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RESEARCH ARTICLE

# Whole Genome Sequencing of *Curtobacterium flaccumfaciens* Strain SHGH, Isolated from the Nodes of *Olea europaea* in Mosul, Iraq

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## ABSTRACT

*Curtobacterium flaccumfaciens* SHGH was associated with Koch's postulates about wilt disease in *Olea europaea* (olive trees) located in Mosul, Iraq. Since olive trees are an important cash crop for Iraq, a whole-genome analysis of SHGH was conducted. The complete genome was sequenced using the IIlumina MiSeq sequencer and uploaded to GenBank under the accession number JAUMSN000000000.1. The circular chromosome consists of 3,834,306 bp, 70.7 percent GC content, 4,176 protein-coding sequences, 53 RNA genes, 7 ribosomal RNA genes, and 42 transfer RNA genes. According to the *in silico DNA-DNA hybridization* technique are 11 strains closely related to the SHGH strain. The 16S rRNA gene sequence analysis showed that the SHGH strain has 100% similarity with *C. flaccumfaciens* LMG 3645 and 99% similarity with the *Curtobacterium allii* 20TX0166 gene over 100 replicates in the bootstrap test. The SHGH strain analysis showed the presence of many genes encoding pathogenicity-associated enzymes such as pectate lyase, glycosyl hydrolase, serine proteinase, beta-1,4-glucanase, and 1,4-beta-xylanase enzymes. Furthermore, the genome analysis of the SHGH strain showed that it contained peptidases, glycosidases, and potential glycopolymer-degrading domains in prophage-derived regions. These help the microbe establish a biofilm and colonize with other microbes to form a microbial community, which is a necessary step for the progression of many bacterial plant diseases, such as bacterial wilt disease.

**Keywords:** Biofilm formation, *Curtobacterium flaccumfaciens*, *Olea europaea*, Virulence associated genes, Whole genome sequencing

## Introduction

With the exception of freezing climates and the Arctic, olive trees are found all over the world and are a regular source of compounds with significant biological qualities such as glycerol compounds, fatty acids and many others. [1,2] *Curtobacterium flaccumfaciens* is a Gram-positive, xylem-inhabiting plant pathogen that causes bacterial wilt in a variety of legume crops, such as dry bean (*Phaseolus vulgaris*), cowpea (*Vignaunguiculata*), mungbean (*Vigna radiata*), and soybean (*Glycine max*). *C. flaccumfaciens* enters the host by contaminated seed, wounds, and natural openings infecting the vascular tissue, hindering water and nutrient transfer. [3] *C. flaccumfaciens* is known in Australia as the source of mungbean tan spots, which can result in yield losses of up to 25%. [3] *C. flaccumfaciens* pv. *flaccumfaciens* is the most dominating pathovar within the species with a wide geographic range and produces economic yield losses on the host plants. [4] The European and Mediterranean Plant Protection Organization has placed *C. flaccumfaciens* on the A2 (high risk) list of quarantine diseases, putting it under rigorous quarantine control and zero tolerance in multiple countries. [5] *C. flaccumfaciens* pv. *flaccumfaciens* is a phytopathogen that is well recognized for its colorful colony variants with yellow, orange, pink, purple, and red colonies on culture media, with

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yellow-pigmented strains being more widespread and virulent compared to other variants.[5] In a recent study (unpublished), *C. flaccumfaciens* is the causative agent of bacterial wilt disease in olive trees; one of the first studies showing the microbe as the causative agent.

The initial soil microbiome and chemical composition are important factors for bacterial wilt disease onset.[6] Bacterial wilt disease caused by SHGH begins at the site of plant damage, like *C. flaccumfaciens* pv. *faccumfaciens* in dry bean plants.[7] From there, the pathogen spreads via the xylem and phloem throughout the plant. As pathogens spread throughout the plant, they break down hemicellulose to infiltrate cell walls, causing wilting and plant death.[8] Individual plant genetics can influence the co-colonizers and biofilm producers propagating the disease.[9] The SHGH strain causes characteristic yellow blotched pigments, as seen in other cash crops in the area.[5] Despite the economic relevance of bacterial wilt disease in the food industry, the causal agent has yet to be explored for genetic characteristics and virulence repertoires. Indeed, *C. flaccumfaciens* pv. *flaccumfaciens* is the most sparsely investigated Gram-positive bacterial plant pathogen in terms of genetic characteristics and pathogenicity factors. This is probably due to a lack of molecular tools for manipulating genes for this Gram-positive actinobacteria.

Currently, there are over 3000 farmers producing ∼7000 tons of olives yearly in Iraq;[10] making this as an important issue for the area. Due to the impact of the SHGH strain on this vital cash crop, its whole genome was sequenced in this study to explore the genes involved in its pathogenicity.

## Materials and methods

### *Isolation of C. flaccumfaciens*

The *Olea europaea* nodes were completely crushed using a ceramic mortar and submerged in physiological normal saline to preserve bacterial viability. Then incubated overnight at 28 °C for 24 h. The nutrient agar medium was prepared by the Neogen/UK (Spanish) manufacturer instructions. To achieve this, 28 g of nutritional agar were dissolved in one liter of D.W. Overnight cultures grown in NA media were incubated for 72 hours at 28 °C, then the growth and color changes were observed.[5]

### *Genome sequencing*

The genome of the isolated bacteria was sequenced using an IIlumina MiSeq next generation sequencer by Psomagen sequencing company/USA.

### *Genome assembly and annotation*

The raw reads were *de novo* assembled to contigs using SPAdes 3.5 bioinformatics tool[11] applying settings of k-mer length of 21,33,55,77. QUAST software[12] was used to generate assembly statistics. The assembled genome was annotated with the Rapid Annotation using Subsystem Technology (RAST) server.[13] The SEED tool[14] was used for predicting functional genes in subsystem categories.

### *Whole genome based phylogenetic tree*

The Type Strain Genome Server (TYGS)[15] was performed to infer the whole-genome-based phylogenetic tree of *C. flaccumfaciens* SHGH and the most closely related strains. The genome in FASTA format was uploaded to the server with the default settings. The tree was inferred with FastME 2.0 program[16] which is integrated within the TYGS.

### *In silico DNA-DNA hybridization (isDDH) analysis*

The Genome-to-Genome Hybridization similarity (GGDH) bioinformatics tool[17] was used to measure *is*DDH values between *C. flaccumfaciens* SHGH and the most closely related strains based on whole genome sequences data.

### *16S rRNA gene phylogenetic tree analysis*

The Nucleotide Basic Local Alignment Search Tool (BLASTn) program was used to search for homology to the *C. flaccumfaciens* SHGH sequence against sequences which are available on the NCBI GenBank database. The phylogenetic tree was constructed by bootstrap (100X) analysis using the MEGA-11 software.[18]

### *Detection of virulence associated genes in C. flaccumfaciens SHGH*

The BLASTp program was used to search for homology of virulence associated genes that are involved in pathogenicity against the *C. flaccumfaciens* SHGH annotated genome.

### *Genome comparisons*

The GView tool[19] was used to align the *C. flaccumfaciens* SHGH genome with the most closely related species to generate image that shows difference and similarity between the sequence of the first bacterium and other sequences as a set of concentric rings.

Table 1. General genome features of *C. flaccumfaciens* SHGH generated using QUAST software and RAST server.

| Feature | Value |
|---|---|
| Genome total length (bp) | 3,834,306 |
| Number of contigs | 873 |
| Largest contig (bp) | 20,188 |
| Smallest contig (bp) | 2003 |
| GC content (%) | 70.7 |
| Total protein-coding sequences (CDSs) | 4176 |
| Number of RNA genes | 53 |
| Number of rRNA genes | 2, 2, 3 (5S, 16S, 23S) |
| Number of tRNA genes | 42 |
| N50 | 4,917 |

## Results and discussion

### Sequencing, genome analysis and closely related strains

The *C. flaccumfaciens* SHGH genome has been deposited at DDBJ/ENA/GenBank under the accession number JAUMSN000000000.1. Final assembly of the *C. flaccumfaciens* SHGH genome resulted in 873 contiguous sequences ranging from 2,003bp to 20,188bp with an average length of 4,917bp. The circular chromosome consists of 3,834,306bp, 70.7% GC content, 4,176 protein-coding sequences, 53 RNA genes, 7 rRNA genes and 42 tRNA genes as listed in Table 1. In all, there are 11 closely associated type strains to SHGH based on *is*DDH. The SHGH genome is relatively large when compared to other closely related *Curtobacterium sp.*, the lowest GC percentage and the largest number of protein-coding sequences when compared to the 11 strains. The smallest nucleotide diversity was with *C. flaccumfaciens* LMG 3645 and *C. flaccumfaciens* CFBP 3418 at a value of 0.06, respectively see Table 2.

*Curtobacterium allii* 20TX0166, *Curtobacterium pusillum* ATCC 19096, *Curtobacterium citreum* JCM 1345, *Curtobacterium citreum* DSM 20528, *Curtobacterium albidum* DSM 20512, *Curtobacterium luteum* DSM 20542, *Curtobacterium luteum* JCM 1480, *Curtobacterium luteum* ATCC 15830 and *Curtobacterium herbarum* DSM 14013 were next closest in relation based off total genome composition, in that order as shown in Fig. 1 Comparing the SHGH genome to closely related *Curtobacterium* sp. showed unique regions in the SHGH genome, as well as segments missing see Fig. 2.

Analysis of the *16S rRNA* gene revealed that the SHGH strain has 100 percent nucleotide similarity with *C. flaccumfaciens* LMG 3645 as well as most of the *Curtobacterium allii* 20TX0166 gene, scoring 99 percent over 100 replicates in the bootstrap test as illustrated in Fig. 3. Additionally, *Curtobacterium oceanosedimentum* ATCC 31317, *Curtobacterium pusillum* DSM 20527, *Curtobacterium luteum* DSM 20542, *Curtobacterium citreum* DSM 20528, *Curtobacterium ammoniigenes* NBRC 101786, *Curtobacterium albidum* DSM 20512, *Curtobacterium herbarum* P 420-07 and *Curtobacterium* sp. Leaf261 all showed greater than 98 percent sequence similarity with the SHGH *16S rRNA* gene as illustrated in Table 3.

The percentage of replicate trees in which the associated strains clustered together in the bootstrap test (100 replicates) are shown next to the branches.

The genome was annotated using the RAST server. The pie chart shows the count of each subsystem feature and the subsystem coverage is displayed using SEED viewer. The green bar of the subsystem coverage corresponds to the percentage of the proteins included in the subsystems while the blue bar corresponds to the percentage of the proteins that are not included in the subsystems see Fig. 4.

### Genes associated with the pathogenicity of C. flaccumfaciens SHGH

There are 31 genes associated with virulence, disease, and defense in the *C. flaccumfaciens* SHGH strain as shown in Fig. 4. The whole genome sequence analysis of the strain SHGH revealed the presence of several genes which encode for enzymes that are associated with pathogenicity. This includes a pectate lyase (protein id: QFS80865.3) with an identity of 100% and 51% to pectate lyase present in the genomes of *C. flaccumfaciens* (accession no. WP_164941293.1) and *Clavibacter michiganensis* (accession no. WP_258059284.1), respectively. The glycosyl hydrolase family 32 (protein id: QFS80892.1) with an identity of 100% to *C. flaccumfaciens* (accession no. WP_164941293.1) was detected. The serine proteinase (protein id: QIH95653.1) with an identity of 100% to that of *C. flaccumfaciens* (accession no. MBO9041541.1) and 44.33% to *Clavibacter michiganensis* (accession no. AWF99952.1) was found.

In addition, the whole genome sequence analysis of *C. flaccumfaciens* SHGH revealed the presence of two genes with their key enzymes potentially associated with the pathogenicity. These enzymes include beta-1,4-glucanase (cellulase) (EC 3.2.1.4) and 1,4-beta-xylanase (EC 3.2.1.8), as seen in other pathogenic endophytes.[20] Moreover, *C. flaccumfaciens* SHGH contains the following enzymes that are suggested to contribute to the pathogenicity[21,22] a putative polysaccharide deacetylase, glycosyl hydrolase, glycodyl transferase and several short-chain dehydrogenases.

Interestingly, SHGH does not have any plasmids or prophages containing the genes for pathogenicity as

**Table 2.** Genome pairwise comparisons of *C. flaccumfaciens* SHGH genome vs. closely related type strain genomes based on isDDH, GC content, $\delta$- value, genome size and number of proteins.

| *C. flaccumfaciens* SHGH vs. type strain genome | Digital isDDH value (%) | Percent G + C (%) | $\delta$-value | Genome size (bp) | Number of proteins |
|---|---|---|---|---|---|
| *C. flaccumfaciens* LMG 3645 | 74.5 | 71.01 | 0.06 | 3,818,932 | 3,584 |
| *C. flaccumfaciens* CFBP 3418 | 74.5 | 70.99 | 0.06 | 3,820,853 | 3,536 |
| *Curtobacterium allii* 20TX0166 | 69.2 | 70.77 | 0.077 | 3,980,909 | 3,738 |
| *Curtobacterium pusillum* ATCC 19096 | 45.9 | 70.87 | 0.199 | 3,600,006 | 3,390 |
| *Curtobacterium citreum* JCM 1345 | 42.5 | 72.04 | 0062 | 3,581,946 | 3,428 |
| *Curtobacterium citreum* DSM 20528 | 42.4 | 71.93 | 0.066 | 3,612,036 | 3,415 |
| *Curtobacterium albidum* DSM 20512 | 41.2 | 71.93 | 0.064 | 3,665,687 | 3,480 |
| *Curtobacterium luteum* DSM 20542 | 39.7 | 71.7 | 0.087 | 3,620,063 | 3,414 |
| *Curtobacterium luteum* JCM 1480 | 39.5 | 71.78 | 0.086 | 3,591,662 | 3,396 |
| *Curtobacterium luteum* ATCC 15830 | 39.4 | 71.81 | 0.084 | 3,693,908 | 3,367 |
| *Curtobacterium herbarum* DSM 14013 | 27.1 | 71.44 | 0.191 | 3,515,806 | 3,328 |

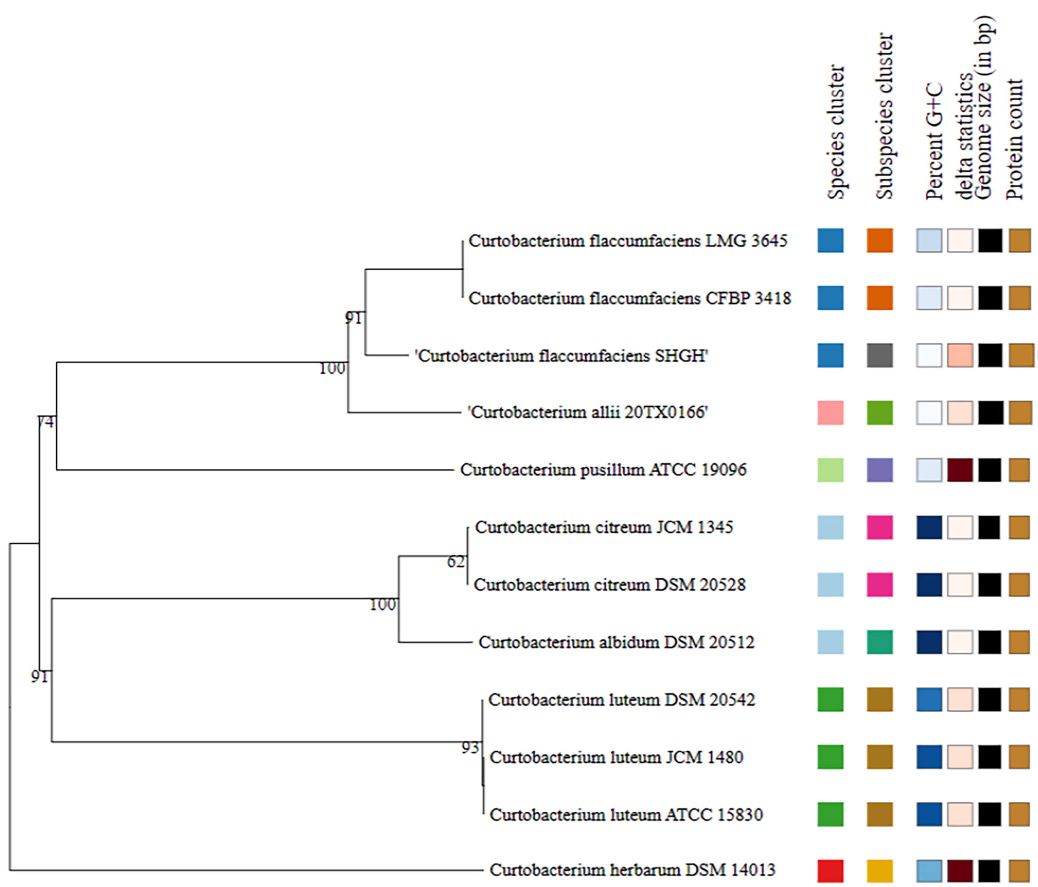


**Fig. 1.** Phylogenetic taxonomy tree of *C. flaccumfaciens* SHGH using TYGS server. The final tree was constructed with FastME 2.0 approach based on balanced minimum evolution method (100X pseudo-bootstrap support values). Labels on leaves are indicated by association to species and subspecies clusters, genomic GC percent, $\delta$-values, overall genome size and total number of proteins.

does *C. flaccumfaciens* P990 and *Curtobacterium* sp. YC1.[23,24] *Curtobacterium* genomes have been shown to have putative glycopolymer-degrading domains, glycosidases and peptidases enzymes,[25,26] which aid in establishing a biofilm and allow the microbe to colonize along with other microbes to form a microbial community, a needed progression for many bacterial plant diseases including bacterial wilt disease.[27] However, like the SHGH strain, other phytopathogenic strains of *Curtobacterium* do not possess any plasmids or prophage sequences and harbor their pathogenic genes on the chromosome, including lytic enzymes, toxins and hormones that disrupt plant cell walls and aid in acquiring nutrients.[28,29]

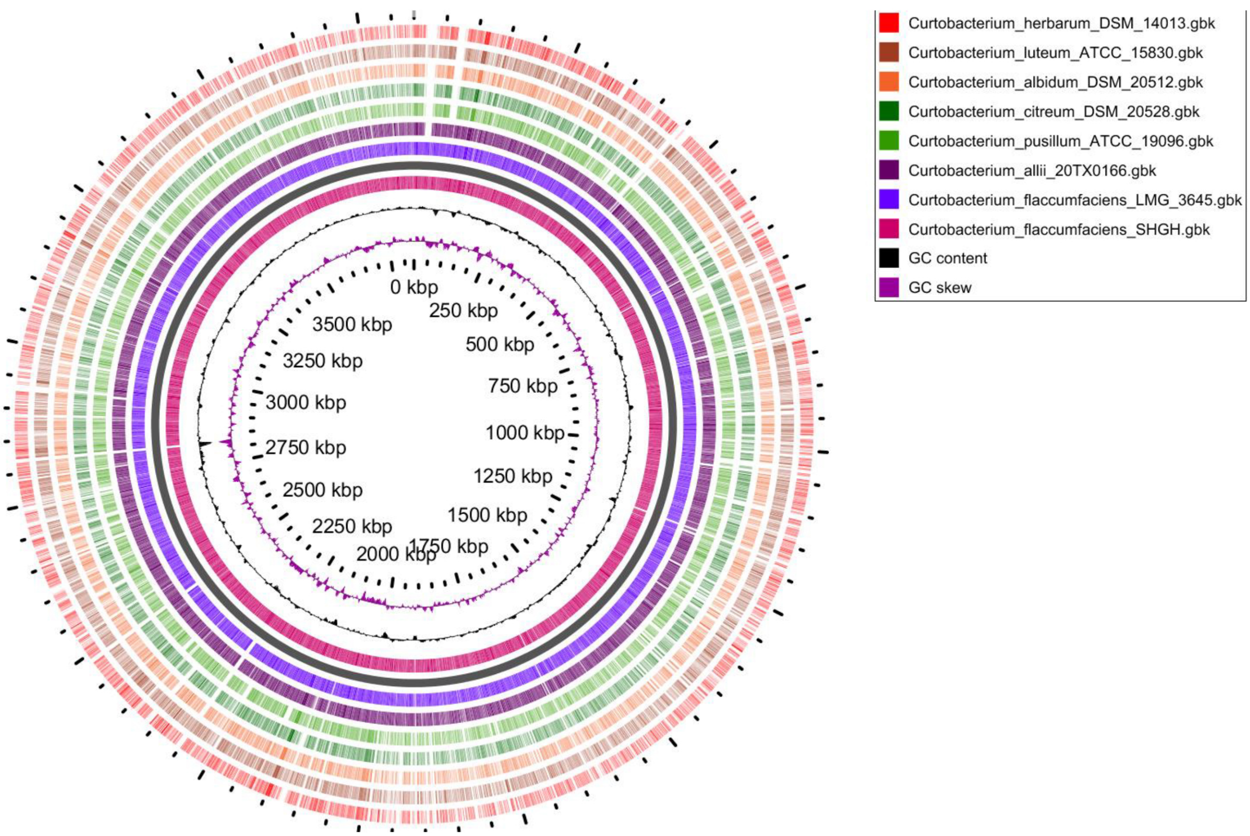


**Fig. 2.** *C. flaccumfaciens* SHGH genome compared against seven most closely related *Curtobacterium* species. The innermost red circle represented the genome of *C. flaccumfaciens* SHGH. The rings showed GC content (black) and GC skew (purple). The next rings represented the genomes of other *Curtobacterium* species which were indicated in different colors. Regions without color within a ring indicated an absence of the region and the difference among the genome sequences.

**Table 3.** Most closely related *Curtobacterium* species with their accession numbers that showed homology with *C. flaccumfaciens* SHGH based on *16S rRNA* sequences retrieved from NCBI database.

| Species name | Strain name | Accession No. | Similarity (%) | Completeness (%) |
|---|---|---|---|---|
| *C. flaccumfaciens* | LMG 3645 | AJ312209.1 | 100.00 | 100.0 |
| *Curtobacterium allii* | 20TX0166 | OK275102.1 | 100.00 | 98.1 |
| *Curtobacterium oceanosedimentum* | ATCC 31317 | EF592577.1 | 99.65 | 100.0 |
| *Curtobacterium pusillum* | DSM 20527 | AJ784400.1 | 99.52 | 100.0 |
| *Curtobacterium luteum* | DSM 20542 | X77437.1 | 99.45 | 100.0 |
| *Curtobacterium citreum* | DSM 20528 | X77436 .1 | 99.31 | 100.0 |
| *Curtobacterium ammoniigenes* | NBRC 101786 | BCSV01000013.1 | 99.24 | 100.0 |
| *Curtobacterium albidum* | DSM 20512 | AM042692.1 | 99.22 | 97.9 |
| *Curtobacterium herbarum* | P 420-07 | AJ310413.1 | 98.82 | 100.0 |
| *Curtobacterium* sp. | Leaf261 | LMMJ01000001.1 | 98.61 | 100.0 |

Recently, *C. flaccumfaciens* has been isolated from cowpeas and other dry bean crops in Iran.[5,30] These were the closest occurrences of *C. flaccumfaciens* causing bacterial wilt disease in Mosul.

A study has shown that the *Curtobacterium* genus confers health benefits to olive trees, particularly by inhibiting the over-growth of more common pathogens, such as *Pseudomonas savastanoi*, which is the causative agent of olive knot disease.[31] Another study has shown that *Curtobacterium* possesses plant growth promoting traits due to its ability to grow under osmotic or salinity stress and can improve plant germination early in development.[32] Most described *Curtobacterium* sp. are not known to cause disease in plants from which they are isolated,[33,34] but *Curtobacterium* were isolated from a variety of plant species[35] indicating that this genus has a potential for broad phyto-pathogenicity when containing

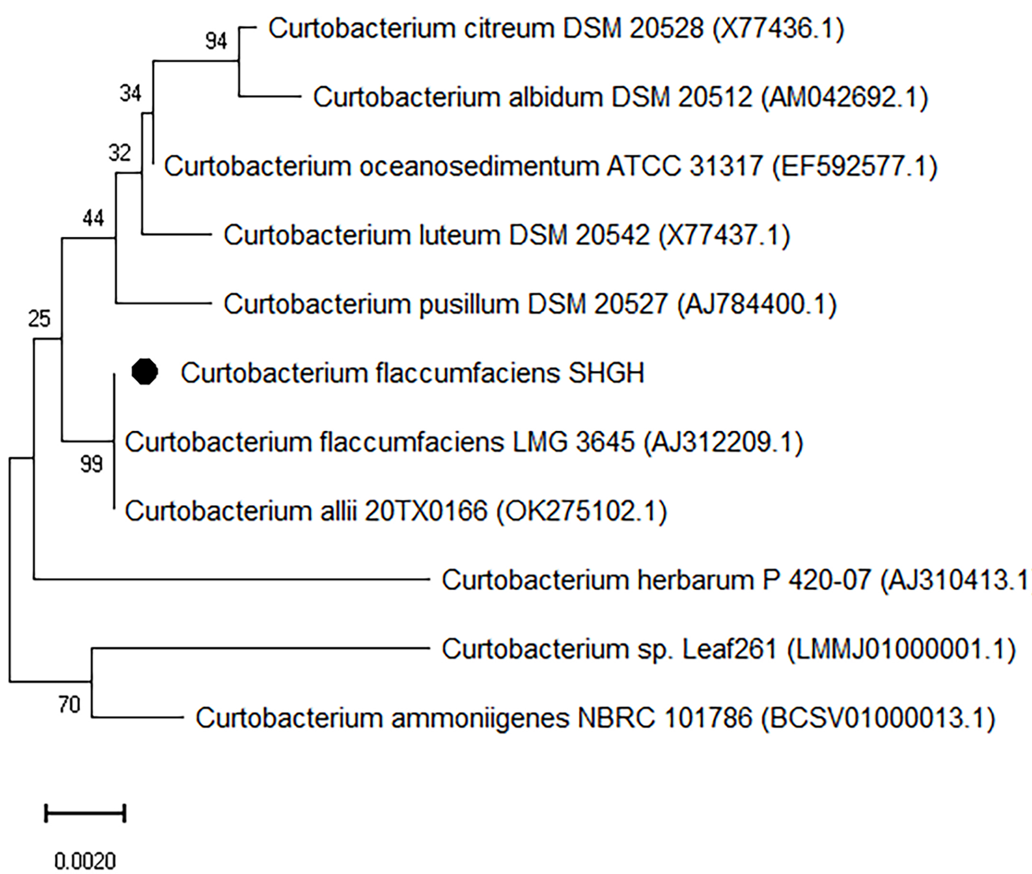


**Fig. 3.** Neighbor-Joining phylogenetic trees showed the relationship between *C. flaccumfaciens* SHGH (indicated in a black circle) and the closely related strains based on *16S rRNA* sequences using MEGA-11 software with a scale length of 0.002.

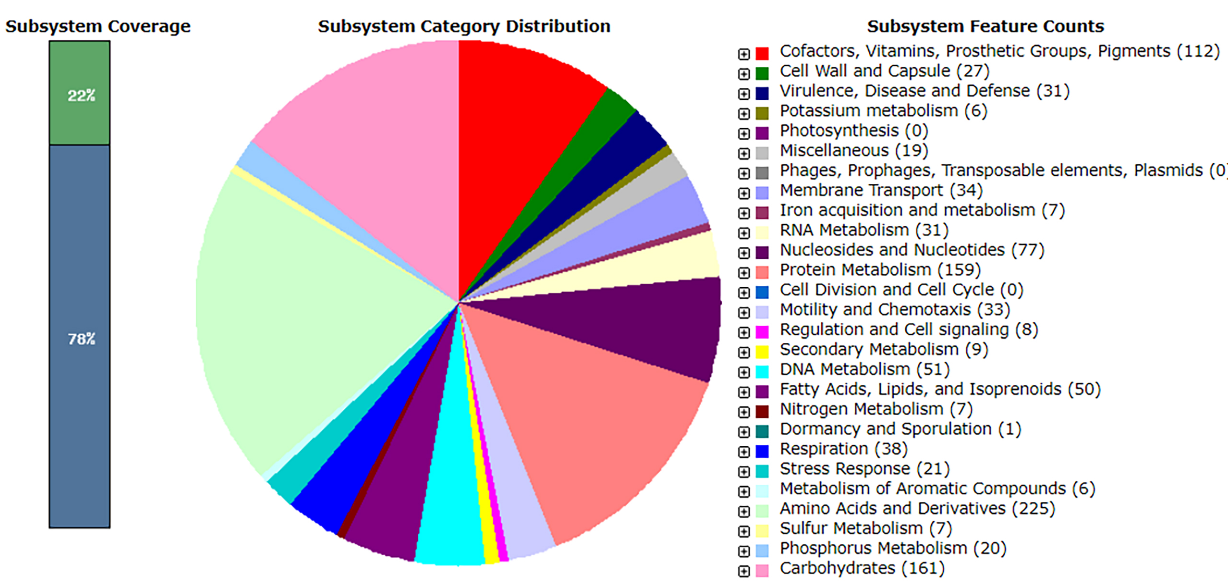


**Fig. 4.** Subsystem category distribution statistics of *C. flaccumfaciens* SHGH.

the needed genes. In *Olea europaea,* the phylum *Actinobacteria* is an "aridity-winner", being more resistant to belowground arid conditions.[36] Since the area around Mosul has a hot semi-arid rainfall climate with less than 0.6 mm of rain from June – September,[37] *Curtobacterium* may assist *O. europaea* in adapting to the dry conditions. Out of 190 *Curtobacterium* sequences deposited on NCBI, only 28 are predicted to be pathogenic strains, leading to uncertainty about the role of *Curtobacterium* sp. in plant pathogenesis worldwide.[38]

## Conclusion

In conclusion, the genome of *C. flaccumfaciens* SHGH strain has been sequenced, analyzed, and accessioned. For proper taxonomic classification, the links between genomic features of *Curtobacterium* and pathogenicity in plants need elaboration and revision. Our contribution here expands the pool of information concerning this complex pathogen.


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## Authors' declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at University of Mosul.

## Authors' contribution statement

We would like to confirm that all authors contributed equally in the design and implementation of this manuscript.

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# تسلسل الجينوم الكامل لسلالة *Curtobacterium flaccumfaciens* SHGH المعزولة من عقد *Olea europaea* في الموصل، العراق

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**الخلاصة**

ارتبطت بكتيريا *Curtobacterium flaccumfaciens* SHGH من خلال مسلمات كوخ بمرض الذبول في أشجار الزيتون *Olea europaea* الموجودة في الموصل، العراق. تم تسلسل الجينوم الكامل وتحميله إلى GenBank تحت رقم الانضمام JAUMSN000000000.1. يتكون الكروموسوم الدائري من 3,834,306 قاعدة نيوكليوتيدية، و70.7% من محتوى GC، و4,176 تسلسلًا لترميز البروتين، و53 جينا من الحمض النووي الرايبي (RNA)، و7 جينات من الحمض النووي الرايبي الريبوسومي (rRNA) ، و42 جينا من الحمض النووي الرايبي الناقل (tRNA). استنادًا إلى تقنية تهجين DNA-DNA سيليكا، هناك 11 سلالة مرتبطة ارتباطًا وثيقًا بسلالة SHGH. أظهر تحليل تسلسل جين 16S rRNA أن سلالة SHGH لها تشابه بنسبة 100% مع جين LMG 3645 *C. flacscumfaciens* وتشابه بنسبة 99% مع جين 20TX0166 *Curtobacterium allii* مع أكثر من 100 تكرار في اختبار التمهيد. كشف تحليل سلالة SHGH عن وجود العديد من الجينات التي تشفر الإنزيمات المرتبطة بالإمراض مثل انزيمات pectate lyase ، glycosyl hydrolase ، serine proteinase ، beta-1,4-glucanase و -1,4-beta xylanase. علاوة على ذلك، أظهر تحليل الجينوم لسلالة SHGH أنها تحتوي على انزيمات peptidases و glycosidases ومجالات تحلل الكلايكوبوليمرالمحتملة potential glycopolymer-degrading domains في المناطق المشتقة من العاثي الاولي. تساعد هذه الميكروبات على تكوين غشاء حيوي والاستعمار مع ميكروبات أخرى لتكوين مجتمع ميكروبي، وهي خطوة ضرورية لتطور العديد من أمراض النباتات البكتيرية مثل الذبول البكتيري.

**الكلمات المفتاحية:** تسلسل الجينوم الكامل، الجينات المرتبطة بالضراوة، الاغشية الحيوية، *Curtobacterium flaccumfacien*، *Olea europaea*.