

Article

Comparative Genomics of a Hemolymph-Derived *Pseudomonas boreofloridensis* Strain RAC1 Reveals Metabolic Versatility and Multidrug Resistance

Rittick Mondal ¹, Atanu Manna ², Pankaj Mandal ³, Amresh Kumar Sharma ⁴, Halil Kurt ^{5,*}
and Amit Kumar Mandal ^{3,*}

¹ Department of Microbiology, Kalinga University, Raipur 492101, Chhattisgarh, India; rittickm98@gmail.com or rittick.mondal@kalingauniversity.ac.in

² Mahatma Gandhi Medical Advanced Research Institute (MGMAIRI), Sri Balaji Vidyapeeth (Deemed to Be University), Puducherry 607402, Puducherry, India; atanuaaa@gmail.com

³ Department of Sericulture, Raiganj University, Raiganj 733134, West Bengal, India; mandalpankaj9749@gmail.com

⁴ Department of Bioinformatics, Kalinga University, Raipur 492101, Chhattisgarh, India; amresharma1@gmail.com

⁵ Department of Medical Biology, Hamidiye International School of Medicine, University of Health Sciences Turkey, Uskudar, Istanbul 34668, Turkey

* Correspondence: halil.kurt@sbu.edu.tr (H.K.); amitmandal08@gmail.com (A.K.M.)

Abstract

Flacherie is a major bacterial disease compromising silkworm health and cocoon productivity; however, the diversity and genomic features of larva-associated pathogens remain poorly characterized. In the present study, we isolated and characterized a Gram-negative bacterium, designated as *Pseudomonas boreofloridensis* strain RAC1, from the hemolymph of silkworm larvae with flacherie symptoms. The isolate exhibited clear swimming motility, indicating an active flagellar system. Biochemical profiling using the VITEK-2 platform revealed broad metabolic capabilities, including utilization of multiple organic acids and amino-acids, related enzymatic activities, highlighting its adaptability under nutrient-variable host conditions. Chemotaxonomic analysis using fatty acid methyl ester (FAME) profiling further supported its identity within the genus *Pseudomonas*. Whole-genome sequencing showed a 4.49 Mb GC-rich genome encoding diverse functional pathways related to central metabolism, aromatic compound degradation, and carbohydrate-active enzymes, suggesting strong ecological flexibility. Importantly, genomic screening identified virulence-associated gene and multiple antimicrobial resistance determinants, dominated by efflux pumps and outer membrane permeability factors. In addition, strain RAC1 contained several mobile genetic elements and three prophage regions, reflecting a highly plastic genome. Pangenome analysis across related *Pseudomonas* strains indicated an open pangenome, driven largely by accessory and cloud genes. Overall, *P. boreofloridensis* RAC1 represents a multidrug-resistant and genomically dynamic strain encoding virulence-associated genes and resistance genes. These genomic features suggest adaptive potential in host-associated environments and require further experimental investigation to evaluate its role in silkworm larval disease.

Keywords: comparative genomics; antibiotic resistance; efflux pumps; CAZymes; silkworm hemolymph



Academic Editors: Vasco Ariston De Carvalho Azevedo and Wolf-Rainer Abraham

Received: 21 May 2026

Revised: 15 July 2026

Accepted: 22 July 2026

Published: 24 July 2026

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1. Introduction

The genus *Pseudomonas* consists of metabolically versatile and highly adaptable bacteria. These microorganisms inhabit a wide range of environments, from soil and marine ecosystems to clinical settings and living hosts. However, natural environmental sources serve as their most common reservoirs for isolation [1]. The ecological origin of bacterial isolates provides important knowledge of their genetic adaptation, host association, and potential biosafety risks. In the present study, the isolate was recovered from the hemolymph of the silkworm (*Bombyx mori* L.) larvae collected from a sericultural field.

The domesticated silkworm (*Bombyx mori*) is an economically important lepidopteran insect that underpins the global sericulture industry. Also, it serves as a crucial experimental model for studies in genetics, immunology, microbiology, and host–pathogen interactions, in addition to its commercial value. Although intensive rearing practices have substantially increased silk production, they also increase larval susceptibility to microbial infections, bringing about significant economic losses [2]. The larval hemolymph comprises the predominant systemic immune compartment, serving as both a circulatory fluid and a reservoir where intruding microorganisms proliferate by disrupting external protective barriers [3]. It contains inducible antimicrobial peptides, hemocytes, and enzymatic defense systems, including the prophenoloxidase cascade, which collectively restrict microbial invasion [4]. Regardless, numerous bacterial pathogens have evolved refined mechanisms to evade these immune responses and successfully colonize the insect hemocoel. Consequently, characterization of hemolymph-associated bacteria is highly relevant to understanding disease development in sericulture and to exploiting *B. mori* as an experimental host for microbial pathogenesis studies [3–5]. Because highly adaptable *Pseudomonas* strains use proteases such as elastase B to bypass silkworm immunity, which leads to systemic infection, hemolymph isolates require comprehensive genomic profiling to assess their pathogenic potential, metabolism, evolution, and biosafety risks [5,6].

Previously, we reported the draft genome sequence and initial genomic resource of *P. boreofloridensis* RAC1 isolated from the hemolymph of *B. mori* larvae exhibiting symptoms of flacherie disease [7]. That study focused primarily on genome assembly, structural annotation, phylogenetic placement, and public deposition of the genomic sequence. However, several important aspects of the organism remained unexplored, including its metabolic versatility, comparative genomic relationships, genome plasticity, mobile genetic elements, antimicrobial resistance genes, virulence-associated features, and adaptive mechanisms that may facilitate persistence within the insect host. Based on previously published genomic resources, our study provides a thorough comparative and functional genomic analysis of *P. boreofloridensis* RAC1. The characterization aims to clarify the biological importance and pathogenic potential of the *P. boreofloridensis* RAC1 within the silkworm-associated environment. Comparative genomic analyses were performed to evaluate its taxonomic status, pangenome composition, metabolic repertoire, virulence genes, mobile genetic elements, and antimicrobial resistance mechanisms. Particular emphasis was placed on subsystem annotation, secondary metabolite biosynthetic gene clusters, carbohydrate-active enzymes (CAZymes), and multidrug efflux systems that may contribute to bacterial persistence within the nutritionally dynamic and immune-reactive silkworm hemolymph. Furthermore, comparative analyses with phylogenetically related *Pseudomonas* species were conducted to investigate metabolic diversification, conservation of flagellar-associated genes, and accessory genome variability associated with ecological adaptation. Collectively, these findings provide important genomic insights into *P. boreofloridensis* RAC1 and establish a foundation for understanding its ecological adaptation and potential interactions with the silkworm host, with implications for disease management in sericulture and the broader application of *B. mori* as a model for host–pathogen interaction studies [4,8]. Nevertheless,

experimental infection studies will be required to determine whether *P. boreofloridensis* RAC1 is a causal agent of flacherie disease or represents an opportunistic colonizer of infected larvae.

2. Material and Methods

2.1. Sampling and Isolation

A total of 12 fifth-instar *Bombyx mori* L. (Nistari race) larvae exhibiting typical symptoms of bacterial flacherie, including lethargy, reduced feeding activity, and body softening, were collected in sterile containers from the silkworm rearing facility at Raiganj University, India. The samples were processed as three independent biological replicates, each consisting of four diseased larvae. The larvae were washed with 70% ethyl alcohol, surface-sterilized with 5% (*w/v*) sodium hypochlorite for five minutes and rinsed three times with sterile deionized water. Hemolymph was extracted into pre-chilled microtubes by rupturing the first abdominal leg of infected larvae (third day of fifth instar) using a sterile, superfine 31G short needle [9]. Hemolymph samples from flacherie-infected Nistari race larvae were spread onto cetrimide agar plates (Sisco Research Laboratories Pvt. Ltd., Mumbai, India). The inoculated plates were incubated overnight at 35 °C to facilitate the growth of mesophilic *Pseudomonas* species. Multiple colonies exhibiting similar *Pseudomonas*-like morphology were observed, and one representative colony from the predominant morphotype was selected and repeatedly subcultured on fresh cetrimide agar plates until a pure culture was obtained. Culture purity was confirmed based on repeated streaking, uniform colony morphology, and Gram staining. The purified isolate, designated as *P. boreofloridensis* RAC1, was subsequently used for biochemical characterization and genomic analyses. These pure cultures were maintained on fresh cetrimide plates and preserved at −80 °C in 20% glycerol [7].

2.2. Motility Assay

The swim motility assay medium was prepared by supplementing the broth medium with 0.3% agar (*w/v*). The medium was then poured into plates and allowed to solidify. The isolated strain was inoculated from liquid culture onto the plates by puncturing the agar to a halfway depth using a sterile needle. The plates were incubated at 37 °C for 24–36 h, a temperature commonly used for the phenotypic characterization of *Pseudomonas* sp. and for evaluating motility under host-relevant conditions [10,11]. The motility was monitored for the formation of bacterial halos as an indicator of swimming motility.

2.3. Biochemical and Chemotaxonomical Traits

Biochemical traits were determined using the VITEK-2 automated system (bioMérieux, Marcy l'Étoile, France). Further, the cellular fatty acid composition of *P. boreofloridensis* RAC1 was analyzed using the MIDI protocol. Fatty acids were extracted, methylated to fatty acid methyl esters (FAMES), and analyzed by gas chromatography (Agilent 6850; Agilent Technologies, Santa Clara, CA, USA) with Sherlock software (MIDI Sherlock™ v6.02). The Aerobic library (RTSBA6 6.21) was used for identification.

2.4. Antibiotics Susceptibility Test

The AST profiling of the study isolate was evaluated by using the disk diffusion method. Standard antibiotic disks sourced from HiMedia (Mumbai, Maharashtra, India) were utilized for the assay. A total of 43 antibiotics with specific disk concentrations were tested, categorized into four primary mechanisms of action: cell wall synthesis inhibitors, protein synthesis inhibitors, nucleic acids synthesis inhibitors, and cell membrane inhibitors. Following incubation, the zones of inhibition against each standard antibiotic disk were

measured in centimeters (cm). The strain's antibiotic susceptibility was consequently recorded as either sensitive (where a measurable zone was observed) or resistant (where no zone of inhibition was observed).

2.5. Genome Data

The draft genome sequence of *P. boreofloridensis* RAC1 used in this study was previously generated and published (GenBank accession NZ_JAUTXS000000000.2; BioProject PRJNA224116; BioSample SAMN36766282; SRA SRR25580540) [7]. Briefly, genomic DNA was sequenced using the Illumina NovaSeq 6000 platform. Raw sequencing reads were quality-assessed using FastQC v0.11.9 and MultiQC v1.9, quality-filtered using fastp v0.20.1, assembled using MEGAHIT v1.2.9, and genome completeness and contamination were evaluated using CheckM (v1.2.3). The original genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP v6.6) as previously described [7]. In the present study, the published genome assembly and all reference genomes were re-annotated using Prokka v1.14.6 to generate standardized gene predictions prior to comparative genomic analyses. Reference genomes of related *Pseudomonas* species, including *P. capeferrum* WCS358, *P. kermanshahensis* SWRI100, *P. urmiensis* SWRI10, *P. wayambapalatensis* RW3S1, *P. xantholysinigenes* RW9S1A, *P. muyukensis* COW39, *P. peradeniyensis* BW13M1, *P. soli* LMG 27941, *P. maumuensis* COW77, *P. promysalinigenes* RW10S1, *P. vlassakiae* RW4S2, *P. fulva* DSM 17717, *P. parafulva* DSM 17004, *P. kurunegalensis* RW1P2, *P. inefficax* JV551A3, *P. asiatica* RYU5, *P. oryzicola* RD9SR1, *P. boreofloridensis* K1, *P. boreofloridensis* K13, and *P. anuradhapurensis* RD8MR3, were retrieved from the NCBI RefSeq database.

2.6. Genomic Characterization

The comparative genome map of *P. boreofloridensis* RAC1 with closely related species was generated using Proksee BLAST-alignment v2.17.0 [12]. Firstly, gene annotation was performed with Prokka [13]. Furthermore, the assembled genomic data underwent annotation and comparative analysis using the Subsystem Technology tool kit (RASTtk). This process facilitated the prediction of genes associated with risk assessment, including virulence factors, antibiotic resistance genes (ARGs), and potential drug targets. Metabolic pathways were reconstructed and compared across related species using METABOLIC v4.0 [14]. Additionally, the MOB-suite (v3.1.9) and Ori-Finder-2022 were used for the prediction of plasmid-associated replicons and to check the number of the origin of replication in a genomic sequence [15,16]. The carbohydrate-active enzymes (CAZymes) were profiled to evaluate carbohydrate utilization potential via METABOLIC v4.0 using the dbCAN3 database [17]. The CAZyme analysis was performed by the following run using dbCAN on annotated protein FASTA (.faa) files of genomes. All protein files were analyzed in protein mode against a locally downloaded dbCAN database (released in 2023) using default CAZyme annotation methods. Then, the CAZyme profile was summarized from the output files for each genome. For comparative analysis, only high-confidence CAZyme hits supported by at least two dbCAN methods were retained.

2.7. Characteristics and Functional Gene Prediction

Antibiotic resistance genes were identified with the deep learning-based tool DeepARG 1.0.2 [18], ResFinder v4 [19], ARG-ANNOT [20], MEGARes 2.0 [21], and Comprehensive Antibiotic Resistance Database (CARD) [22]. Further, the CRISPR-Cas system [23], mobile genetic elements [24], bacteriophage-specific genes (PHASTEST) [25], and Phigaro [26] were predicted using the Proksee web-based server. Moreover, a curated reference dataset of flagella-associated genes (structural, motor, regulatory, and export genes) was prepared from annotated *Pseudomonas* reference genomes. Orthologs were identified in each genome using BLAST-based similarity searches (BLASTp/tBLASTn). A binary gene

matrix was generated, where detected genes were scored as present (1) and missing genes as absent (0). The resulting presence/absence matrix was visualized as a clustered heatmap in R using the pheatmap package v1.0.12 [27].

2.8. Pan-Genome Analysis

Pan-genome analysis of *P. boreofloridensis* RAC1 and related species was performed using the anv i v8 pangenome program [28]. Briefly, the genomes of the species to be compared were downloaded in fasta format from the NCBI genome database. The annotations of the downloaded genomes were performed using the NCBI COG database and transferred to the pangenome database for comparison. The determination of genome ANI similarities was accomplished through the utilization of the pyANI program v0.2.13.1 [29] while the construction of the phylogenetic tree was achieved by employing ANI values. Here, genes that were present in all genomes were designated as core genes, while those present in only one genome were classified as singletons. The ANI similarity cutoff value of 88% was utilized in this study.

3. Results and Discussion

3.1. Biochemical and Chemotaxonomical Analysis

Preliminary isolation of the bacterium and its staining revealed that the bacteria were Gram-negative in nature. Further, the bacterial motility assay revealed the swimming motility forming the halo surrounding its inoculation position (Supplementary Figure S1), consistent with the characteristic flagellum-mediated movement reported for multiple *Pseudomonas* spp. [30]. Flagella-driven swimming plays an important role in environmental fitness and early surface/host colonization, and is commonly regulated through conserved flagellar gene networks in *Pseudomonas* [31,32].

The biochemical characterization of the *P. boreofloridensis* RAC1 by the VITEK-2 automated system reveals that the strain tested positive for GGT and exhibited arylamidase activity towards L-proline and tyrosine, indicating efficient amino acid metabolism and peptide turnover (Supplementary Table S1). Such enzymatic activities are common to *Pseudomonas* species and contribute to nutrient acquisition in host-associated and nutrient-variable environments [33]. Moreover, the study strain demonstrated the positive utilization of citrate, succinate, malate, and lactate, along with alkalization of lactate and succinate, reflecting a fully functional tricarboxylic acid (TCA) cycle and respiratory metabolism, which is the ability to utilize diverse organic acids as carbon and energy sources [34]. Further, the positive utilization of coumarate also suggested the possible capability to metabolize aromatic or plant-derived compounds. The biochemical profile of *P. boreofloridensis* RAC1 supports the strains' metabolic adaptability, capable of persisting in fluctuating environments such as silkworm hemolymph, where nutrient availability and host immune pressures vary dynamically. Comparison of the FAME profile against the TSBA6 library of the Sherlock® MIDI System indicated a closest match to members of the genus *Pseudomonas*, with a high Similarity Index (typically >0.6 is considered a good match, though the exact value is system-generated). The fatty acid profile of the *P. boreofloridensis* RAC1 is consistent with its closely related species. The prevalent fatty acid composition of *P. boreofloridensis* RAC1 is palmitic acid (16:0) 27.02%, summed feature 2 14.76%, summed feature 8 14.21%, and cis-9,10-methylene palmitic acid (17:0 cyclo) 10.35% (Supplementary Table S2). Additionally, the summed feature 8 content of the *P. boreofloridensis* RAC1 is aligned with *P. soli* LMG 27941, further describing the similar genus-level fatty acid content. Furthermore, the palmitic acid content of the *P. boreofloridensis* RAC1 is also aligned with the *P. soli* LMG 27941 and *P. asiatica* RYU5. The prevalent fatty acid composition in *Pseudomonas* could possibly provide structural integrity, stress protection, membrane fluidity, endotoxin, etc.,

which is a consistent composition for the *Pseudomonas* genera [35]. The resulting profile is diagnostic for specific microbial groups. The dominance of straight-chain saturated (16:0, 18:0), unsaturated (Summed Features 2, 3, and 8), and cyclopropane fatty acids (17:0 cyclo, 19:0 cyclo ω 8c) is a hallmark of Gram-negative bacteria, specifically aligning with the genus *Pseudomonas* [36]. Furthermore, the other less abundant fatty acid compounds include 18:00, Summed Feature 3, 19:0 cyclo ω 8c, 15:1 ω 5c, 17:1 iso ω 5c, and 12:1 3-OH, and 20:2 ω 6,9c represents other cellular functions in *Pseudomonas* spp. While FAME profiling provides robust genus-level identification and suggests a species complex, conclusive species-level designation requires complementary genomic analysis to resolve closely related species within this metabolically versatile and ecologically important genus.

3.2. General Genomic Features

The total genome length was 4,494,347 bp, distributed across 38 contigs, with an N50 contig size of 199,969 bp and an L50 value of 7, indicating a moderately contiguous assembly with good genomic coverage. The genome exhibited a G + C content of 63.5%, which is consistent with the GC-rich nature of *Pseudomonas* species, typically ranging from 60% to 67%. Genome annotation predicted a total of 4116 genes, including 3992 coding DNA sequences (CDSs), 57 miscellaneous RNAs, 7 rRNAs, 59 tRNAs, and 1 tmRNA. The presence of a complete set of tRNAs and rRNAs suggests a well-represented and functionally competent translational machinery (Supplementary Table S3; Supplementary Figure S2B). The comparison of the *P. boreofloridensis* RAC1 and its closely related genome sequences was also presented in the form of a circular map (Supplementary Figure S2A).

P. boreofloridensis RAC1 exhibited the highest ANI value of 98.35% and a dDDH value of 86.8% with *Pseudomonas boreofloridensis* K13, both exceeding the recommended species boundary thresholds of $\geq 95\%$ for ANI and $\geq 70\%$ for dDDH (Figure 1) [37,38]. In contrast, ANI and dDDH values against all other reference *Pseudomonas* strains remained below 87% and 30%, respectively, well below the recommended species boundary criteria. These genomic indices provide strong evidence that strain RAC1 belongs to the species *Pseudomonas boreofloridensis*.

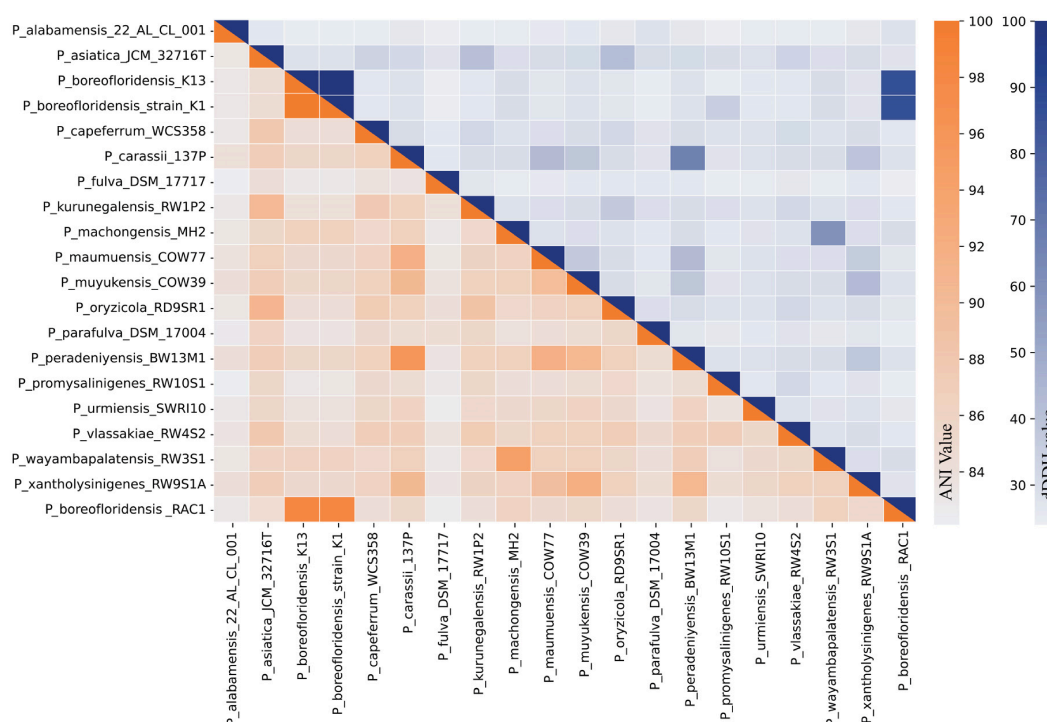


Figure 1. Pairwise ANI and dDDH values for *P. boreofloridensis* RAC1 and its closely related genomes.

Plasmid screening using MOB-suite [16] did not identify any plasmid-associated replicons or contigs, indicating the absence of detectable plasmids in *P. boreofloridensis* RAC1. Furthermore, Ori-Finder [15] predicted a single chromosomal origin of replication (oriC) located at positions 340,217–340,805 bp on contig JAUTXS020000037.1, adjacent to the *dnaA* and *dnaN* genes, supporting the presence of a single chromosome, resulting in no possible chance for horizontal gene transfer.

Annotation through the PATRIC database identified 3242 characterized proteins and 842 putative or hypothetical proteins, indicating that ~79% of the predicted coding sequences could be assigned putative functions based on existing databases. The remaining fraction represents hypothetical proteins, which may include strain-specific or novel functional genes, potentially contributing to a niche adaptation or unique metabolic capabilities. Through functional mapping through KEGG, a total of 2291 genes have been assigned to 1862 unique KEGG numbers using KofamScan. The identified genes were found to be associated with a total of 241 pathways, and 54 complete modules were present. Pathway annotation revealed the strain's diverse metabolic potential, including pathways involved in carbon metabolism, amino acid biosynthesis, and environmental adaptability. Such metabolic versatility is a hallmark of the *Pseudomonas* genus, allowing members to thrive in varied ecological niches, including soil, water, and plant-associated environments.

Antibiotic resistance profiling revealed four resistance genes in CARD, reflecting intrinsic resistance mechanisms. These genes likely encode efflux pumps, and multidrug transporters, which are commonly found in *Pseudomonas* species and contribute to their well-known multidrug-resistant phenotype. The difference in the number of resistance genes detected between CARD and PATRIC may arise from database coverage and annotation criteria. Analysis against the TCDB database identified 75 transporter genes, encompassing various transporter families involved in nutrient uptake, metal ion transport, and multidrug efflux. The presence of seven drug target genes predicted through the Therapeutic Target Database (TTD) provides insight into possible molecular targets for antibacterial compound development. Further, genomic information suggested the isolated strain confers resistance to antibiotics mainly through efflux pump.

Overall, the genomic features of *P. boreofloridensis* RAC1 are consistent with other *Pseudomonas* genomes, such as *P. aeruginosa* PAO1 and *P. putida* KT2440, in terms of genome size, GC content, and gene number. However, the unique combination of virulence genes, antibiotic resistance genes, and transport systems highlights its potential ecological flexibility and clinical significance. Additionally, the distribution of subsystems is described, representing key biological and metabolic processes.

Metabolic pathway comparisons revealed that *P. boreofloridensis* RAC1 harbors a wide repertoire of functional genes compared with related species (Figure 2). KEGG-based annotation and METABOLIC profiling identified enrichment in pathways for aromatic compound degradation, nitrogen utilization, and secondary metabolite biosynthesis [14]. These traits point to enhanced ecological adaptability, enabling *P. boreofloridensis* RAC1 to exploit a broad range of environmental substrates and persist in fluctuating conditions during silkworm development.

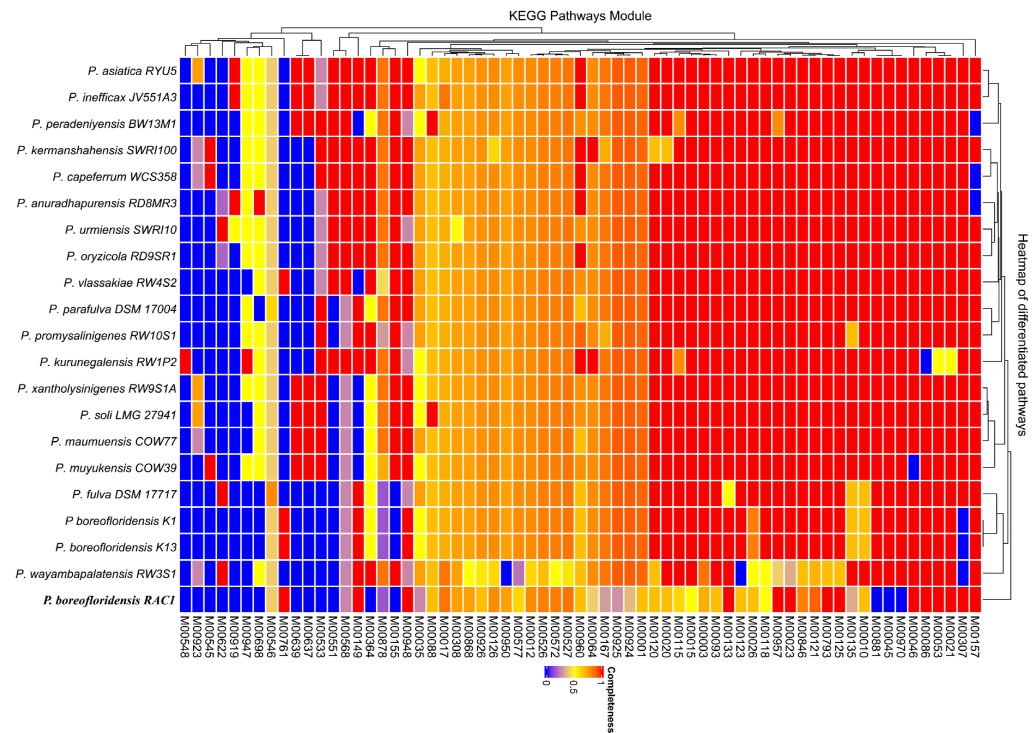


Figure 2. Heatmap comparing major metabolic pathways in *P. boreofloridensis* RAC1 and closely related species based on KEGG annotation and METABOLIC analysis, highlighting enrichment of aromatic compound degradation and secondary metabolite biosynthesis pathways in *P. boreofloridensis* RAC1. Carbohydrate-active enzyme (CAZyme) profiling further distinguished *P. boreofloridensis* RAC1 from its relatives (Figure 3). The genome encoded an expanded set of glycoside hydrolases and polysaccharide lyases, suggesting enhanced capacity to degrade plant-derived polysaccharides. Such metabolic flexibility may facilitate colonization of nutrient-rich host environments while also supporting survival under oligotrophic conditions. The coexistence of oligotrophic traits with broad catabolic versatility indicates an adaptive strategy well suited for feast-and-famine dynamics in the silkworm larvae.

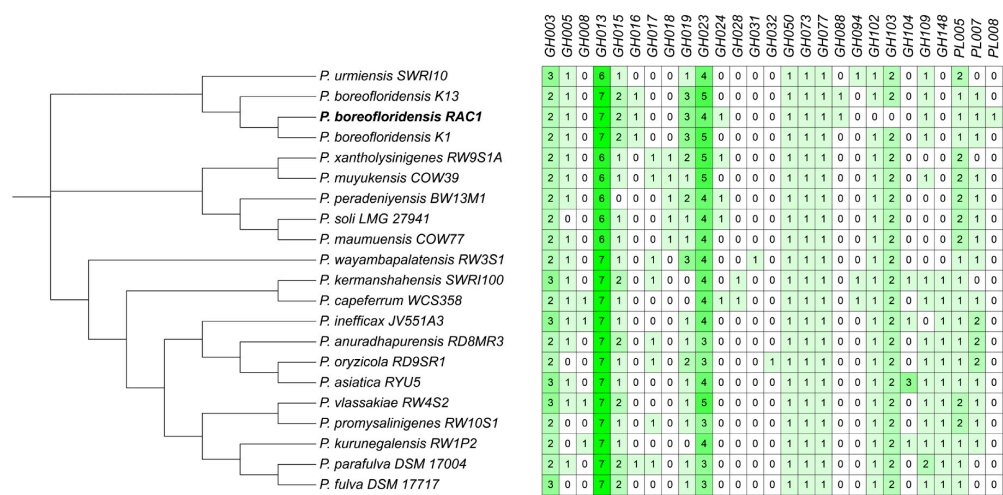


Figure 3. Carbohydrate-active enzyme (CAZyme) profiles across genomes, demonstrating the expanded carbohydrate utilization potential of *P. boreofloridensis* RAC1 (Deep green indicates a higher abundance of CAZyme family enzymes, while white indicates a lower abundance).

3.3. Flagellar-Associated Genes

The comparative analysis of flagellar-associated genes in *P. boreofloridensis* RAC1 and closely related species revealed a largely conserved flagellar gene cassette, supporting the

multidrug-resistant [40]; therefore, *P. boreofloridensis* RAC1 was categorized as an MDR isolate. Detailed AST results have been reported previously (Supplementary Table S1 in Mondal et al., 2024) [7].

Genomic screening for antibiotic resistance genes highlighted the multidrug-resistant potential of *P. boreofloridensis* RAC1 (Figure 5). DeepARG analysis detected a wide array of efflux pumps, β -lactamases, and other resistance factors spanning multiple antibiotic classes. In this instance, the AMR database search did not recover any classical horizontally acquired resistance genes. The study isolate did not contain any plasmid sequences, indicating low chance of plasmid-mediated horizontal gene transfer of antimicrobial resistance genes. This finding is consistent with the genomic AMR profiling. The presence of these genes aligns with *P. boreofloridensis* RAC1’s isolation from a silkworm larvae affected by flacherie, where antibiotic exposure may have created selective pressure. These findings also reinforce concerns about the dissemination of antimicrobial resistance traits in environmental *Pseudomonas* populations.

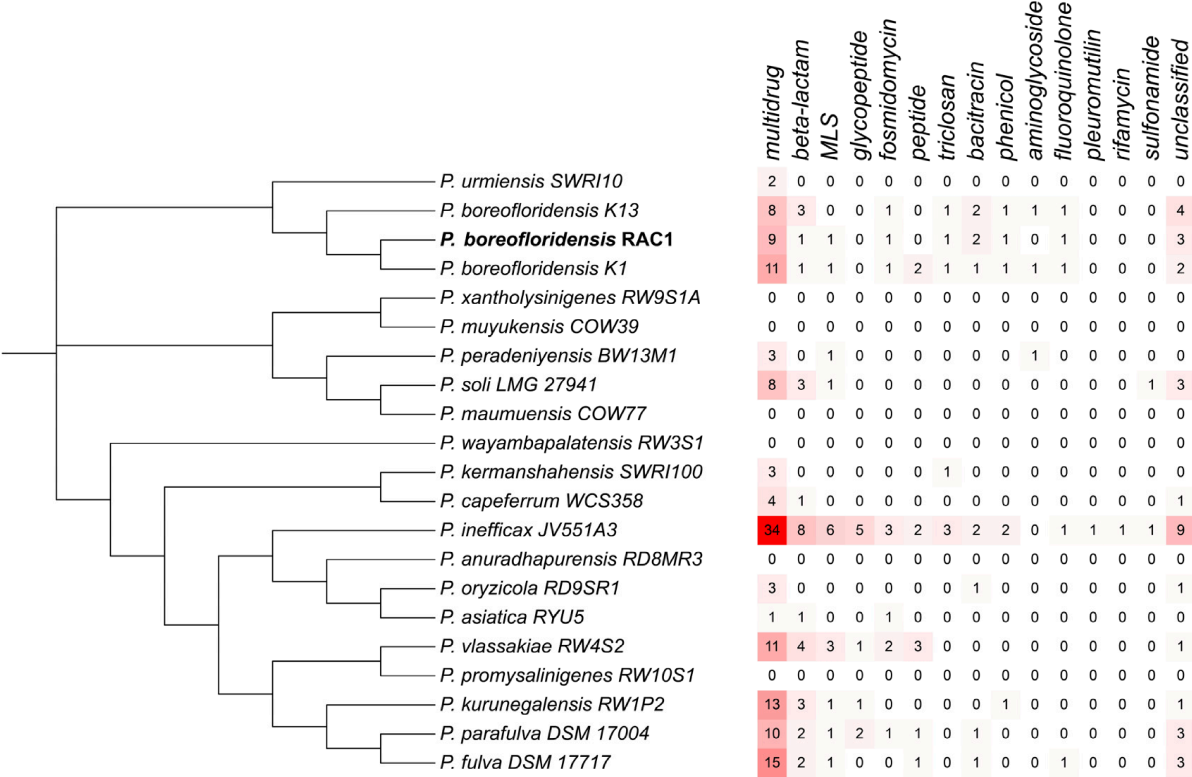


Figure 5. Distribution of antibiotic resistance genes predicted by DeepARG, highlighting the multidrug-resistant potential of *P. boreofloridensis* RAC1 (Red denotes a higher number of AMR-associated genes, whereas white denotes no AMR-associated genes).

The whole-genome analysis of the *P. boreofloridensis* RAC1 revealed a wide array of antimicrobial-resistance genes, suggesting a multifactorial resistance strategy typical of this genus. The detection of the catalase–peroxidase gene *katG* indicates a potential role in oxidative-stress defense and antibiotic activation, while the presence of multiple target-associated genes such as *alr*, *adl*, *gyrA*, *gyrB*, *folA*, and *murA* implies possible target modification or protection mechanisms (Table 1). The identification of FabG, *fabV*, and HtdX highlights antibiotic-target replacement pathways that could bypass inhibitory effects of certain antimicrobials.

Table 1. The presence of the antibiotic's resistance genes in the genomic sequence of *P. boreofloridensis* RAC1.

AMR Mechanism	Genes/Proteins
Antibiotic activation enzyme	KatG
Antibiotic target in susceptible species	Alr, Ddl, dxr, EF-Tu, <i>folA</i> , Dfr, <i>folP</i> , <i>gyrA</i> , <i>gyrB</i> , Iso-tRNA, <i>kasA</i> , MurA, <i>rho</i> , S10p
Antibiotic target replacement protein	FabG, fabV, HtdX
Efflux pump conferring antibiotic resistance	EmrAB-TolC, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexEF-OprN, MexEF-OprN system, MexHI-OpmD, MexJK-OprM/OpmH, TriABC-OpmH
Gene conferring resistance via absence	<i>gidB</i>
Protein altering cell wall charge conferring antibiotic resistance	GdpD, PgsA
Protein modulating permeability to antibiotic	OccD1/OprD, OccD2/OpdC, OccD3/OpdP, OccD6/OprQ, OccK8/OprE, OprB, OprD family, OprF
Regulator modulating expression of antibiotic resistance genes	OxyR

The resistance determinants identified in the genome of *P. boreofloridensis* RAC1 showed substantial evidence for its multidrug-resistant phenotype. In particular, the presence of multiple efflux systems (MexAB-OprM, MexEF-OprN, MexHI-OpmD, MacAB, and MdtABC) together with porin-associated proteins and regulatory genes suggests that intrinsic resistance mechanisms play a major role in the observed antimicrobial resistance against fluoroquinolones, chloramphenicol, and trimethoprim antibiotics [41,42]. Notably, the presence of resistance-associated genes does not necessarily imply their expression under the tested conditions, and further transcriptomic or functional studies would be required to establish a direct genotype–phenotype relationship. The *ttgABC-ttgR* module (Supplementary Table S4), first characterized in the solvent-tolerant *Pseudomonas putida* DOT-T1E, mediates the efflux of ampicillin, carbenicillin, tetracycline, nalidixic acid, and chloramphenicol at a basal regulation of TtgR [43]. In our present study, *P. boreofloridensis* RAC1 is congruent with the observed ampicillin/penicillin-class resistance, while sensitivity to nalidixic acid and tetracycline again indicates the pump is not induced under the conditions tested. Moreover, the presence of the *yajC*, an associated resistance-class profile spanning cephalosporins, glycopeptides, oxazolidinones, penicillin-class β -lactams, phenicols, rifamycin, and tetracycline [22], aligned with the study isolate phenotypic resistance to shared efflux-associated contribution to tested antibiotics. So, the presence of efflux-pump genes and outer membrane exclusion of the study isolate associated with flacherie-symptomatic *Bombyx mori* plausibly reflects an ecologically linked, genus-level trait rather than recent antibiotic selection pressure alone.

P. boreofloridensis RAC1 contains multiple copies of mobile genetic elements in its genomic sequence, indicating an actively evolving genome potentially having horizontal gene transfer capability. We identified multiple genes associated with integration/excision (27), phage-specific function (59), and conjugative transfer (12), suggesting ongoing genomic rearrangements and gene flux (Supplementary Figure S3). The presence of these elements is consistent with reports that *Pseudomonas* species maintain highly plastic genomes driven by IS-mediated recombination and adaptive acquisition of functional genes, including antimicrobial resistance [44].

Prophage prediction and annotation of *P. boreofloridensis* RAC1 using PHIGARO identified three bacteriophage-derived genomic regions comprising phage-specific genes and pVOG-supported annotations [26,45]. Two regions were classified as Siphoviridae and one as Myoviridae, indicating exposure of *P. boreofloridensis* RAC1 to phylogenetically distinct tailed bacteriophages. The largest prophage-like region was detected on contig JAUTXS020000020_1 (nt 5767-39588) and showed a structured gene block dominated by phage-related ORFs. Importantly, the co-localization of terminase and portal genes supports a conserved DNA packaging module, a hallmark of tailed dsDNA bacteriophages [46]. Gene orientation in this region revealed largely co-directional transcription, consistent with modular prophage architecture. Further, a second smaller Siphoviridae-like region occurred on contig JAUTXS020000024_1 (nt 848-7421) and contained mainly tail- and assembly-associated genes, suggesting a partial prophage remnant. In contrast, a Myoviridae-like prophage region in the same contig (nt 110900-133621) contained clustered ORFs associated with lysis, capsid/coat, tail, and assembly, indicating retention of key structural and lytic modules typical of tailed phages (Supplementary Figure S4).

Therefore, the presence of multiple prophage regions with conserved genes and directional gene clusters indicates that prophage acquisition has contributed to the genomic diversification of *P. boreofloridensis* RAC1 and may influence fitness through prophage-encoded accessory functions. The antibiotic susceptibility profile of *P. boreofloridensis* RAC1 revealed complete resistance to all 17 antibiotics tested across multiple classes, including β -lactams (ampicillin, penicillin G, amoxycylav, cefoxitin, cephalothin, oxacillin, cloxacillin, methicillin), glycopeptides (vancomycin, teicoplanin), protein-synthesis inhibitors (fusidic acid, linezolid, lincomycin), and nucleic-acid synthesis inhibitors (rifampicin, co-trimoxazole, sulphatriad, nitrofurantoin). This broad-spectrum resistance pattern strongly indicates a robust intrinsic resistance background, characteristic of *Pseudomonas* spp., which often possess low outer-membrane permeability and multiple multidrug efflux systems that limit antibiotic entry and accumulation [47]. However, the observed resistance profile likely reflects both the detected resistance-associated factors and the intrinsic resistance mechanisms naturally present in *Pseudomonas* spp., suggesting a possible multidrug-resistant phenotype.

The genomic sequence of the *P. boreofloridensis* RAC1 contains two copies of *adeF*, a component associated with the AdeFGH RND efflux pump family in non-fermenting Gram-negative bacteria. Furthermore, the presence of the outer membrane protein, *oprM* and *oprJ* possibly provides further resistance without the presence of the Mex- inner membrane proteins [48]. In *Acinetobacter baumannii*, *adeF* overexpression significantly contributes to resistance to chloramphenicol, fluoroquinolones, tetracyclines, and trimethoprim [49]. Moreover, the presence of the *mdtABC*, *marR*, *emrAB* multidrug efflux pump operons and regulators, along with the *fsr* and *abaF* genes, indicates a genomic basis for fosfomycin resistance. Collectively, the identified antimicrobial resistance determinants indicate a broad resistance-associated genomic repertoire in *P. boreofloridensis* RAC1, which may contribute to its persistence and pathogenic potential within the silkworm host environment. Moreover, the presence of *clpK1* in *P. boreofloridensis* RAC1 encodes a stress-tolerance AAA family ATPase, which is predominantly recognized as a virulence and heat-shock survival gene in *Enterobacteriaceae* (Supplementary Table S5). Future studies incorporating clinically relevant antipseudomonal antibiotics, including carbapenems, aminoglycosides, fluoroquinolones, polymyxins, and antipseudomonal cephalosporins, will further strengthen the phenotypic resistance characterization of *P. boreofloridensis* RAC1. Although *P. boreofloridensis* RAC1 was recovered from the hemolymph of flacherie-infection symptomatic silkworm larvae and encodes several putative virulence-associated genes, the present study does not establish a causal relationship between *P. boreofloridensis* RAC1 and flacherie disease. Future studies

involving controlled infection experiments and Koch's postulates-based validation are required to determine its pathogenic potential.

3.5. Pangenome Analysis

Pangenome analysis was performed to characterize the genomic diversity across the 21 *Pseudomonas* strains included in this study (Supplementary Table S6). A total of 102,142 genes has been identified across 21 genomes, and 13,412 of these have been designated as gene clusters (Figure 6A). A total of 2123 genes have been delineated as core genomes which represent the conserved genetic backbone essential for fundamental cellular functions, while 5231 genes have been identified as singletons. Additionally, an accessory genome (i.e., set of genes present in only some genomes, but not all) constituted a substantial proportion of the pangenome, consisting of 3604 shell genes (26.9%) and 7685 cloud genes (present in $\leq 15\%$ genomes) (57.3%). Shell genes typically encode niche-adaptive traits or strain-specific variations, whereas cloud genes represent highly variable or horizontally acquired elements, including mobile genetic elements and plasmid-associated loci. The high number of cloud genes indicates considerable genomic plasticity within the *Pseudomonas* population [8]. Each strain contributed a varied number of unique genes to the pangenome.

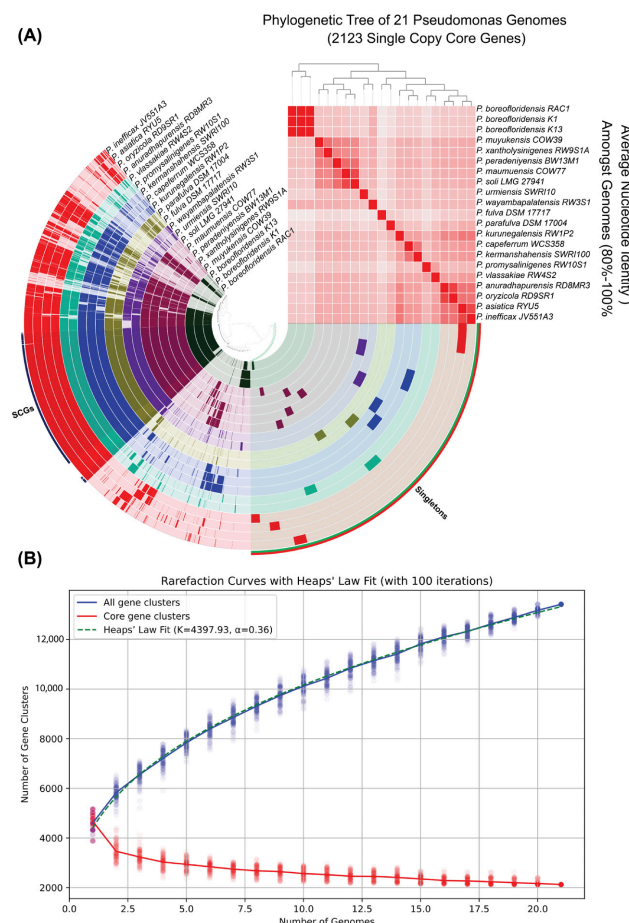


Figure 6. The pangenome of *P. boreofloridensis* RAC1. (A) Each one of the 13,412 gene clusters is represented by a radius that originates from 21 genomes. Gene clusters are sorted based on the distribution of genomes. The phylogenetic tree in the upper right corner of the figure is calculated according to ANIb values, and the genomes in the ring are sorted accordingly. SCGs (single-copy gene clusters) and singletons are highlighted; (B) Pan and core genome sizes. Blue represents the pan genome size, while red represents the core genome size.

Pangenome estimation using Heaps' law gave an α (alpha) value of 0.36 for the 21 *Pseudomonas* genome (Figure 6B). Since, $\alpha < 1$, this clearly indicates an open pangenome, meaning new genes continue to appear as more genomes are added [50].

Functional annotation of core genes revealed enrichment in essential metabolic pathways, including carbohydrate metabolism, amino acid biosynthesis, transcription, translation, and DNA repair. In contrast, accessory genes were predominantly associated with environmental stress response, antimicrobial resistance, virulence factors, transport systems, host-specific cross talking genes, and hypothetical proteins. Cloud genes contained most of the mobile genetic elements, including insertion sequences, prophage-related proteins, and conjugation machinery.

4. Conclusions

In this study, *P. boreofloridensis* RAC1 isolated from the silkworm larvae was confirmed as a Gram-negative, motile bacterium with strong metabolic versatility as evidenced by biochemical profiling and FAME-based chemotaxonomic signatures consistent with the genus *Pseudomonas*. Whole-genome sequencing revealed a 4.49 Mb GC-rich genome encoding diverse pathways for carbon utilization, aromatic compound degradation, and carbohydrate-active enzymes, supporting ecological adaptability in nutrient-variable host environments. The genome also harbored virulence-associated genes and an extensive repertoire of antimicrobial resistance genes, predominantly associated with multidrug efflux and reduced membrane permeability, corroborating the observed broad-spectrum phenotypic resistance. Furthermore, the presence of mobile genetic elements and three prophage regions (Siphoviridae- and Myoviridae-like) highlights active genome plasticity and potential horizontal gene transfer. Pangenome analysis indicated an open genome structure dominated by accessory and cloud genes, suggesting continued acquisition of adaptive traits. Overall, *P. boreofloridensis* RAC1 was isolated from flacherie-affected silkworm larvae and represents a genomically flexible, multidrug-resistant *Pseudomonas* strain with traits that may confer ecological persistence and host-associated fitness in silkworm larval environments. However, experimental infection studies are required to establish whether this bacterium contributes to the development of flacherie disease.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/bacteria5030043/s1>, Figure S1: Swimming motility of *P. boreofloridensis* RAC1 was evaluated on soft agar medium (0.3% agar), forming the circular bacterial halo radiating from the point of inoculation after incubation at 37 °C for 24–36 h indicated positive swimming motility; Figure S2: Circular genomic map and subsystems information of *P. boreofloridensis* RAC1. (A) from outer to inner rings, the contigs, CDS on the forward strand, CDS on the reverse strand, RNA genes, CDS with homology to known antimicrobial resistance genes, CDS with homology to known virulence factors, GC content and GC skew. The colors of the CDS on the forward and reverse strand indicate the subsystem; (B) The subsystem is based on the set of proteins that together implement a specific biological process or structural complex; Figure S3: The circular Pie-chart representing the Mobile Genetic Elements presents in the genomic sequence of *P. boreofloridensis* RAC1; Figure S4: Presence of the bacteriophage-specific genes with the orientations in genomic sequence of *P. boreofloridensis* RAC1; Table S1: The biochemical characteristics of *P. boreofloridensis* RAC1; Table S2: Cellular fatty acid contents of *P. boreofloridensis* RAC1 and related members of the genus *Pseudomonas*. Strains: 1, *P. boreofloridensis* RAC1; 2, *P. soli* LMG 27941; 3, *P. fulva* DSM 17717; 4, *P. parafulva* DSM 17004; 5, *P. asiatica* RYU5 (Note- ND: Not determined); Table S3: Genome statistics and description of the *P. boreofloridensis* RAC1 (some data has obtained from our previous study [7]); Table S4: Genome-wide AMR genes present in *P. boreofloridensis* RAC1 search by AMRFinderPlus, CARD, ResFinder, and ARG-ANNOT; Table S5: Virulence gene of *P. boreofloridensis* RAC1 determined by VirulenceFinder-3.2.1 through CGE web-based tool; Table S6. Characteristics

of the analyzed *Pseudomonas* genomes, including isolation source, geographic location, type strain status, and assembly quality metrics (completion, redundancy, contig count, and total length).

Author Contributions: R.M. and A.M. wrote the main manuscript text, A.M., P.M. and A.K.S. prepared figures and tables, H.K. and A.K.M. supervise and review the main manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article and its Supplementary Materials. Additional data supporting the findings of this study are available from the corresponding author upon reasonable request.

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

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