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## Article

# Genome Assembly, Pan-Genome Analysis, Taxonomic Re-Assignment, and Biosynthetic Gene Clusters of a Plant Growth-Promoting Bacterium, *Streptomyces cavourensis* SAI-25

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**Abstract:** A *Streptomyces* strain SAI-25, isolated from the rice rhizosphere, was previously reported to have several plant growth-promoting (PGP) and biocontrol potential. Since the previous studies were targeted for particular traits, this study focused on mining the genome to explore its full biosynthetic potential, which were unknown so far. To address that, its genome was sequenced to obtain a chromosome-level assembly, which was analyzed to identify its secondary metabolite potential, taxonomic positioning, genes/pathways responsible for PGP and biocontrol traits, strain-specific genes, and potential enzymes for biosynthesis of a diketopiperazine class compound, Cyclo(Trp-Phe), previously reported in this strain. Sixteen Biosynthetic Gene Clusters (BGCs) out of thirty-two predictions were annotated to diverse functions; majority with biocontrol properties. The strain was reclassified to another species, *S. cavourensis*. The pan-genome analysis showed the presence of a relatively higher number of unique genes in the SAI-25 strain. Genes/pathways identified for the PGP and biocontrol traits were largely similar to those of other *Streptomyces* species. The potential BGCs that might be involved in the biosynthesis of Cyclo(Trp-Phe) were also predicted. Thus, the presence of BGCs with diverse functions, beneficial for agricultural and industrial purposes, highlights its potential.

**Keywords:** *Streptomyces*; plant-growth promotion; secondary metabolites; whole-genome sequencing; pan-genome; biosynthetic gene clusters

## 1. Introduction (Rationale, Current Status, Gaps, Objectives, Overview of the Work)

The increasing costs and negative effects of synthetic chemicals used in crop production necessitate the adoption of biological options of crop production and protection, such as crop residues, farmyard manures, composts, and plant growth-promoting (PGP) bacteria. The use of PGP bacteria for improving agricultural production, soil, and plant health has become one of the attractive strategies for developing sustainable agricultural systems in many parts of the world due to their eco-friendliness, low production cost, and minimizing consumption of non-renewable resources [1].

PGP bacteria are found mostly in soil, compost, fresh and marine water, and play an important role in the PGP, plant protection, decomposition of organic materials, and produce secondary metabolites of commercial interest. There is a growing interest in the use of secondary metabolites, such as toxins, proteins, hormones, vitamins, amino acids, and antibiotics, from microorganisms, for the control of plant pathogens, as these are readily degradable, highly specific, and less toxic to nature [2].

PGP actinomycetes such as *Streptomyces* and their secondary metabolites were reported widely as an excellent alternative for improving nutrient availability, enhancing root and shoot growth, nitrogen fixation, grain and stover yields, solubilisation of inorganic minerals, and protecting against plant pathogens of agriculturally important crops [3–6]. The attributes may be due to the production of antibiotics, chitinase, cellulase, lipase, hydrocyanic acid, siderophore, phytohormones,  $\beta$ -1, 3-glucanase production, and ACC-deaminase [7]. The PGP potential of *Streptomyces* strains was well documented in tomato, wheat, rice, bean, chickpea, pigeonpea, sorghum, and pea [1,2,7–18].

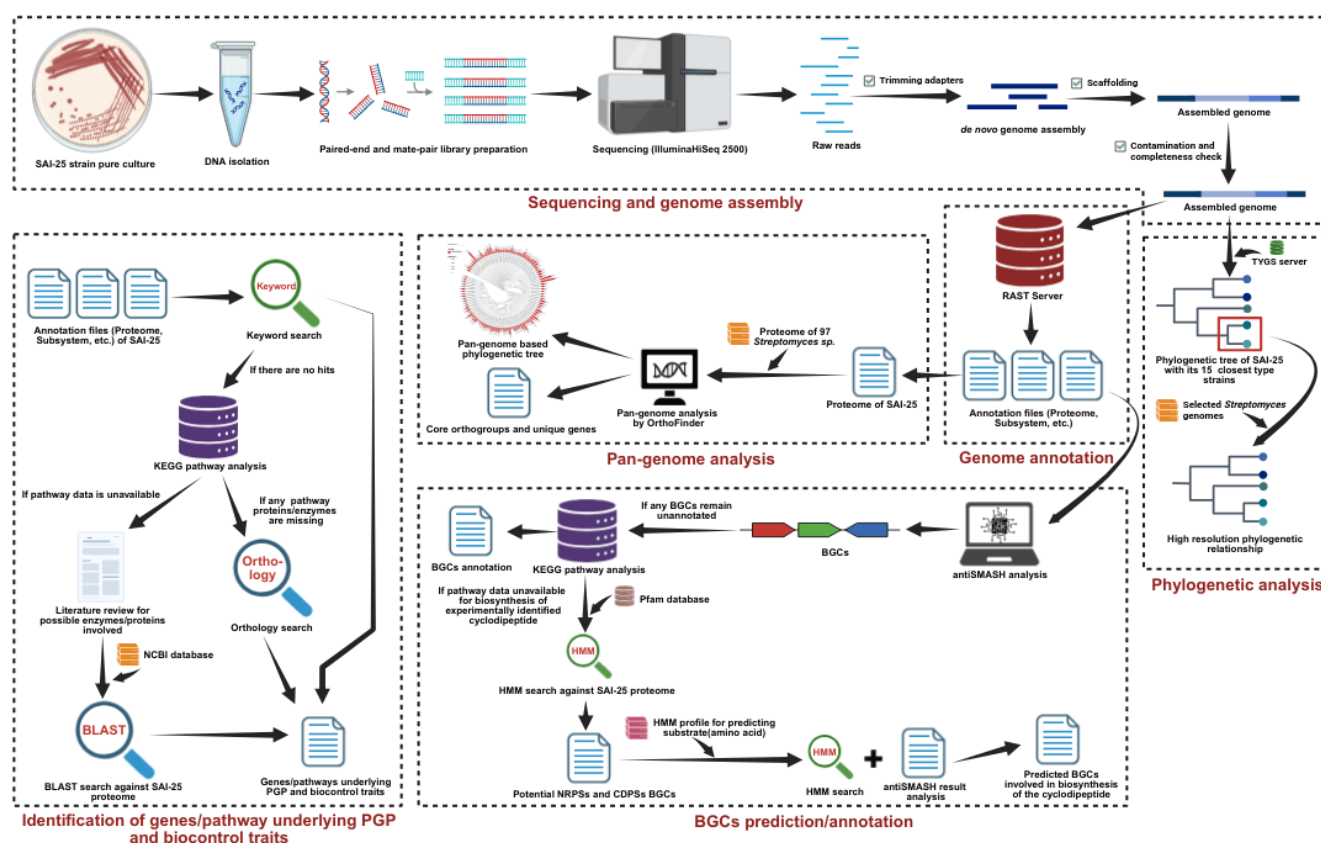
While screening selected rhizospheric isolates for entomopathogenic/insecticidal activities *in vitro* and under greenhouse conditions, [19] reported one of them, namely SAI-25, as a promising candidate, given its strongest activity against lepidopteran insects such as *Helicoverpa armigera*, *Spodoptera litura*, and *Chilo partellus*. Based on the similarity to the 16s rRNA sequence database, this strain was assigned to *Streptomyces griseoplanus* [19]. On further investigation, a cyclodipeptide was identified from SAI-25, namely cyclo(Trp-Phe), which showed insecticidal properties, such as antifeedant, insecticidal, and pupicidal activity, against *H. armigera* [17]. Furthermore, spectral analysis of the cell-free extracellular extract of SAI-25 by FTIR confirmed the presence of alcohols, amines, phenols, and protein, which not only played the role of stabilizing agent while synthesis of silver nanoparticles, but also proved as a base for the development of *Streptomyces* mediated nanoparticle biopesticide due to its antifungal activity against charcoal rot pathogen, *Macrophomina phaseolina* [20].

Several such strains or isolates, which were characterized for having a few plant growth-promoting features, have been further examined for the underlying genes or pathways, which include a couple of studies by the authors on multiple isolates from *Streptomyces* and *Amycolatopsis* genera [14,21]. Although the entomopathogenic, antifungal properties and identification of an insecticidal metabolite (a diketopiperazine class compound) make SAI-25 a very promising PGP bacteria, the genetic and genomic basis of the known features, as well as its potential to synthesize other secondary metabolites, remain unexplored.

The current study aimed to identify the genes and pathways underlying the PGP/biocontrol traits and to predict the secondary metabolite biosynthetic potential of SAI-25 by genome mining. To answer this question, deep sequencing of the genome sequence of this isolate was done to obtain a genome assembly of SAI-25. The genome sequence was compared with existing *Streptomyces* genomes to confirm its taxonomic classification. The pan-genome analysis of complete genomes of the *Streptomyces* genus helped in identifying the core and unique genes, as well as their functional importance. Further, the annotation of the genome assembly was mined for genes or gene clusters involved in the biosynthesis of secondary metabolites, with more emphasis on the ones that have already been reported in SAI-25.

## 2. Materials and Methods

The workflow followed in the current study is illustrated in Figure 1. The details are elaborated in the subsequent sections.



**Figure 1.** Schematic diagram showing the workflow followed in the current study. This figure was created using the BioRender tool (<https://BioRender.com>).

### 2.1. Microbial Strain Used in This Study

The previously identified *S. griseoplanus* strain, namely, SAI-25 (GenBank accession number: KF770901), isolated from a rice rhizospheric field at Karnataka, India, demonstrated previously for its plant growth promotion (PGP) and entomopathogenic traits against *Helicoverpa armigera*, *Spodoptera litura*, and *Chilo partellus* [17,19,20], was used in this study.

### 2.2. Culture of PGP Strain and Genomic DNA Isolation

DNA of SAI-25 was isolated as per the protocols mentioned in [14]. In brief, SAI-25 was inoculated in starch casein broth followed by incubation for 120 h at 28° C. After the incubation period, the culture was centrifuged at 10,000g for 10 min at 4 °C, and subsequently the pellet (cells) were washed twice with STE buffer (sucrose 0.3 M, Tris/ HCl 25 mM and Na<sub>2</sub>EDTA 25 mM, pH 8.0). After discarding the supernatant, the pellet (1 g) was then re-suspended in 8.55 ml STE buffer and 950 µl lysozyme (20 mg/ml STE buffer), and subsequently incubated for 30 min at 30 °C. Thereafter, 500 µl of SDS (10%; w/v) and 50 µl of protease (20 mg/ml) were added, and the mixture was then kept for one hour at 37 °C. After the incubation, 1.8 ml of NaCl (5 M) was added with gentle mixing to avoid shearing the DNA, and 1.5 ml of CTAB (10%; w/v) in 0.7 M NaCl (CTAB/NaCl solution) was then incubated for 20 min at 65 °C. After the addition of CTAB, the subsequent steps were performed at room temperature. The lysate was subjected to two sequential extractions using an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1; by vol), followed by centrifugation at 13,000g for 10 min. The extraction of the aqueous phase was carried out using chloroform/isoamyl alcohol (24:1, by volume), and then transferred to a tube, followed by the addition of 600 µl of propan-2-ol, and DNA was spooled out after 10 min. Alternatively, it was recovered by centrifugation at 12,000g for 10 min. The pellet was washed twice using ethanol (70%; v/v), followed by vacuum drying, and then finally dissolved in 2 ml of TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0). RNase A (50 mg/ml) was



added, followed by incubation at 37 °C for two hours. The sample was then again extracted with phenol as described above. DNA was then re-precipitated from the aqueous phase by adding 100 µl of 3 M sodium acetate (pH 5.3) and 600 µl of propan-2-ol. The resulting DNA pellet was then washed with ethanol (70%; v/v), dried, and subsequently resuspended in the TE buffer. The purity of the SAI-25 DNA was assessed by agarose gel electrophoresis and then quantified using NanoDrop.

### 2.3. Library Preparation and Sequencing

The genomic DNA thus extracted was processed to prepare libraries for whole-genome shotgun sequencing as described in our previous work [21]. Briefly, high-quality genomic DNA of ~5 µg (devoid of any contamination and exhibiting an A260/280 ratio between ~1.8 - 2.0, with DNA concentration  $\geq 100$  ng/µl) was sent to AgriGenome Labs (Kochi, India) for library preparation and next-generation sequencing using the Illumina platform. The genomic DNA was fragmented to obtain two types of libraries: i) a paired-end library with an insert size of 300 bp; and ii) a mate-pair library with an insert size of 5 Kbp. The quality of DNA fragment libraries was validated by tapestation and subsequently sequenced on the Illumina HiSeq2500, generating ~9.905 million paired-end reads (100 bp  $\times$  2) and ~6.242 million mate pair (250 bp  $\times$  2) reads.

### 2.4. Genome Assembly and Annotation

The whole genome sequenced paired- and mate-pair reads (Encoding: Illumina 1.9) of the bacterial genome were cleaned (by removing adapter and primer sequences, etc.) using Trimmomatic [22]. The pre-processed paired- and mate-pair reads were *de novo* assembled using SOAPdenovo V2.04 and SPAdes V3.10.1 assemblers [23]. Two assemblies were assessed and compared by using QUAST, followed by discarding the contigs having a length of <500bp and coverage <5 [24]. The GapCloser was used to close the gaps that emerged during the scaffolding process by the *de novo* assembler, using the abundant pair relationships of short reads [25]. To check the contamination, resulting scaffolds were subjected to NCBI BLAST database search, and scaffolds aligning to anything other than the *Streptomyces* genus were discarded. The scaffold sequences were subjected to Pathosystems Resource Integration Center (PATRIC V3.6.9) [26] and KmerFinder-3.2 [27] searches to find the closely related genomes. To perform reference genome-based reordering, genome sequences of *Streptomyces cavourensis* strain TJ430, *Streptomyces* sp. CFMR 7, *Streptomyces cavourensis* strain 1AS2a, and *Streptomyces atratus* SCSIO-ZH16 were obtained from the NCBI microbial genomes database, followed by scaffolding using Multidraft-Based Scaffold (MEDUSA) [28]. The completeness of gene space was estimated using BUSCO v5.2.2 [29], where the lineage dataset selection was streptomycetales\_odb10. The annotation of the assembled genome of the SAI-25 strain was performed using the RAST (Rapid Annotation using Subsystem Technology) server (<https://rast.nmpdr.org/rast.cgi>) via the RASTtk pipeline (accessed in December 2024)[30].

### 2.5. Phylogenetic Relationship of SAI-25

To identify its taxonomic position in the *Streptomyces* genus, the genome sequence of the SAI-25 strain was uploaded to the Type (Strain) Genome Server (TYGS) (accessed in February 2025)[31]. TYGS identified the closest type strains by constructing two phylogenetic trees based on (i) 16S rDNA gene sequences and (ii) whole genome sequences.

To obtain the taxonomic position of SAI-25 at a higher resolution, the species closest to SAI-25 in the TYGS phylogenetic tree, along with appropriate outgroups, were selected for further examination. The proteomes of i) all the strains belonging to the closest species, with completeness  $\geq 99\%$ , and having scaffold or higher-level assembly, ii) the three next closest type-strains in the TYGS phylogenetic tree, and iii) *Peterkaempferia griseoplana*, formerly *Streptomyces griseoplanus* [32], were retrieved from the NCBI database (accessed in March 2025). A phylogenetic tree was constructed based on the number of overlapping orthogroups among the above strains using the OrthoFinder tool (Version 3.0.1b1)[33].

## 2.6. Pan-Genome Analysis

The proteome (.faa) of all the species belonging to the *Streptomyces* genus with at least chromosome-level assembly and completeness of  $\geq 98.5\%$  were retrieved from the NCBI database (accessed in February 2025). Additionally, two more filtering criteria were applied: (i) inclusion of only reference genomes, RefSeq-annotated genomes, and those derived from type material, and (ii) exclusion of atypical genomes and metagenomically assembled genomes (MAGs). The proteome (.faa) of 98 species, including the SAI-25 strain and 97 other *Streptomyces* species retrieved from the NCBI database, were used as input for OrthoFinder (Version 3.0.1b1) to identify the unique genes of each species of the pangenome and the core orthogroups (orthogroups consisting of orthologs coming from all the pangenome species).

Genes not assigned to any orthogroups, and those belonging to SAI-25-specific orthogroups, were labelled as unique genes in this study. The unique genes of the SAI-25 strain were further analyzed to get functional insights using multiple strategies: the KEGG PATHWAY database (<https://www.genome.jp/kegg/>) via 'Automatic KO assignment and KEGG mapping service' (BlastKOALA Version 3.1) [34] and 'KEGG Mapper – Reconstruct' [35,36], InterPro search [37], and NCBI BLAST followed by Reciprocal Best BLAST [38] if no subsystem information was available in RAST annotation.

## 2.7. Identification of Biosynthetic Gene Clusters (BGCs) in SAI-25 Strain

Genome-wide identification of secondary metabolite biosynthesis gene clusters (BGCs) was performed using antiSMASH 7.1.0 [39]. The regions that showed very little or no similarity to any known clusters were further analyzed using the KEGG PATHWAY database (described above).

## 2.8. Identification of Potential Genes/Enzymes Responsible for Biosynthesis of an Insecticidal Diketopiperazine Derivative, Cyclo(Trp-Phe)

The SAI-25 strain was reported to have activity of one of the Cyclodipeptides (CDPs), Cyclo(Trp-Phe). The Cyclodipeptides are typically synthesised by two unrelated biosynthetic enzyme families: by non-ribosomal peptide synthetases (NRPSs) or by cyclodipeptide synthases (CDPSs). To identify NRPS and CDPSs members in SAI-25, the Hidden Markov Models (HMMs) profiles of NRPS' three domains, namely, i) Adenylation(A)-domain (responsible for binding and activation of amino acids; Pfam-ID: PF00501), ii) peptidyl carrier protein(PCP)-domain or the Thiolation(T)-domain (for loading the activated amino acid onto this by A-domain; Pfam-ID: PF00550), and iii) Condensation(C)-domain (for catalysing the peptide bond formation between two T-domain bounded amino acids; Pfam-ID: PF00668); and CDPSs enzyme family (Pfam-ID: PF16715), were downloaded from the Pfam database [40–42]. The *hmmsearch* (HMMER 3.3.2)(<http://hmmer.org>) for all the above HMM profiles was carried out against the SAI-25 strain proteome, constraining the e-value to  $\leq 0.01$ .

To predict the amino acid substrate(s) that the NRPSs would bind to, the SAI-25 proteins, showing significant hits to all three domains mentioned above, were selected. The A-domain sequences of such proteins were extracted and examined for two amino-acid substrates (tryptophan and phenylalanine) using substrate-binding specific HMM profiles from the "Non-Ribosomal Peptide Synthetase Substrate Predictor" database (<https://nrpssp.usal.es/download.php>) [43].

Alternatively, the BGCs prediction tool not only identified NRPSs but also their amino acid substrates and products. Thus, the results of antiSMASH were analyzed to look for any NRPSs whose predicted substrates were tryptophan and/or phenylalanine.

## 2.9. Genes/Pathways Underlying PGP Features

Seven plant growth-promoting (PGP) and biocontrol traits (Siderophore+, chitinase+, cellulase+, lipase+, protease+, indole-3-acetic acid+, and hydrocyanic acid+) were observed in SAI-25 strain through biochemical assays (Table 1). Potential genes and pathways associated with the above-mentioned PGP and biocontrol traits were identified as described in our previous work [21]. Briefly,

using the KEGG PATHWAY database, keyword searches in the RAST annotation, BLAST searches against the SAI-25 strain proteome, and pan-genome-wide BLAST searches to detect possible orthologs that the KEGG PATHWAY database may have missed.

3. Results

3.1. Features of Genome Assembly of *Streptomyces* sp. SAI-25:

An isolate from the rice rhizospheric soil was earlier characterized for having several plant-growth-promoting and biocontrol features (Table 1)[19]. To examine the genetic or genomic basis of PGP features and to explore its biosynthetic potential, its whole genome was deeply sequenced. A total of ~51 million paired-end and ~135 million mate-pair raw reads were generated after sequencing, which were reduced to ~49 million and ~104 million reads, respectively, after their quality check (Table 2).

Table 1. *Streptomyces* sp. SAI-25 culture information.

GenBank accession no.:	KF770901
Source of isolation:	Rice rhizosphere soil
Temperature tolerance:	20–40°C
PGP and biocontrol traits:	Siderophore+, chitinase+, cellulase+, lipase+, protease+,
indole-3-acetic acid+ and hydrocyanic acid+	
Entomopathogenic	Helicoverpa armigera, Spodoptera litura and Chilo partellus
Metabolite identified:	Cyclo(Trp-Phe), a diketopiperazine derivative with insecticidal activity on H. armigera.

Table 2. Summary of raw and cleaned reads used in assembly generation.

	Paired-end (100 bp)	Mate-pair (250 bp)
Raw reads	50,83,396	1,34,97,102
Clean reads	48,81,906	1,03,71,537

After *de novo* assembly of clean reads followed by scaffolding, a single scaffold of ~7.7 million bp in length was obtained. Standard assembly statistics showed a high GC content of 72.1%, a characteristic of Actinomycetes, and a very minimal number of anonymous nucleotides (0.25%) (Table 3).

To assess the quality of genome assembly, out of 1579 reference Benchmarking Universal Single-Copy Orthologs (BUSCOs) derived from 145 genomes belonging to order streptomycetales, 1567 (99.2%) were complete (C), two were completely duplicated (D), four were fragmented (F) and eight were missing (M) in the SAI-25 strain assembly (Figure S1). The complete BUSCOs over 99% indicated a very high degree of completeness of the generated assembly.

The annotation of the SAI-25 genome showed that it has 6,923 coding sequence regions (88.91%) with a mean length of 990 bp. It also consisted of 74 tRNA genes with a mean length of 76 bp, three rRNA genes with a mean length of 1,595 bp, 152 repeats with a mean length of 127 bp, and 81 CRISPR spacers with a mean length of 32 bp. A total of 4,560 (65.86%) proteins were assigned functional annotation, while 2,363 (34.13%) proteins were assigned as hypothetical (Tables 3, S1; Figure 2a).

Table 3. Genome assembly and annotation statistics.

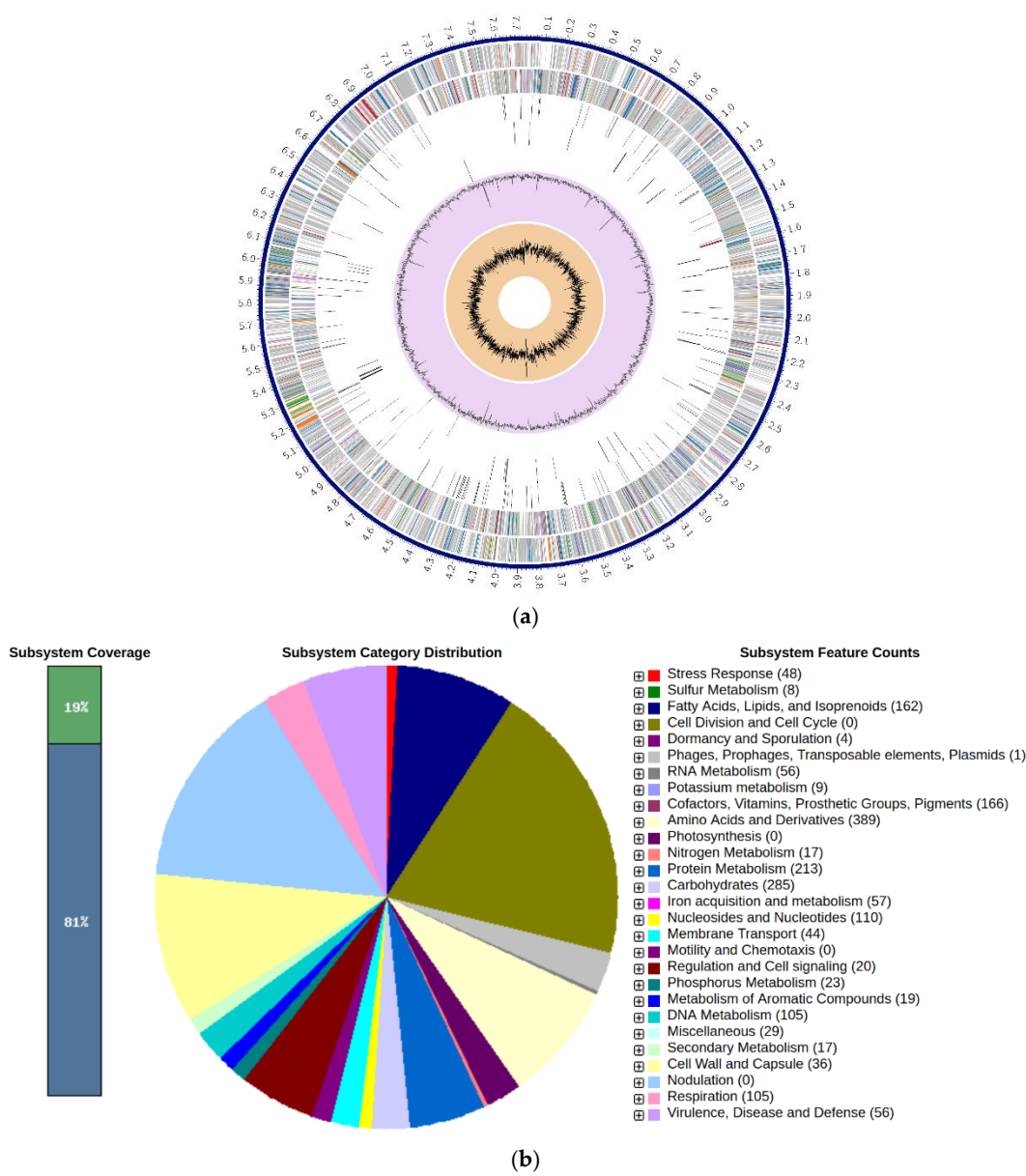
	Features	Value
Assembly details	Contig count	1
	Genome length	7,733,723 bp
	No. of plasmids	1
	Total no. of non-ATCG bases	19,290 (0.25%)

Annotated genome	Number of Ns per 100kb	249.43
	GC content	72.12%
	Contig L50	1
	Contig N50	7,733,723
	Coding density	88.91%
	Coding seq. count	6,923
	Coding seq. mean length	989.9 bp
	tRNA gene count	74
	tRNA mean length	76.01
	rRNA gene count	3
	rRNA mean length	1,595.33
	Count of repeats	152
	Repeat mean length	126.87
	CRISPR spacer count	81
	CRISPR spacer mean length	32.42
Proteins	Count of Hypothetical proteins	2,363 (34.13%)
	Count of proteins with functional assignment	4,560 (65.86%)
	Count of proteins with EC number assignment	1,207

Among the 6,923 coding sequence regions, only 1,269 (19%) were classified into subsystems, comprising 1,212 non-hypothetical proteins and 57 hypothetical proteins. Amino acids and derivatives (389) were the most predominant subsystem feature, followed by carbohydrates (285), protein metabolism (213), cofactors, vitamins, prosthetic groups, pigments (166), fatty acids, lipids, and isoprenoids (162), nucleosides and nucleotides (110), DNA metabolism (105), respiration (105), iron acquisition and metabolism (57), virulence, disease, and defense (56), RNA metabolism (56), stress response (48), membrane transport (44), cell wall and capsule (36) and others (Figure 2. b). Several genes associated with antibiotic resistance, drug targets, transporters, and virulence factors



were identified (Table 4). Antibiotic resistance genes, along with their associated antimicrobial resistance (AMR) mechanisms, were also identified (Table 5).



**Figure 2. (a)** Visualization of genome assembly and key features. The distribution of different genome features was provided as a circular graphical display. This includes, from outer to inner rings, the scaffolds, 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. **(b)** Summary of subsystems annotated by the RAST online server.

**Table 4.** Specialty genes.

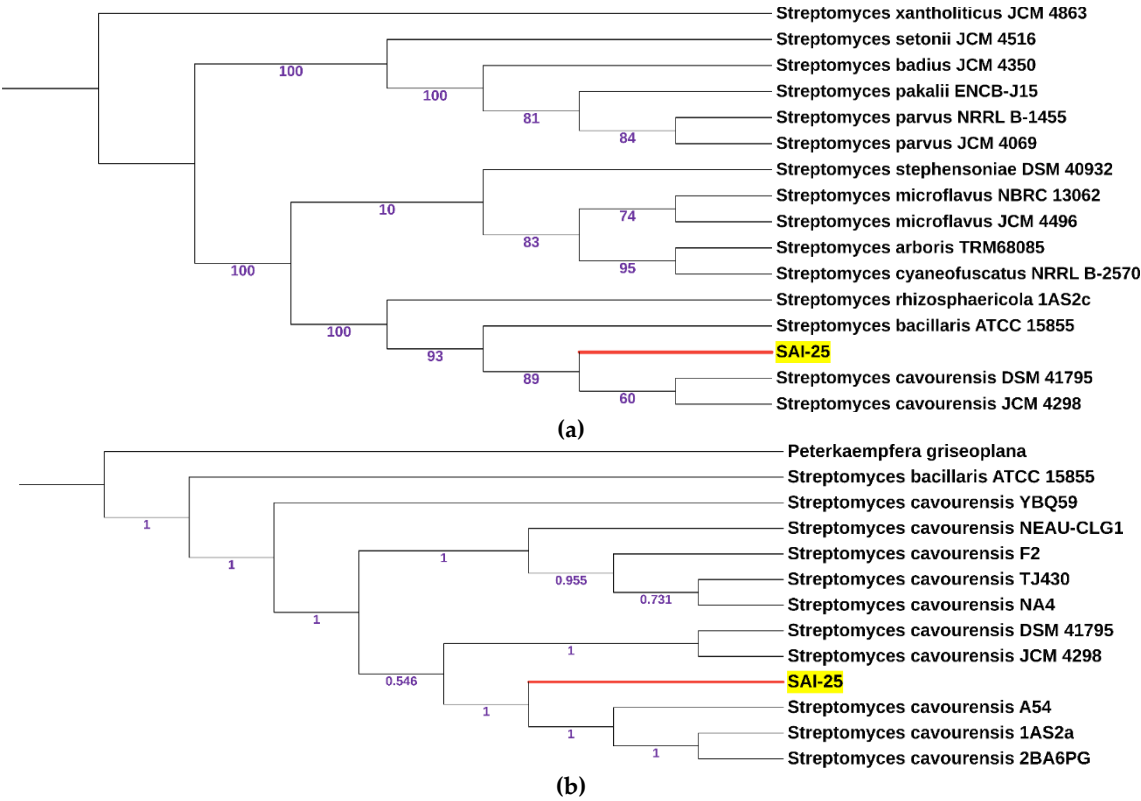
Property	Source DB	No. of genes
Antibiotic resistance	PATRIC	48
Drug targets	Drug Bank	6
Drug targets	TTD	1
Transporter	TCDB	36
Virulence factors	PATRIC_VF	3

**Table 5.** Antibiotic resistance genes.

AMR mechanism	Genes
Antibiotic activation enzyme	katG
Antibiotic inactivation enzymes	AAC(2')-I
Antibiotic target in susceptible genes	Alr,Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, FabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
Antibiotic target replacement protein	FabG, HtdX
Efflux pump conferring antibiotic resistance	CmIV family, Otr(C)
Gene conferring resistance via absence	gldB
Protein-altering cell wall charge	GdpD, MprF, PgsA
Regulator modulating expression of antibiotic resistance genes	LpqB, MtrA, MtrB, OxyR

3.2. Phylogenetic Relationship and Taxonomic Positioning of SAI-25

The SAI-25 strain, on its isolation, was initially assigned to *Streptomyces griseoplanus* based on the similarity of its amplified 16s rRNA sequence to database sequences (GenBank ID: KF770901) [19]. However, a recent study has reclassified this species to a novel genus in the family Streptomycetaceae, namely *Peterkaempfera* [32]. This necessitated a re-examination of the phylogenetic relationship of SAI-25. Comparison of the complete gene sequence of 16S rRNA extracted from the SAI-25 genome against the database of type strains indicated that it is closest to two type strains belonging to *Streptomyces cavourensis* (Figure S2). The high bootstrap value of the branch leading to the clade containing SAI-25 was high (83 out of 100), but a few other branches had lower bootstrap values (Figure S2). A whole-genome comparison with the genomes of type strains gave a similar relationship where the SAI-25 strain was still closely related to *Streptomyces cavourensis*, with an even higher bootstrap value (89 out of 100) (Figure 3. a). Thus, the phylogenetic tree obtained from TYGS (Figure 3. a) confirms that SAI-25, instead of genus *Peterkaempfera*, belongs to the genus *Streptomyces*.

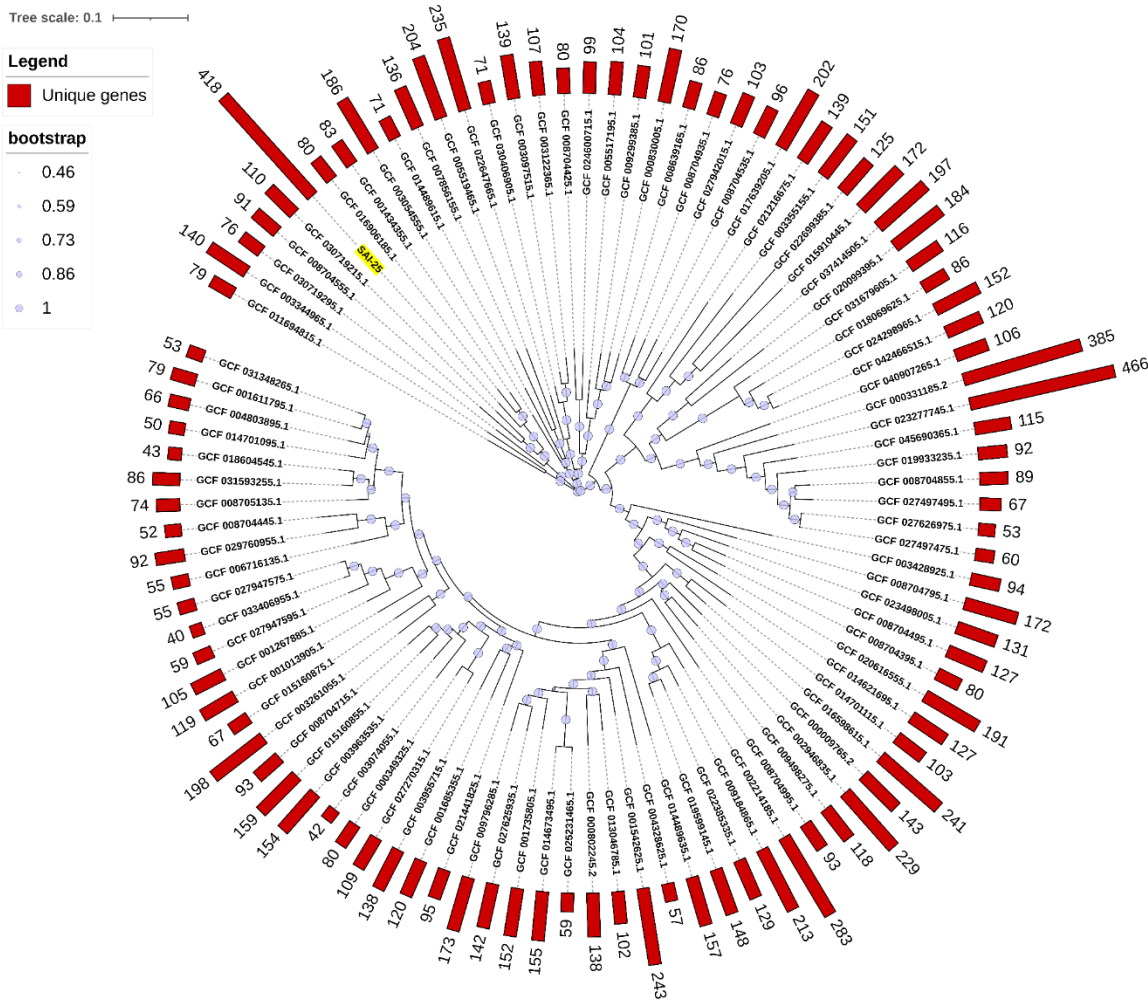


**Figure 3. (a)** Phylogenetic tree based on whole genome sequences of the SAI-25 strain and the closest type strains in the TYGS server. **(b)** The phylogenetic tree based on the number of overlapping orthogroups among the strains of the species that was closest to SAI-25 in the TYGS phylogenetic tree, with completeness  $\geq 99\%$ , and having scaffold or higher level assembly along with appropriate outgroups (the three closest type-strains in the TYGS phylogenetic tree and *Peterkaempferia griseoplana*, formerly *Streptomyces griseoplanus*)(as per Figure 3. a) by OrthoFinder. The phylogenetic trees were visualised by the iTOL server (Interactive Tree Of Life) [44].

To resolve the taxonomic positioning, the phylogenetic tree involving strains of *Streptomyces cavourensis* along with appropriate outgroups (three next closest type-strains in the TYGS phylogenetic tree, and *Peterkaempferia griseoplana* (formerly *Streptomyces griseoplanus*)) showed that SAI-25 was relatively more closer to a few strains of *S. cavourensis* than its remaining strains, indicating that SAI-25 belongs to *cavourensis* species (Figure 3. b). It is possibly a novel strain as evident by its phylogenetic position, being surrounded by the strains of *Streptomyces cavourensis* with a high bootstrap value (1 out of 1)(Figure 3. b).

3.3. Core Ortho-Groups and Unique Genes of SAI-25

In addition to the establishment of phylogenetic relationships, a pan-genome analysis was conducted to examine the core and unique gene sets. Comparison of proteomes of 97 representative *Streptomyces* species along with SAI-25 gave a total of 15,225 orthogroups and 11,686 unassigned genes (see methods)(Table S2). Among the 15,225 orthogroups, 1,695 orthogroups (11.13%) were present in all the 98 species (core set), and 248 orthogroups comprising of orthologs belonging to a single species (species-specific orthogroups) (1.63%) were obtained (Table S3). A total of 418 unique genes (second highest in the pangenome) were observed in the SAI-25 strain (Figure 4).



**Figure 4.** The phylogenetic tree based on the pan-genome analysis involving 97 good quality genome assemblies of *Streptomyces* and SAI-25 (highlighted in yellow)(see methods). Red bars indicate the number of unique genes. The phylogenetic tree was visualised by the iTOL server.

When these 418 unique genes were further analysed for functional characterisation by BLAST search in ‘Non-Redundant’ database, followed by ‘Reciprocal Best BLAST’; 147 out of 418 unique genes still showed partial but significant homology with genes/proteins belonging to *Streptomyces* genus, with query and subject coverage  $\geq 50\%$  and e-value  $\leq 0.01$ . Only 4 of the remaining 271 unique genes were functionally annotated (Table 6).

**Table 6.** Functional annotation of unique genes of the SAI-25 strain.

ID	Annotation/Function	Source	Evidence
fig 1472664.5.peg.3363	Ribokinase (EC 2.7.1.15)	RAST server	Code: idu(2);D-ribose_utilization idu(2);Deoxyribose_and_Deoxynucleoside_Catabolism
fig 1472664.5.peg.4738	PE-PGRS FAMILY PROTEIN	RAST server	Not provided
fig 1472664.5.peg.5531	Xanthine dehydrogenase, molybdenum binding subunit (EC 1.17.1.4)	RAST server	Code: icw(2);Purine_Utilization icw(2);Xanthine_dehydrogenase_subunits
fig 1472664.5.peg.1814	ligA protein [ <i>Mycobacterium pseudoshottsii</i> JCM 15466]	NCBI BLAST followed by Reciprocal Best BLAST	Accession: GAQ32343.1 e-value: 2.36E-04 Alignment length: 461 Percentage identity: 32.936 Query coverage (fig 1472664.5.peg.1814): 81% Subject coverage (GAQ32343.1): 85%

3.4. Secondary Metabolite Potential of SAI-25

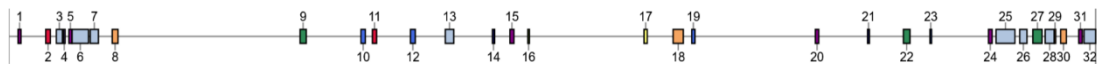
A total of 32 biosynthetic gene cluster (BGC) regions were predicted using antiSMASH (Figure 5). Of these, 13 regions exhibited  $\geq 81\%$  similarity to known clusters. These 13 regions were responsible for biosynthesis of three siderophores (griseobactin, coelichelin and desferrioxamine B) [45–47]; geosmin, which not only tends to give earthy smell to soil but also regulates seed germination and acts as a chemical repellent/attractant to predators (nematodes and protists) and insects [48]; naringenin, a flavonoid which alleviates abiotic stress (osmotic and salinity stress) and also contributes to pathogen resistance in plants [49–52]; ectoine, an osmoprotectant which alleviates cadmium-induced stress in plants [53,54]; AmfS, whose derivative acts as extracellular morphogen for onset of aerial-mycelium [55]; keywimysin, a lasso peptide whose biological function remains unknown [56]; bafilomycin B1, a macrolide antibiotic which inhibits vacuolar-type ATPase (V-ATPase) [57,58]; 10-epi-HSAF (along with its analogues) which shows antifungal activities against plant pathogens [59]; valinomycin, a potassium ionophore which demonstrates a diverse spectrum of biological activities (antibacterial, antifungal, insecticidal, etc.) [60]; montanastatin, a cancer cell growth inhibitory cyclooctadepsipeptide [61]; alkylresorcinol, a polyketide which exhibits a wide range of bioactivities (antimicrobial, anti-cancer, antilipidemic, antioxidant, etc.) [62]; and isorenieratene, a natural antioxidant and photo/UV damage inhibitor [63] (Table 7)(Table S4).

The regions that had very little or no similarity to any known cluster were subjected to KEGG PATHWAY analysis (see methods). Out of 19 such regions, only three regions (Region 18, 20, and 21) were found to be part of the existing biosynthetic pathways for secondary metabolites. Region 18 was responsible for biosynthesis of type II polyketide backbone (Figure S3), region 20 for terpenoid backbone biosynthesis (Figure S4), and region 21 for D-amino acid metabolism (Figure S5).

Complete pathways for the biosynthesis of two more metabolites were discovered while examining the SAI-25 genome/proteome for genes and pathways associated with the seven PGP and



biocontrol traits (discussed in section 3.6, Table 1): (-)-Germacrene D, an aphid repellent sesquiterpenoids [64], and (+)-Caryolan-1-ol, an antifungal volatile [65] (Figure S6).



**Figure 5.** Distribution of BGC regions within the genome of SAI-25 as predicted by antiSMASH. The color of the boxes has no relevance to their function.

**Table 7.** Metabolites produced by BGC regions and their corresponding functions. Asterisk (\*) indicates the BGCs annotated using the KEGG database, otherwise the rest were annotated by antiSMASH.

Metabolites	Biosynthetic gene cluster	Functions	References
Geosmin	Region 5	Regulates seed germination and acts as a chemical repellent/attractant to predators (nematodes and protists) and insects	[48]
Griseobactin	Region 6	Siderophore	[45]
Coelichelin	Region 7	Siderophore	[46]
Naringenin	Region 8	Alleviates abiotic stress (osmotic and salinity stress) and also contributes to pathogen resistance in plants	[49–52]
Desferrioxamine B	Region 11	Siderophore	[47]
Ectoine	Region 16	An osmoprotectant that alleviates cadmium-induced stress in plants	[53,54]
AmfS	Region 17	Whose derivative acts as an extracellular morphogen for the onset of aerial mycelium	[55]
Biosynthesis of type II polyketide backbone	Region 18*	It is utilised for the biosynthesis of type II polyketide products	Figure S3
Keywimysin	Region 19	A lasso peptide whose biological function remains unknown	[56]
Terpenoid backbone biosynthesis	Region 20*	It is utilised in sesquiterpenoids and triterpenoids biosynthesis	Figure S4
D-Amino acid metabolism	Region 21*	It plays a role in the production of D-proline, which is utilised for biosynthesis of linatine (a vitamin B6 antagonist)	Figure S5 and [66]
Bafilomycin B1	Region 25	A macrolide antibiotic that inhibits vacuolar-type ATPase (V-ATPase)	[57,58]
10-epi-HSAF and its analogues	Region 26	Shows antifungal activities against plant pathogens	[59]
Valinomycin and Montanastatin	Region 28	Valinomycin is a potassium ionophore which demonstrates a diverse spectrum of biological activities (antibacterial, antifungal, insecticidal, etc.), and Montanastatin is a cancer cell growth inhibitory cyclooctadepsipeptide	[60,61]
Alkylresorcinol	Region 30	A polyketide which exhibits a wide range of bioactivities (antimicrobial, anti-cancer, antilipidemic, antioxidant, etc.)	[62]
Isorenieratene	Region 31	A natural antioxidant and photo/UV damage inhibitor	[63]

3.5. Potential Genes/Enzymes Responsible for Biosynthesis of an Insecticidal Diketopiperazine Derivative, Cyclo(Trp-Phe)

Since the SAI-25 strain was earlier reported to have activity of one of the Cyclodipeptides (CDPs), Cyclo(Trp-Phe), so the SAI-25 genome was searched for the two biosynthetic enzyme families: Non-ribosomal peptide synthetases (NRPSs) and cyclodipeptide synthases (CDPSs). While



no hits were observed for CDPs enzyme family, a total of 15 proteins showed hits to all three domains of NRPSs. Out of these 15 proteins, 1 had three A-domains, 6 had two A-domains, and 8 had single A-domain. Based on the RAST annotation, 8 of the above mentioned proteins were recognised as polyketide synthase modules and related proteins; 3 were identified as hypothetical proteins; 3 were noted as siderophore biosynthesis non-ribosomal peptide synthetase modules; and 1 was annotated as capsular polysaccharide biosynthesis fatty acid synthase, WcbR. All 15 proteins identified above were part of one or the other BGCs as per the results of the antiSMASH (Table 8). The A-domains of all 15 proteins were predicted to have affinity for multiple amino acids (including tryptophan and phenylalanine)(Table S5). Only one protein (ID: fig|1472664.5.peg.5776) was predicted by the antiSMASH to utilize phenylalanine and tryptophan, along with 3 more substrates (Ph-Gly, Tyr, and bOH-Tyr).

**Table 8.** Details of the 15 proteins belonging to the SAI-25 strain that were identified to be a NRPSs.

Protein ID	RAST annotation	Biosynthetic gene cluster	Number of A-domains
fig 1472664.5.peg.6606	hypothetical protein	Region 28	2
fig 1472664.5.peg.6542	Polyketide synthase modules and related proteins	Region 27	2
fig 1472664.5.peg.481	Siderophore biosynthesis non-ribosomal peptide synthetase modules	Region 7	3
fig 1472664.5.peg.6541	Siderophore biosynthesis non-ribosomal peptide synthetase modules	Region 27	2
fig 1472664.5.peg.429	Siderophore biosynthesis non-ribosomal peptide synthetase modules	Region 6	2
fig 1472664.5.peg.5776	Polyketide synthase modules and related proteins	Region 22	1
fig 1472664.5.peg.2757	Polyketide synthase modules and related proteins	Region 13	1
fig 1472664.5.peg.6452	Capsular polysaccharide biosynthesis fatty acid synthase WcbR	Region 26	1
fig 1472664.5.peg.6605	hypothetical protein	Region 28	2
fig 1472664.5.peg.2758	Polyketide synthase modules and related proteins	Region 13	1
fig 1472664.5.peg.6533	Polyketide synthase modules and related proteins	Region 27	1
fig 1472664.5.peg.392	Polyketide synthase modules and related proteins	Region 6	1
fig 1472664.5.peg.6862	Polyketide synthase modules and related proteins	Region 32	2
fig 1472664.5.peg.2755	Polyketide synthase modules and related proteins	Region 13	1
fig 1472664.5.peg.5774	hypothetical protein	Region 22	1

3.6. Genes/Pathway Underlying PGP Features

The SAI-25 strain genome was analysed to identify the pathways/genes responsible for the seven experimentally validated PGP and biocontrol traits (Table 1).

A siderophore group nonribosomal peptides biosynthesis pathway was observed to be present in the SAI-25 strain (Figure S7; Table S6). Additionally, 3 BGC regions responsible for the production of siderophores (griseobactin, coelichelin and desferrioxamine B) were also observed to be present in the SAI-25 strain (Table 7).

Enzymes having a role in chitin metabolism, such as Chitinase (eleven copies), Chitodextrinase (one copy), beta-glycosyl hydrolase (four copies), beta-N-acetylglucosaminidase (one copy), and endochitinase (one copy) were identified in the SAI-25 strain genome (Figure S8; Table S7). Cellulolytic enzymes such as endoglucanase, glycoside hydrolase, cellulose 1,4-beta-cellobiosidase, and beta-glucosidase were also present in the SAI-25 strain genome (Figure S9; Table S8). Several kinds of lipases and proteases, which play a major role in plant growth and protection, were identified in the SAI-25 strain genome (Table S9; Table S10).

The Indole-3-acetamide (IAM) pathway is one of the extensively studied pathways for the biosynthesis of Indole-3-acetic acid (IAA). In this pathway, tryptophan is converted to IAM, which is then hydrolysed to IAA. Two major enzymes are required for this pathway: tryptophan monooxygenase, which converts tryptophan to IAM, and indole-3-acetamide hydrolase for hydrolysis of IAM into IAA [67]. BLAST search of tryptophan monooxygenase protein sequence against SAI-25 strain proteome showed presence of its ortholog. KEGG PATHWAY analysis identified two proteins that can convert IAM into IAA (Figure S10; Table S11).

Three enzymes, hydrogen cyanide synthase subunit HcnA, hydrogen cyanide synthase subunit HcnB, and hydrogen cyanide synthase subunit HcnC are required for biosynthesis of hydrocyanic acid [68]. Through pan-genome-wide BLAST, one ortholog of HcnA, one ortholog of HcnB, and three orthologs of HcnC were identified (Table S12).

## 4. Discussion

### 4.1. SAI-25's Chromosome-Level Assembly with High Completeness Will Be a Valuable Resource for *Streptomyces* Genome Mining

The NCBI genome database currently has 11,237 *Streptomyces* genomes, out of which only 1,357 (~12%) are at chromosome level or higher assembly level (<https://www.ncbi.nlm.nih.gov/datasets/genome/>; accessed on April 2025). The single linear genome of SAI-25, with completeness >99% and total gap size of <20 Kb, makes it among the good quality assemblies. For a meaningful and accurate pan-genome analysis or genome mining for BGCs, the quality of assembly plays a key role, in particular, the completeness and extent of fragmentation [69].

Even for the high-quality assembly, concerns regarding the relatively higher number of hypothetical genes were raised earlier [69], and even in this genome assembly, a slightly higher fraction of hypothetical genes were observed (34%). Among the set of unique genes, surprisingly, the majority of them were hypothetical genes, indicating the limitations of annotation pipelines when it comes to species-specific genes.

### 4.2. Phylogenetic Analysis Corrected the Species Name from *S. griseoplanus* to *S. cavourensis*

When the SAI-25 strain was first reported for its biocontrol and PGP features, it was initially assigned to *S. griseoplanus* species based on the 16S rRNA sequencing [19]; the taxon has recently been re-assigned to a separate genus, namely, *Peterkaempferia griseoplanus*, as per the NCBI taxonomy database [32]. However, the availability of whole genome sequences led to the re-examination of the taxonomic assignment, and *S. cavourensis* emerged as the species based on comparison with whole genomes of type strains as well as that of strains of the species that were closest to the SAI-25.

*S. cavourensis* strains have earlier been isolated, largely from soil, and almost all were found, either experimentally or computationally, to have potential for biosynthesis of several active compounds or secondary metabolites [70–73]. For instance, *S. cavourensis* strain 1AS2a, isolated from the wheat rhizosphere in the Brazilian Neotropical savanna, has a genome size similar to SAI-25 (~7.6 Mb), with a similar number of BGCs (n=30), and also exhibited strong antimicrobial activities [71].

As far as biosynthetic potential of different strains of *S. cavourensis* are concerned, the TJ430 strain isolated from mountain soil from China had proteins related to antibiotic synthesis, and tolerance or detoxification of metals [70]. Another strain, TN638, isolated from an industrial waste soil, was detected having three cyclodipeptides or diketopiperazine (DKP) derivatives, and four macrotetrolides [72]; both groups of compounds showed strong antibacterial activity against *A. tumefaciens* ATCC 23308 and *S. typhimurium* ATCC 14028.

### 4.3. Presence of Sixteen Annotated and the Same Unannotated BGCs Highlights Its PGP and Industrial Potential

Large-scale genome mining of *Streptomyces* genomes (n=1,110) has shown that *Streptomyces* bacteria carry BGCs in the range of 8–83 per genome, which weakly correlated with the genome size [74]. The SAI-25 genome was predicted to have 32 BGCs, which was close to the mean for this genus.

The *S. griseoplanus* SAI-25 used in this study exhibited insecticidal properties such as antifeedant, insecticidal, and pupicidal activity against *H. armigera*. The SAI-25 was also reported to have antifungal activity against the charcoal rot of sorghum pathogen *Macrophomina phaseolina* [20]. The BGC prediction in the SAI-25 genome showed the presence of several metabolites with biocontrol properties. For example, Valinomycin, a potassium ionophore, reportedly has a diverse spectrum of biological activities, such as antibacterial, antifungal, and insecticidal [60]. The BGC was observed for another broad-spectrum biocontrol agent, Bafilomycin B1, a macrolide antibiotic. The Bafilomycin B1 and C1 from *S. cavourensis* NA4 showed significant inhibitory activities against a variety of *Fusarium* spp. and *R. solani*, while being inactive against *Setosphaeria turcica* [75]; thus, they could be used as potential biocontrol agents for soil-borne fungal diseases of plants. Yet another antifungal metabolite, a polycyclic tetramate macrolactams (PTMs) type 10-epi-HSAF, showed modest antifungal properties [59].

Besides the BGCs for biocontrol, several others were annotated for plant-growth promotion. The SAI-25 genome has BGCs for biosynthesis of several siderophores, such as Griseobactin, Coelichelin, and Desferrioxamine B, indicating their role in mineral mobilisation. In addition to siderophore, a few metabolites having a role in abiotic stress were also found (Naringenin, Ectoine, etc.). Beyond the agriculturally important secondary metabolites, SAI-25 was predicted to have BGCs with even chemotherapeutic potential, such as Alkylresorcinol and Montanastatin. However, these two metabolites didn't overlap with the list of 38 BGCs with known chemotherapeutic potential found in *Streptomyces* species [74]. Surprisingly, the most common chemotherapeutic gene cluster in *Streptomyces*, namely, the macrolide FD-891, was missing in SAI-25.

#### 4.4. Limited Success in Prediction of Genes/BGCs for Cyclo(Trp-Phe) Biosynthesis Opens the Scope for Further Characterization

SAI-25 strain was earlier reported to produce an insecticidal cyclodipeptide, cyclo(Trp-Phe) [17], and the BGC prediction in the SAI-25 genome also showed the presence of a few distinct classes of cyclopeptides, such as Montanastatin, a cyclooctadepsipeptide (CODP). The computational prediction of genes/BGCs for the cyclo(Trp-Phe) could only narrow down to a few candidate genes, thus remaining incomplete. Although cyclodipeptides have been reported in other strains of this species, such as *S. cavourensis* TN638 [72], and strains of other species, such as *S. leeuwenhoekii* NRRL B-24963 [76], eight different strains of *Streptomyces* [77]; however, the substrate diversity among the cyclodipeptides could be one of the main challenges for their characterization.

#### 4.5. Limitations and Future Directions

Only 4 out of the 271 unique genes identified in the SAI-25 strain could be annotated, indicating the need for a more effective annotation pipeline or software. Moreover, a huge number of hypothetical genes were also found in the genome annotation. Although the SAI-25 strain has the potential to biosynthesize various secondary metabolites with a broad range of biological functions, the extent of their production and the specific conditions that stimulate their biosynthesis require further investigation. In addition, a few of the BGCs remain unannotated including the partially annotated BGC for Cyclo(Trp-Phe). The anti-pesticidal and anti-fungal activity of SAI-25 should be further explored as an alternative pest management tool that can help in exploring its utility in sustainable agriculture.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Abbreviations

The following abbreviations are used in this manuscript:

10-epi-HSAF	10-epi-heat-stable antifungal factor
A-domain	Adenylation-domain
AMR	Antimicrobial resistance
BGC	Biosynthetic Gene Cluster
BLAST	Basic Local Alignment Search Tool
BlastKOALA	Automatic KO assignment and KEGG mapping service
boH-Tyr	β-hydroxytyrosine
bp	base pairs
BUSCO	Benchmarking Universal Single-Copy Orthologs
CDPs	Cyclodipeptides
CDPSs	Cyclodipeptide Synthases
CDS	Coding DNA Sequence
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
.faa	FASTA format for Amino Acid sequences
FTIR	Fourier Transform Infrared
HMMs	Hidden Markov Models
IAA	Indole-3-Acetic Acid
IAM	Indole-3-acetamide
InterPro	Integrative Protein Signature Database
iTOL	Interactive Tree Of Life
KEGG	Kyoto Encyclopedia of Genes and Genomes
NCBI	National Center for Biotechnology Information
NRPSs	Non-Ribosomal Peptide Synthetases
Pfam	Protein Families And Motifs

PGP	Plant Growth Promotion
PGPB	Plant Growth Promoting Bacteria
Phe	Phenylalanine
Ph-Gly	Phenylglycine
RAST	Rapid Annotation using Subsystem Technology
Trp	Tryptophan
TYGS	Type (Strain) Genome Server
Tyr	Tyrosine
WGS	Whole genome Shotgun Sequencing

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