their growth, survival and pathogenicity in gastrointestinal tract.
- (a) Type IV pili are essential for bacterial colonization and pathogenicity in gastrointestinal tract;
 - (b) Flagella are vital for bacterial pathogenicity to gastrointestinal tract; flagella are involved in the motility and chemotaxis functions in intestinal colonization and pathogenicity in bacterial gastroenteritis; flagellar structure, assembly, biosynthesis regulation, gene clustering; constituents and the signal pathway of flagellar chemotaxis;
 - (c) The virulence factors urease, SodB, DnaK, Elongation factor Tu vital for bacterial growth, survival and pathogenicity in the gastrointestinal tract.
- 3) The virulence factors Mip, ClpP, ClpC, DnaK, GroEL, MgtC and Icl are vital for intracellular survival and infectivity of *C. kerstersii* to lung.

Tab. 2 VFGs in genome of *C. kerstersii* 121606 (not discussed)

表 2 *C. kerstersii* 121606 基因组中的 VFGs (未讨论)

Classification of Virulence Factors	VFGs	121606	Virulence Factors
Acyllhomoserine lactone synthase	<i>hdtS</i>		<i>N</i> -acyllhomoserine lactone synthase
Alcaligin	<i>alcE</i>		Putative iron-sulfur protein
Alginate	<i>algU</i> (<i>algZ/fimS</i>)		Alginate biosynthesis protein AlgZ/FimS
Alginate biosynthesis	<i>algC</i>		Phosphomannomutase AlgC
Alginate regulation	<i>algP/algR3</i>		Alginate regulatory protein AlgP/AlgR3
Alginate regulation	<i>algW</i>		2-alkenal reductase
Alginate regulation	<i>algW</i>		Trypsin domain protein
Alginate regulation	<i>mucD</i>		S1C protease Do subfamily peptidase MucD
Alginate regulation	<i>mucP</i>		Protease MucP
Capsule	<i>rmlA</i>		Putative Glucose-1-phosphate thymidyltransferase
Capsular polysaccharide	<i>rmlB</i>		dTDP-glucose 4,6-dehydratase
Capsular polysaccharide	<i>rmlC</i>		dTDP-6-deoxy- <i>D</i> -xylo-4-hexulose-3,5-epimerase
Capsular polysaccharide	<i>wbfV/wcvB</i>	—	Predicted UDP-glucose 6-dehydrogenase
Capsular polysaccharide	<i>wbjD/wecB</i>		UDP- <i>N</i> -acetylglucosamine 2-epimerase
Capsular polysaccharide	<i>wecC</i>		UDP- <i>N</i> -acetyl- <i>D</i> -mannosaminuronate dehydrogenase
Polysaccharide capsule	<i>BC5275</i>		UTP-glucose-1-phosphate uridylyltransferase
Polysaccharide capsule	<i>gtaB</i>	—	UTP-glucose-1-phosphate uridylyltransferase
Capsule	<i>lipA</i>		Capsule polysaccharide modification protein
Capsule	<i>oppF</i>		Oligopeptide ABC transporter, permease component
Capsule	<i>uppS</i>		Undecaprenyl diphosphate synthase
Capsule biosynthesis and transport	<i>kpsF</i>		Arabinose-5-phosphate isomerase
Capsule	<i>M3Q_296</i>	—	Hypothetical protein
Capsule	<i>ABK1_0097</i>	—	Transposase
K1 capsule	<i>kpsD</i>		KpsD
K1 capsule	<i>kpsT</i>		KpsT
CupA fimbriae	<i>cupA1</i>		Fimbrial subunit CupA1
CupA fimbriae	<i>cupA3</i>		Usher CupA3
CupD fimbriae	<i>cupD2</i>		Putative pili assembly chaperone CupD2

Continue table
续表

Classification of Virulence Factors	VFGs	121606	Virulence Factors
CupD fimbriae	<i>cupD4</i>	—	Fimbrial subunit CupD4
CupD fimbriae	<i>cupD5</i>		Putative pili assembly chaperone CupD5
Type 2 and 3 fimbriae	<i>fimX</i>	—	Fimbrial protein
LOS	<i>galE</i>		UDP-glucose 4-epimerase
LOS	<i>kdsB</i>		3-deoxy- <i>D</i> -manno-octulosonate cytidyltransferase
LOS synthesis	<i>kdtA/waaA</i>		3-deoxy- <i>D</i> -manno-octulosonic-acid transferase
LOS	<i>lpxA</i>		UDP- <i>N</i> -acetylglucosamine acyltransferase
LOS	<i>lpxB</i>	—	Lipid- <i>A</i> -disaccharide synthase
LOS	<i>lpxC</i>		UDP-3- <i>O</i> -acyl <i>N</i> -acetylglucosamine deacetylase
LOS	<i>orfM</i>		Deoxyribonucleotide triphosphate pyrophosphatase
LPS	<i>acpXL</i>		Aacyl carrier protein
LPS	<i>fabZ</i>		3R-hydroxymyristoyl ACP dehydratase
LPS	<i>gtrB</i>		Bactoprenol glucosyl transferase
LPS	<i>hisF</i>		Imidazole glycerol phosphate synthase subunit HisF
LPS O-antigen (P ₁ aeruginosa)	<i>hisH2</i>		Glutamine amidotransferase
LPS	<i>kdsA</i>		2-dehydro-3-deoxyphosphooctonate aldolase
LPS	<i>lpxD</i>		LpxD
LPS	<i>kdtB</i>		Lipopolysaccharide core biosynthesis protein
Exopolysaccharide	<i>mrsA/glmM</i>		Predicted phosphomannomutase
Exopolysaccharide	<i>pgi</i>		Glucose-6-phosphate isomerase
Catalase-peroxidase	<i>katG</i>		Catalase-peroxidase-peroxynitritase T KatG
CcmC	<i>ccmC</i>		Cytochrome C-type biogenesis protein CcmC, putative lyase for CcmE
CdpA	<i>cdpA</i>	—	Cyclic di-GMP phosphodiesterase
Copper exporter	<i>ctpV (zntA)</i>		Cation transport ATPase, ZntA
Copper exporter	<i>ctpV</i>		Probable metal cation transporter P-type ATPase CtpV
Cpi-1a + Cpi-1 (SPI-1 like)	<i>armR</i>		Two-component response regulator
Cya	<i>cyaB</i>		Cyclolysin secretion ATP-binding protein
Cytochrome c maturation (ccm) locus	<i>ccmB</i>		ABC transporter involved in cytochrome c biogenesis, CcmB subunit
Cytochrome c maturation (ccm) locus	<i>ccmF</i>		Cytochrome C-type biogenesis protein CcmF
EVP (E ₁ tarda virulent protein)	<i>evpH</i>		Type VI secretion system protein EvpH
FadE29	<i>fadE29</i>		Acyl-CoA dehydrogenase domain-containing protein
Fibronectin-binding protein	<i>Rv1837c</i>		Malate synthase G
Fibronectin-binding protein	SSU98_1513		Phosphopyruvate hydratase
Fibronectin-binding protein	<i>oprF</i>		Outer membrane protein OprF
Glutamine synthesis	<i>glnA1</i>		Glutamine synthetase, type I
Hcp secretion island-1 encoded type VI secretion system (H-T6SS)	<i>PFLU6009</i>		Putative ABC transport system, ATP-binding protein
Histone-like protein (Hlp)/laminin-binding protein (LBP)	<i>ML1683</i>		Histone-like protein
IlpA	<i>IlpA</i>		Immunogenic lipoprotein A
LetA/LetS two component	<i>letA</i>		Legionella transmission activator LetA

Continue table			
续表			
Classification of Virulence Factors	VFGs	121606	Virulence Factors
Leucine synthesis	<i>leuD</i>		3-isopropylmalate dehydratase, small subunit
Mg ²⁺ transport	<i>mgtB</i>		Hypothetical protein MgtB
Magnesium transport	<i>mgtC</i>		Mg ²⁺ transport P-type ATPase C MgtC
MprA/B	<i>mprA</i>		Response regulator MprA
MtrCDE	<i>mtrC</i>		Membrane fusion protein MtrC
MtrCDE	<i>mtrD</i>		Drug efflux protein MtrD
MtrCDE	<i>mtrE</i>		Outer-membrane lipoprotein MtrE
MtrCDE	<i>mtrE</i>	—	Multiple transferable resistance system protein MtrE
Mycobactin	<i>mbtI</i>		Anthranilate synthase component I
Mycolic acid trans-cyclopropane synthetase	<i>cmaA2</i>		Cyclopropane-fatty-acyl-phospholipid synthase
MymA operon	<i>adhD</i>	—	Putative zinc-type alcohol dehydrogenase AdhD aldehyde reductase
MymA operon	<i>fadD13</i>		Fatty-acid-CoA ligase
Nucleoside diphosphate kinase	<i>ndk</i>		Nucleoside diphosphate kinase
O-antigen	<i>ddhD</i>		CDP-6-deoxy-delta-3,4-glucoseen reductase
O-antigen	<i>YPA_2067</i>		Histidine transport system permease protein HisQ
Pantothenate synthesis	<i>panC</i>		Pantoate-beta-alanine ligase
Pantothenate synthesis	<i>panD</i>		Aspartate 1-decarboxylase precursor panD
PbpG	<i>pbpG</i>		PbpG
PhoP/R	<i>phoP</i>		Two component transcriptional regulator, winged helix family
PhoPQ	<i>phoP</i>		Transcriptional regulatory protein PhoP, regulator of virulence determinants
Proline synthesis	<i>proC</i>		Pyrroline-5-carboxylate reductase
Purine synthesis	<i>purC</i>		Phosphoribosylaminoimidazole-succinocarboxamide synthase PurC
Pyoverdine	<i>pvdJ</i>		Thermophilic carboxylesterase Est2
Pyoverdine	<i>PA2383</i>		Transcriptional regulator
Recombinational repair protein	<i>recN</i>		DNA repair protein RecN
RegX3	<i>regX3</i>		Sensory transduction protein RegX3
RelA	<i>relA</i>		GTP pyrophosphokinase pppGpp synthetase I stringent stress response RelA
RicA	<i>ricA</i>		Rab2 interacting conserved protein A
SP41/UgpB	<i>ugpB</i>		Glycerol-3-phosphate-binding periplasmic protein precursor
Streptococcal plasmin receptor/GAPDH	<i>plr/gapA</i>		Glyceraldehyde-3-phosphate dehydrogenase
T2SS	<i>exeE</i>		Type II secretory pathway, ATPase Pule-like protein
T3SS	<i>ABB77417</i>		MCP-like protein
T3SS-Bsa	<i>bprA</i>		HNS-like regulatory protein BprA
T4SS effectors	<i>CbuG_0446</i>		Hypothetical protein
T4SS effectors	<i>CbuK_1823</i>		GatB/Yqey domain protein
T4SS effectors	<i>coxDFB4</i>	—	17 u common-antigen
T4SS effectors	<i>coxH3</i>		Alpha/beta hydrolase
Thioquinolobactin	<i>qbsC</i>		QbsC

Continue table
续表

Classification of Virulence Factors	VFGs	121606	Virulence Factors
Thioquinolobactin	<i>qbsH</i>		QbsH
Ton system	<i>exbB</i>		Biopolymer transport protein ExbB
Ton system	<i>exbD</i>		Biopolymer transport protein ExbD
Trehalose-recycling ABC transporter	<i>sugC</i>		ABC transporter-like protein SugC
Trehalose-recycling ABC transporter	<i>sugC</i>		Putative sugar-transport ATP-binding protein ABC transporter SugC
Trehalose-recycling ABC transporter	<i>sugC</i>		Carbohydrate ABC transporter ATP-binding protein, CUT1 family
Tryptophan synthesis	<i>trpD</i>		Anthranilate phosphoribosyltransferase
Two-component system	<i>bfmR</i>		Putative response regulator activator in two-component regulatory system OmpR family
Vulnibactin	VVA1298		Phospho-2-dehydro-3-deoxyheptonate aldolase

Notes: “—” indicates that VFGs absent in the genome of *C. kerstersii* 121606, but present in our another two *C. kerstersii* strains (12322-1, 202149); keeping “—” in table is convenient for later comparative analysis with *C. kerstersii* 12322-1 and 202149.

注: “—”表示VFG不存在于*C. kerstersii* 121606的基因组中,但存在于另外两个*C. kerstersii*菌株(12322-1, 2002149)中;在表中保留“-”便于以后与*C. kerstersii* 12322-1和202149进行比较分析。

3 Discussion

As described above, *C. kerstersii* mainly causes intra-abdominal infections, and the gastrointestinal tract is its natural habitat for survival and virulence. Since we also isolated a *C. kerstersii* strain (12322-1) from the sputum of our patient, it is possible that the respiratory tract is also a habitat for its survival and virulence. The correlation of VFGs with bacterial survival, growth in humans, and infectivity of *C. kerstersii* in the gastrointestinal tract and lungs was exemplified by analyzing the following VFGs.

3.1 *C. kerstersii* Possesses Multiple Iron Acquisition Systems Vital for Bacterial Survival and Virulence

3.1.1 Iron Sources in Host are Vital for Bacterial Survival

Iron is essential for almost all organisms^[3-4]. In the human body, iron exists as ferric (Fe³⁺) and ferrous (Fe²⁺) ions, as well as iron-heme coordinated with protoporphyrin IX^[5]. The concentrations of free iron (mostly Fe²⁺) and insoluble iron (Fe³⁺, with extremely low solubility of 10⁻¹⁸ mol · L⁻¹) are negligible^[5-6]. Heme-iron (Fe²⁺) incorporated into hemoglobin (Hb) in red blood cells is the main form of circulating iron, accounting for approximately 80% of all human iron^[5]. The main circulating non-heme iron in the human bloodstream includes transferrin (Fe³⁺) and lactoferrin (Fe³⁺)^[5]. *C. kerstersii* has several iron acquisition mechanisms, which may confer higher survival ability and pathogenicity^[7].

3.1.2 Yersiniabactin Siderophore System Sequesters Irons From Transferrin and Lactoferrin and Delivers Them to Cytosol and is an Essential Virulence Factor

The Yersiniabactin siderophore is made up of one salicylate, one thiazoline, and two thiazolidine rings^[4]. It has an extremely high affinity for ferric (Fe³⁺) ions ($K_a=4\times10^{-36}$ L · mol⁻¹)^[8]. Yersiniabactin efficiently removes Fe³⁺ ions from transferrin and lactoferrin^[4], then binds to the TonB-dependent outer membrane receptor-Psn in *Yersinia pestis*^[4] and FyuA in *Y. enterocolitica*^[9] to pass through the outer membrane into the periplasm^[4]. It subsequently delivers Fe³⁺ ions to the cytosol via the inner membrane ABC transporter, which is composed of two inner membrane fused-function permease/ATP-binding proteins, YbtP and YbtQ^[4], followed by the reduction of Fe³⁺ to Fe²⁺.

The yersiniabactin siderophore promotes the growth of *Y. enterocolitica* and *Escherichia coli* under iron-restricted conditions^[9]. It is also a virulence factor for other highly pathogenic bacteria, such as *Y. pseudotuberculosis*, *Y. enterocolitica* biotype IB, and extraintestinal pathogenic *E. coli* (ExPEC)^[10]. Additionally, the yersiniabactin siderophore-based iron transport system is essential for the pathogenesis of bubonic and pneumonic plague^[4, 11], which is a flea-borne zoonosis caused by *Y. pestis*^[12].

Yersiniabactin is synthesized through a nonribosomal peptide/polyketide mechanism involving several proteins, including high-molecular-weight protein 1 (HMWP1), HMWP2, YbtD, YbtT, YbtE, YbtS, and YbtU. Its secretion requires an inner membrane exporter encoded by a four-gene operon (ybtPQXS)^[4]. Most of the genes required for yersiniabactin biosynthesis (*ybtT*, *ybtE*, *ybtS*), transport (*fyuA*, *ybtP*, *ybtQ*) and the regulation (*ybtA*) are located within a *Yersinia* high-pathogenicity island (HPI)^[4, 13]. This island is also crucial for the virulence of uropathogenic *E. coli* (UPEC), the main causative agents of non-nosocomial urinary tract infections, particularly in the highly inflammatory infection environment of high-titer mouse cystitis^[14].

C. kerstersii possesses the yersiniabactin siderophore system, as it contains genes encoding for the yersiniabactin siderophore system: (a) *fyuA*; (b) *ybtQ/ybtP*, which are overlapping genes, with each encoding half of an inner membrane ABC transporter containing an amino-terminal membrane-spanning permease domain and a carboxy-terminal ATPases domain^[15]; and (c) *irp2*, which encodes the putative peptide synthetase-HMWP2 for the siderophore yersiniabactin^[16]. Laboratory verification of the presence of these yersiniabactin biosynthesis genes contained in HPI in *C. kerstersii* genome would help clarify its pathogenicity to patients.

3.1.3 Haemophilus Iron Transport System Functions in Assimilation of Iron From Human Transferrin

C. kerstersii may be able to utilize another classic high-affinity, TonB-independent iron acquisition system-called Hit (Haemophilus Iron Transport) ABC. This system is composed of a periplasmic iron-binding protein (HitA), a cytoplasmic permease (HitB), and a nucleotide-binding protein (HitC) found in *Haemophilus influenza*^[17]. These components could potentially allow *C. kerstersii* to assimilate iron from human transferrin, as *hitA* and *hitC* were predicted in the *C. kerstersii* 121606 genome.

3.1.4 FbpABC Transporter Functions in Periplasm-to-Cytosol Transport of Iron

Together with hitABC, the prediction of *fbpC* in the *C. kerstersii* 121606 genome suggests that it has an additional periplasmic protein-dependent iron ABC transporter, FbpABC. This transporter consists of the ferric binding protein (FbpA), a homologue of HitA, FbpB, a cytoplasmic permease, and FbpC protein, a nucleotide binding protein^[18]. It is required for iron uptake from the host ferric binding proteins^[19].

Additionally, *C. kerstersii* 121606 contains: (a) iron compound ABC transporter permease protein gene, *fatD*; (b) Fe³⁺ ABC transporter ATP-binding protein gene, *vctC*; (c) iron-negatively regulated virulence gene, *irgA*, and d) its negative regulator gene, *fur*^[20]. Fur was reported to sense intracellular iron amount and maintain iron homeostasis in *Bacillus subtilis*^[21]. Fur might also be regulator of *hitA* as it was a regulator of *afuA*, which encodes a homologue of HitA^[22].

3.1.5 Molecular Chaperones are Involved in Iron Uptake

Helicobacter pylori is the major pathogen of gastritis^[23] and requires iron for growth^[24]. Bacterial GroEL, a homologue of human heat shock protein 60 (Hsp60), is a well-characterized molecular chaperone that prevents aggregation and facilitates proper protein folding in bacteria^[25]. It, along with urease (discussed below), are the major surface-exposed proteins of *H. pylori*^[26]. GroEL can

assist in iron acquisition for *H. pylori* through specific binding with iron extracellularly and scavenging iron from heme^[27], as well as in the maturation of metalloenzymes by supporting the insertion of a metal cluster into an apoprotein, such as Molybdenum-iron protein^[28]. GroEL and another bacterial molecular chaperone, DnaK, the main homologue of human Hsp70 in bacteria^[29], were also shown to bind to lactoferrin^[30]. The prediction of *groEL* and *dnaK* in *C. kerstersii* 121606 genome indicated that this bacterium had these iron acquisition mechanisms.

3.1.6 Hemolysins Lyse Erythrocyte Cells and Release Heme Into Blood and Act as Vital Virulence Factors

C. kerstersii 121606 possesses the genes (*hly*Ⅲ, *hlyA*) that encode hemolysins capable of lysing erythrocyte cells, releasing Hb into the blood serum, and converting it into oxidized Hb (metHb), in which ferric heme is loosely bound and can be utilized by bacterial heme uptake systems^[5]. It has been reported that haemolysin Ⅲ (encoded by *hly*Ⅲ) from *B. cereus* can form 3 - 3.5 nm transmembrane pores in diameter on the erythrocyte membrane and lyse them^[31]. HlyA (110 ku, listeriolysin O)^[32] can also lyse erythrocytes^[33] and a wide spectrum of cells, such as granulocytes, monocytes, and endothelial cells^[33]. When produced by the intramacrophage *Listeria monocytogenes*, a food-borne pathogen causing listeriosis with a mortality rate of approximately 20%^[32], HlyA can break the endosome membrane and help this bacterium escape the hostile endosome environment^[34]. HlyA is verified as a unique and essential virulence factor for virulent *L. monocytogenes*^[32] and plays a vital role in *E. coli*-caused extraintestinal diseases^[35].

3.1.7 Heme Uptake Pathways

These findings of the genes *hutC*, *hutD*, *bhuT*, *hmuV* in *C. kerstersii* 121606 indicated that *C. kerstersii* had multiple heme uptake systems.

Gram-negative bacteria are found to have two heme uptake mechanisms^[5] to transport of host heme, heme proteins (Hb, hemopexin) into cytosol, one is through the heme scavenging proteins-hemophores, another is by binding outer membrane receptors, then heme is transported across the bacterial outer membrane via a TonB-dependent outer membrane receptor into the periplasm, then transferred to the periplasmic heme binding protein (PBP), which transmits heme to the inner membrane ABC transporter complex, finally translocated into the cytoplasm^[5, 36-37].

The prediction of the genes, *hutC*, encoding a putative inner membrane permease, and *hutD*, encoding a putative ABC-transporter ATPase, which form the heme/Hb ABC transporter-hutCD^[36-38] in *C. kerstersii* 121606, and *bhuT*, encoding the periplasmic heme-binding protein which functions as HutB^[37], indicated that they might own the TonB dependent heme uptake mechanism^[36], but whether another component-the outer membrane heme receptor encoding gene *hutA* is in *C. kerstersii* strains requires experimental evidence.

The presence of *hmuV* in *C. kerstersii* 121606, which encode a ATPase, suggested the possible presence of another heme ABC transport system-HmuTUV, if the genes encoding other two components-the periplasmic binding protein (*hmuT*), and the permease (*hmuU*) are verified to be present in *C. kerstersii* by experiment^[39].

C. kerstersii may also possess HasA-type hemophore-mediated heme uptake system, a two component of a ABC exporter-HasDEF, which exports a heme acquisition protein, HasA^[40], as *C. kerstersii* 121606 contained the genes *hasD*, encoding ABC transporter ATP-binding protein, and *hasE*, encoding ABC transporter periplasmic substrate-binding protein^[40].

3.1.8 Heme Biosynthesis Pathway

In addition to heme uptake, *C. kerstersii* may also have the ability to biosynthesize heme. The heme biosynthetic pathway starts from GltX-catalyzed conversion of *L*-glutamate to charged glutamyl-

tRNA^{Glu}, which acts as substrate of glutamyl-tRNA reductase (HemA), the latter works sequentially with glutamate-1-semialdehyde aminotransferase (HemL) to synthesize 5-aminolevulinic acid (ALA). Subsequently, ALA is used to synthesize uroporphyrinogen **III** by porphobilinogen synthase (HemB), then heme by uroporphyrinogen decarboxylase (HemE) and protoporphyrin ferrochelatase (HemH)^[41]. Prediction of the genes encoding HemB, HemC (porphobilinogen deaminase), HemE, HemH (ferrochelatase), HemL and HemN (coproporphyrinogen **III** oxidase) in *C. kerstersii* 121606 suggests that it possesses this heme biosynthesis pathway^[42].

3.1.9 Iron Acquisition Proteins are Potential Drug and Vaccine Targets

The iron acquisition proteins play an essential role in the pathogenesis of causative bacteria. They have been identified as potentially novel antibiotic and vaccine targets to combat infections. For example, FyuA^[43], HitA^[44], GroEL and DnaK^[45] are the potential vaccine candidates against bacteria.

3.2 Yersiniabactin Siderophore System Also Acts as Chelator of Cu²⁺, Zn²⁺, Ni²⁺ and Plays Corresponding Important Role in Bacterial Survival in Environment and Virulence in Human

Yersiniabactin is also a chelator of Cu²⁺^[46], Zn²⁺^[16]. Copper serves as both a nutrient and a toxin during bacterial infections. Yersiniabactin efficiently scavenges extracellular Cu²⁺ in the form of a Cu²⁺-Yersiniabactin complex. It then uses the same Fe³⁺-Yersiniabactin transport proteins to facilitate copper import and release in UPEC. This supports copper-dependent enzyme activity in low copper conditions, such as cuproenzymes^[47], and helps UPEC resist copper toxicity in higher copper conditions^[47] by preventing Cu²⁺ reduction to the more bactericidal Cu⁺^[46]. Additionally, it protects UPEC against phagocytic killing by catalyzing superoxide dismutation intraphagocytically with the superoxide dismutase (SOD)-like activity of the Cu²⁺-Yersiniabactin complex^[46].

Zn²⁺ is a necessary micronutrient during bacterial infections. The yersiniabactin siderophore also plays a crucial role in acquiring Zn²⁺ and causing lethal pathogenesis in mouse pneumonic plague^[16]. In addition to Fe³⁺, Cu²⁺, and Zn²⁺, yersiniabactin also has a high affinity for chelating Ni²⁺, Cr³⁺, Co³⁺, and Ga³⁺^[14], as well as Co²⁺ and Pd²⁺^[48]. This promotes the survival of bacteria in heavy metal-polluted environments.

3.3 *C. kerstersii* Possesses Many Virulence Factors Crucial for Their Growth, Survival and Pathogenicity in Gastrointestinal Tract

3.3.1 Type IV Pili are Essential for Bacterial Colonization and Pathogenicity in Gastrointestinal Tract

As proteinaceous hair-like appendages, pili are essential for bacterial adherence to human gut cells^[49]. Type IV pili (T4P), which are approximately 5-8 nm in diameter and several micrometers in length, have three subtypes: Type IVa pili (T4aP), Type IVb pili (T4bP), and Type IVc pili (T4cP)^[50]. They are predicted to be produced by about 30% of the known gut microbiome^[49]. T4P promotes the intestinal colonization of enterohemorrhagic *E. coli* (EHEC) O157:H7, a food-borne pathogen of hemorrhagic colitis^[51], enterotoxigenic *E. coli* (ETEC), the most common bacterial pathogen of diarrhea in developing countries and traveler's diarrhea^[52], and enteropathogenic *E. coli* (EPEC)^[53]. It also promotes the survival, virulence, and transmission of EHEC O157:H7^[51], invasion of epithelial cells, hemagglutination of erythrocytes, biofilm formation, and twitching motility of EHEC O157:H7^[51].

The biogenesis process of Type IV pili in Gram-negative bacteria begins with the attachment of an ATPase-PilB to the cytoplasmic ring formed by PilM and the platform protein PilC^[49]. PilM, along with the subunits PilN, PilO, and PilP, forms an inner membrane alignment complex called PilMNOP. This complex has a cage-like ring in the inner membrane and periplasm, which is formed by conformational changes in PilN and PilO resulting from the attachment of PilB to PilM. Then, the

platform protein PilC incorporates the major pilin subunit PilA after the removal of the leader peptide by the prepilin peptidase PilD. PilD also methylates PilA or minor pilins, such as pilE, into the growing pilus based on the PilMNOP alignment complex. Afterward, PilA is translocated through the outer membrane pore formed by PilQ to elongate the pilus^[49]. The cytoplasmic ATPase PilB drives pilin assembly, while the PilT ATPase drives disassembly or retraction. Both of them can reversibly associate with the PilC protein^[49]. In the final step, the minor pilins are synthesized in a similar way to PilA and create a priming complex for pilin formation at the pilus tip and along the pilus^[49].

The presence of the Type IV pili biogenesis genes encoding PilA, PilB^[54], PilC, PilD, PilE, PilJ {a minor pilin and a methyl-accepting chemotaxis protein (Mcp)-like chemosensor, which directly interacts with the major pilin subunit PilA and regulates surface-induced gene expression and pathogenicity^[54]}, PilG {a polytopic membrane protein dedicated to assist transformation of bacteria by binding DNA and interacting with the N-terminal region of PilQ, which can also bind DNA^[55] and essential for twitching motility^[56]}, PilH {an ABC transporter homologue required for the biogenesis of any bacterial pilus type^[57]}, PilM, PilN, PilQ {a secretin working in concert with PilG to bind DNA during T4P-mediated transformation^[55]}, PilR {the response regulator of a two (PilS and PilR)-component transcriptional regulatory system dedicated to activate transcription of pilA^[58]}, PilT, PilT2, PilU {also a retraction ATPase motor^[54]}, PilZ {which can stimulate T4P formation or regulate T4P-dependent motility^[59]} in *C. kerstersii* 121606 indicated that *C. kerstersii* possessed the virulence factor T4aP possibly involved in the pathogenicity of *C. kerstersii* in intestinal diseases^[49], such as peritonitis, appendicitis, as mentioned above.

3.3.2 Flagella are Vital for Bacterial Pathogenicity to Gastrointestinal Tract

3.3.2.1 Flagella are Involved in Motility and Chemotaxis Functions in Intestinal Colonization and Pathogenicity in Bacterial Gastroenteritis

Flagella are approximately 20 nm in diameter^[60]. The possession of rapid, darting motility in viscous milieus conferred by the polar flagella is an exclusive characteristic of virulent *Campylobacter jejuni* and *E. coli*^[61], the major pathogen of acute human bacterial gastroenteritis worldwide^[62-63], and are required for intestinal mucus colonization^[61], a key pathogenicity determinant in intestinal infection by *C. jejuni*^[64-65] and *H. pylori*^[66]. Additionally, they function in secreting virulence proteins, microcolony formation, biofilm formation and escape of the innate immune response^[61]. Chemotaxis conferred by polar flagella is also critical for colonization and pathogenicity in the establishment of gastrointestinal infections by *C. jejuni*^[61-62] and *H. pylori*^[66].

3.3.2.2 Flagellar Structure, Assembly, Biosynthesis Regulation, Gene clustering

Flagella are composed of approximately 20 000-30 000 protein subunits, consisting of over 20 different types of proteins^[67]. They comprise: (a) the base located in the cytoplasm and inner membrane, which is composed of the flagellar type III secretion system (T3SS), a membrane-embedded export gate composed of six transmembrane proteins-FlhA, FlhB, FliO, FliP, FliQ, and FliR within the central membrane patch of the ring, the water-soluble ATPase ring complex composed of three cytoplasmic proteins, FliH, FliI, and FliJ^[68-71], the inner membrane MS ring (a homomultimer of flagellar motor switch protein FliF)^[68, 72], the cytoplasmic C ring or switch complex {which is formed by two motor/switch proteins FliM and FliN^[68, 73] and functions as the flagellar switch and also aids in secretion^[66]}, and the motor {formed by multimers of the motor/switch protein FliG^[68, 72]} attached to MS ring^[68]; (b) the coaxial periplasmic rod {a helical cylinder containing four flagellin proteins-FliE, FlgB, FlgC, and FlgF in the proximal part and FlgG in the distal part^[66-67]} and the associated ring structures; (c) the surface-localized hook structure (a helical assembly of about 120 FlgE subunits) spanning the periplasm and outer membrane; and (d) the extracellular filament {composed pri-

marily by the major flagellin, FlaA, with the minor flagellin, FlaB^[61, 66] or FliC^[67]. The rod is surrounded by: (a) three disk structures, consisting of the basal (composed of FlgP), the medial paralyzed flagellum protein A (PflA), and the proximal disk (composed of PflB); (b) the motor components of the base including MotA and MotB (encoded by *motA*, *motB*), which forms a proton channel^[74]; (c) the stators of flagellum in periplasm that interact with FliG and function to link proton flow and generation of flagellar rotation torque^[66, 68, 72]; (d) the periplasmic P ring (FlgI) in peptidoglycan; and (e) the L ring (FlgH) in outer membrane^[66, 68].

Flagellar assembly begins at the base, then progresses to the hook, and finally to the filament^[70, 75]. The peptidoglycan-hydrolyzing activity of the FlgJ protein (peptidoglycan hydrolase) is necessary for flagellar rod formation^[76]. To assemble the hook and filament from the cell surface, their component proteins are transported from the cytoplasm to the distal end of the growing structure by T3SS^[66]. Chaperones FlgN, FliS, and FliT assist in the transfer of flagellin subunits to T3SS from the cytoplasm^[69]. The initial entry of the flagellin subunits into the narrow pore of the export gate is facilitated by the ATPase ring complex and powered by proton motive force, which is controlled via the specific interaction of FlhA with FliJ^[69]. The scaffolding protein FlgD is also essential for flagellar hook assembly in *Salmonella typhimurium*^[77]. Its export requires FliE, a flagellar basal body protein, and an adaptor protein located between the MS ring and rod substructures^[68, 78]. The flagellar number is controlled by the antiactivator FleN through binding to the enhancer of the flagellar gene, *fleQ*, a multidomain sigma-54 (σ^{54}) factor^[79].

Flagellar biogenesis is tightly controlled by the activities of σ^{54} and σ^{28} ^[66]. For instance, the two-component signal transduction system, FlgSR, is composed of the FlgS sensor kinase and the FlgR response regulator. This system recognizes a regulatory checkpoint in the MS ring and rotor to activate the σ^{54} -dependent expression of flagellar rod and hook genes, and ultimately the σ^{28} -dependent flagellins and *fed* gene expression^[72].

In the genome, a number of flagellar genes are structurally organized as operons, with a master and several secondary levels of gene expression regulation^[80]. Examples include the *flgGHIJKL* operon^[81], the *flgBCDEF* operon, whose genes are transcribed as a single mRNA in a σ^{54} -dependent way in *Rhodobacter sphaeroides*^[82], the *flhBAE* operon in *Y. enterocolitica*^[83], the *flmA(flaA)B* operon, and the *flmGH* operon in *Caulobacter crescentus*^[84]. Another example of a master regulator in flagellum biogenesis is the *flhD* operon, which encodes two genes, *flhD* and *flhC* in *E. coli*. This operon produces the FlhD/FlhC complex, which is a transcriptional activator required for the transcription of the three class II operons-*fliA*, *flhB*, and *fliL*^[85]. Additionally, as a subunit of RNA polymerase and a homologue of σ^{28} , the FliA protein activates flagellin synthesis by transcribing the *fliC* gene^[86].

The prediction of the flagellar components and biogenesis genes in *C. kerstersii* 121606 indicates that *C. kerstersii* possesses flagella with intact motility function.

3.3.2.3 Constituents and Signal Pathway of Flagellar Chemotaxis

The signal transduction proteins of chemotaxis include the core components. These consist of chemoreceptors, which sense environmental stimuli and transduce this signal to mediate rotation of flagella. The two-component system is composed of the histidine kinase CheA and its cognate response regulator CheY^[87]. The phosphorylated form of CheY, regulated by ligand binding to the chemoreceptors, interacts with the FliM component of the flagellar motor switch to cause clockwise motor rotation^[88]. The CheW coupling protein physically conjugates chemoreceptors to CheA^[66]. Other components include the phosphatases CheZ and FliY, which promote CheY dephosphorylation, the chemoreceptor modification enzymes CheR (a methyltransferase that transfers methyl groups from S-adenosine

sylmethionine to glutamate residues on the cytoplasmic domains of the chemoreceptors Mcps), and CheB (a methylesterase), that governs adaptation responses to sustained ligand levels by methylating or demethylating glutamyl residues of chemoreceptor respectively^[66]. The chemoreceptor glutamine deamidase CheD deamidates the Mcps essential for chemoreceptors to effectively transduce signals to the CheA kinase in *B. subtilis*^[87] and increases the receptor kinase activity or enhances CheC phosphatase activity to regulate the CheY levels in *B. subtilis*^[89]. Some bacteria have multiple homologues of bacterial chemotaxis and chemosensing genes, such as *tsr*, *tar/cheM*, and have a great ability to respond to a wide variety of compounds, like *Chromobacterium violaceum*^[74], an extremely virulent opportunistic pathogen ubiquitously distributed in water and soil^[90].

The prediction of *cheA*, *cheB*, *cheD*, *cheR*, *cheW*, *cheY*, *cheY1*, *tsr* and *tar/cheM* in our *C. kerstersii* 121606 suggested that their flagella have full chemotaxis function.

As a whole, the prediction of almost all of the flagellar components, biogenesis, motility, and chemotaxis genes in *C. kerstersi* 121606 indicates that they have fully functional flagella, which contributes to their high pathogenicity in the gastrointestinal tract.

3.3.3 Virulence Factors Urease, SodB, DnaK, Elongation Factor Tu Vital for Bacterial Growth, Survival and Pathogenicity in Gastrointestinal Tract

Survival, colonization and pathogenicity of *H. pylori* in gastric acidic environment depends on the abundantly produced nickel-dependent urease, which hydrolyses urea into ammonia to create a pH neutral microenvironment^[26] and block the phagosome-lysosome fusion to resist against the host immune system^[91]. Most bacterial ureases are made of a ($\alpha\beta\gamma$)₃ (encoded by *ureA*, *ureB*, *ureC*) trimer^[92]. Urease also requires different sets of accessory proteins, such as UreE, UreF, UreG, UreH and UreI^[93] or UreD, UreE, UreF and UreG^[94-95], to coordinate nickel ions into the UreABC subunits. Considering that the urease encoding genes predicted through VFDB database might be not complete, we manually searched the annotated genome of *C. kerstersii* 121606, and found that it contained the genes encoding the subunit α , β , γ , and the accessory proteins UreD, UreE, UreF, UreG, meaning that *C. kerstersii* could express functional urease.

The survival, colonization, and pathogenicity of *H. pylori* in the gastric acidic environment depend on the abundantly produced nickel-dependent urease. This enzyme hydrolyzes urea into ammonia to create a pH-neutral microenvironment^[26] and blocks the phagosome-lysosome fusion to resist the host immune system^[91]. Most bacterial ureases are composed of a ($\alpha\beta\gamma$)₃ trimer (encoded by *ureA*, *ureB*, *ureC*)^[92]. Urease also requires different sets of accessory proteins, such as UreE, UreF, UreG, UreH, and UreI^[93], or UreD, UreE, UreF, and UreG^[94-95], to coordinate nickel ions into the UreABC subunits. Considering that the urease-encoding genes predicted through the VFDB database might not be complete, we manually searched the annotated genome of *C. kerstersii* 121606 and found that it contained the genes encoding the subunits α , β , γ , and the accessory proteins UreD, UreE, UreF, UreG, indicating that *C. kerstersii* could express functional urease.

SodB, an iron-cofactored superoxide dismutase, is necessary for *H. pylori* to combat the oxidative stress produced by neutrophils and macrophages^[96] while colonizing the host gastric mucosa^[97].

DnaK is found on the cell surface of several pathogens, such as human probiotic intestinal microbiota *Lactobacillus salivarius*, and *Bifidobacterium animalis* subsp. *lactis*^[98]. It can be upregulated by bile salts in the gastrointestinal tract of mammals to enhance the adaptation and colonization of *B. animalis* subsp. *lactis* in the gut bile environment by acting as a high affinity receptor of host plasminogen^[98].

The elongation factor thermal unstable Tu (EF-Tu, encoded by *tuf*) is a translational GTPase (G protein), that delivers aminoacyl-tRNAs (aa-tRNAs) to the A-site of the ribosome^[99] and has ad-

ditional adhesive moonlighting functions^[100]. EF-Tu on bacterial cell surfaces can bind to membrane receptors and fibronectin, a key component of the extracellular matrix (ECM) proteins on the surface of host cells^[100]. It can also bind to human intestinal cells and mucins, indicating the important role of EF-Tu in gut colonization for the probiotic bacterium, *L. johnsonii*^[101]. The secreted EF-Tu from *H. pylori* also promotes adherence and invasion to host cells during pathogenesis^[102].

The above analyses collectively indicate the potential of survival and pathogenicity of *C. kerstersii* in the gastrointestinal tract. These data help to explain the fact that nearly all reported *C. kerstersii* infections are gut diseases.

3.4 Virulence Factors Mip, ClpP, ClpC, DnaK, GroEL, MgtC and Icl are Vital for Intracellular Survival and Infectivity of *C. kerstersii* to Lung

Legionella pneumophila is a Gram-negative, facultative intracellular bacterium^[103] and the causative agent of Legionnaires' disease, which presents as pneumonia^[104]. It is able to survive and multiply in lung macrophages^[103] and Type I and II pneumocytes^[105]. The Legionella Mip (macrophage infectivity potentiator) protein, a homodimeric lipoprotein of 24 ku located on the bacterial surface and on host membranes^[106-107], is necessary for the early intracellular infection step of *L. pneumophila* to alveolar macrophages^[108] and the human phagocytic cell line U937^[109], intracellular multiplication^[110], and the full virulence of *L. pneumophila* in the lungs of guinea pigs and the spread to the spleen after intratracheal inoculation^[108]. It also facilitates *L. pneumophila* to pass through the collagen IV-containing ECMs or lung epithelial cell lines, and disseminate within the lung tissue and spread to the spleen^[111].

ClpP is a 21.6 ku serine-type protease in *E. coli* that is highly conserved in prokaryotes and eukaryotes^[112]. It plays a similar role in bacterial pathogenicity in the lungs. ClpP is necessary for the colonization of *Streptococcus pneumoniae* in the nasopharynx, survival in the lungs of mice after intranasal challenge, and intracellular survival in murine macrophages^[113]. ClpP must bind with the Hsp100/Clp family of molecular chaperones, ClpA, or ClpX, or ClpC, to form the Clp protease ClpAP, ClpXP, or ClpCP complex in order to degrade aggregated protein^[112]. Therefore, it is understandable that ClpC ATPase is also found to promote the intracellular survival of the facultative intracellular pathogen *L. monocytogenes* in macrophages and survival in host tissues^[114].

Similar to the ClpC ATPase, two other chaperones, DnaK and GroEL, play important roles in the survival and pathogenicity of bacteria. The stress-induced DnaK homologue-Hsp70 is involved in protein folding, proteostasis control^[29], and removal of aggregated proteins^[115]. It has been verified to be essential for intramacrophage survival of the intracellular pathogen *S. enterica* serovar Typhimurium, invasion of epithelial cells, leading to systemic infection^[116], and intramacrophagic replication of *Brucella suis*^[117].

MgtC is also a vital virulence factor for several intracellular pathogens, such as *Burkholderia cenocepacia*^[118], *M. abscessus*^[119], both of them being important respiratory tract pathogens in patients with cystic fibrosis, *Mycobacterium tuberculosis*^[120], *S. enterica* serovar Typhimurium^[121], and *Bordetella pertussis*, the causative agent of whooping cough^[122]. It also promotes bacterial survival of *B. cenocepacia*^[118], *M. abscessus*^[119], and *M. tuberculosis*^[120] within macrophages through postponing the phagolysosomal fusion of bacteria^[118]. These functions of *mgtC* are also observed in *P. aeruginosa*, a highly lethal extracellular pathogen in cystic fibrosis patients with persistent pulmonary infections^[123], during the intramacrophage phase or when there is a limitation of Mg²⁺, impairing host phagocytic functions^[124].

Isocitrate lyase (Icl), an enzyme indispensable for the metabolism of fatty acids, has been to promote the persistence of *M. tuberculosis* in activated macrophages and its virulence in mice^[125]. It is al-

so indispensable for the pathogenesis of *Pseudomonas aeruginosa* in the rat lung infection model^[126]. These predicted VFGs in *C. kerstersii* 121606 indicate that *C. kerstersii* might be an intracellular pathogen, especially for lung macrophages and Type I and II pneumocytes. This helps to explain the infectivity of *C. kerstersii* in our patient's lung.

In summary, these exemplified VFGs helped us understand the ability of intracellular survival, growth in humans, and the infectivity of *C. kerstersii* in the gastrointestinal tract and lungs of the reported cases and our patients. Some of them might act as novel drug and vaccine targets.

4 Conclusion

C. kerstersii possessed a wide range of VFGs to demonstrate its status as an intracellular pathogen and to elucidate its pathogenicity in the reported gastrointestinal tract infection and a case of lung infection encountered in our clinical work. These findings also offer potential targets for antibiotic innovation and vaccine development to address the rising incidence of *C. kerstersii* infections.

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