In silico analysis of the chemotactic system of Agrobacterium tumefaciens

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Abstract
Agrobacterium tumefaciens is an efficient tool for creating transgenic host plants. The first step in the genetic transformation process involves A. tumefaciens chemotaxis, which is crucial to the survival of A. tumefaciens in changeable, harsh and even contaminated soil environments. However, a systematic study of its chemotactic signalling pathway is still lacking. In this study, the distribution and classification of chemotactic genes in the model A. tumefaciens C58 and 21 other strains were annotated. Local blast was used for comparative genomics, and hmmer was used for predicting protein domains. Chemotactic phenotypes for knockout mutants of ternary signalling complexes in A. tumefaciens C58 were evaluated using a swim agar plate. A major cluster, in which chemotaxis genes were consistently organized as MCP (methyl-accepting chemotaxis protein), CheS, CheY1, CheA, CheR, CheB, CheY2 and CheD, was found in A. tumefaciens, but two coupling CheW proteins were located outside the ‘che’ cluster. In the ternary signalling complexes, the absence of MCP atu0514 significantly impaired A. tumefaciens chemotaxis, and the absence of CheA (atu0517) or the deletion of both CheWs abolished chemotaxis. A total of 465 MCPs were found in the 22 strains, and the cytoplasmic domains of these MCPs were composed of 38 heptad repeats. A high homology was observed between the chemotactic systems of the 22 A. tumefaciens strains with individual differences in the gene and receptor protein distributions, possibly related to their ecological niches. This preliminary study demonstrates the chemotactic system of A. tumefaciens, and provides some reference for A. tumefaciens sensing and chemotaxis to exogenous signals.

DATA SUMMARY
Using the keyword ‘Agrobacterium tumefaciens’ in the National Center for Biotechnology Information genome database, Agrobacterium tumefaciens and some Agrobacterium radiobacter strains (A. tumefaciens biovars) were retrieved. Considering the genome sequencing level and related literature report, 21 A. tumefaciens strains (C58, P4, 1D1609, cherry 2E–2–2, 186, Ach5, B, CCNWGS0286, DSM30147, F2, GW4, H13, K599, S33, S2, LMG140, LMG125, N273, LBA4404, 5A and WRT31) and A. radiobacter K84 were selected. Related genome sequences, 16S rRNA and protein sequences were downloaded from www.ncbi.nlm.nih.gov/genome. No new sequence data was generated in this work. An Hidden Markov Model (HMM) model for methyl-accepting chemotaxis proteins (PF00015) and 77 existing ligand-binding domain models (Table S1, available with the online version of this article) were downloaded from the Pfam database (http://pfam.xfam.org/).

INTRODUCTION
Chemotaxis can help micro-organisms under nutrition stress search for suitable living environments, and is important for host–microbe symbiotic processes, such as host invasion [1], biofilm formation [2] and cell adhesion [3]. The movement of bacteria towards degradable environmental pollutants is related to chemotaxis, and could improve the degradation rates and bioavailability of some pollutants, and effectively promote bioremediation of environmental pollutants [4, 5]. The chemotactic system of bacteria (often called the Che system) is composed of a chemoreceptor and core proteins. Genes encoding core proteins always occur in clusters in bacterial genomes, and mostly in one operon.
Chemoreceptors, also known as methyl-accepting chemotaxis proteins (MCPs), are front-end signal acceptors, which specifically sense chemokine effectors and transmit sensed signals to downstream proteins of the Che system [6]. In most bacteria, core proteins are highly conserved, but different MCPs could recognize specific chemoeffectors. The most extensively studied Che system is the Escherichia coli system [7, 8], composed of five MCPs (Tar, Tsr, Trg, Aer and Tap) and six core proteins (CheA, CheB, CheR, CheW, CheY and CheZ).

Agrobacterium tumefaciens, a Gram-negative alphaproteobacterium, is abundant in soil. Agrobacterium has a complicated taxonomic classification [9], which was derived from the evolutionary relationships between the 16S rRNA marker, recA genes and other conserved housekeeping genes using important public databases such as the National Center for Biotechnology Information (NCBI), SILVA [10], RDP [11] and IMG [12] databases. The well-accepted classification includes three biovars: A. tumefaciens species complex (biovar I), Agrobacterium rhizogenes (biovar II) and Agrobacterium vitis (biovar III) [13]. As a common plant pathogen, A. tumefaciens was initially identified as the cause of plant tumours. In A. tumefaciens, Braun first proposed, in 1947, the tumour-inducing principle that meant that some of the bacterial components might be tumorigenic factors [14]. DNA fragments of a tumour-inducing (Ti) plasmid were later validated to be the tumour-inducing principle delivered by A. tumefaciens into plant cells [15]. T-DNA and most virulence genes are located on the Ti plasmid. A. tumefaciens is capable of transferring part of its virulent Ti plasmid into plant cells and integrating the segments into the host genome, thereby implementing genetic transformation of host cells. To increase the T-DNA transfer frequency of A. tumefaciens, various A. tumefaciens mutants were constructed to increase vir gene expression or remove adverse factors from Agrobacterium–plant interactions. For example, super-Agrobacterium ver. 4, containing the ACC deaminase (acdS) and GABA transaminase (gabT) genes, was validated as a more effective and powerful tool for plant genetic engineering than the previously developed strains [16].

T-DNA transfer mediated by A. tumefaciens has been widely used in plant transgenic technology [17], and the microbe’s chemotaxis is directly associated with the onset of its transgenic mechanism. The first step in the A. tumefaciens infection process is to recognize suitable infection sites in plant hosts by chemotactic activity [18], and previous studies of chemotaxis in A. tumefaciens have focused on the identification of attractant types, rarely investigating the chemotaxis mechanism. Some organic compounds secreted by plant callus could be attractants of A. tumefaciens and induce the expression of oncogenes [19, 20]. A. tumefaciens is reported to be sensitive to many sugars, phenols and amino acids [21, 22], some of which strongly induce chemotaxis and tumorigenesis [23–25]. However, these studies are scattered and lack systematic research [26, 27].

**Impact Statement**

Chemotaxis is the phenomenon by which motile microorganisms sense the presence and/or concentration of chemicals and move towards favourable attractants or avoid harmful repellents. The first step in the Agrobacterium tumefaciens infection and transgenic process is driven by chemotactic activity. By comparative genomics analysis, one chemotactic operon was found to exist in A. tumefaciens, and the order of chemotactic genes was generally MCP (methyl-accepting chemotaxis protein), CheS, CheY1, CheA, CheR, CheB, CheY2 and CheD, except for GW4 and B2 strains. Two CheW coupling proteins in A. tumefaciens were outside the chemotactic operon. Formation of the ternary signalling complex (MCP-CheA-CheW) was the basis for chemotaxis in A. tumefaciens C58. In both CheA single mutation strains and CheWs double deficient strains chemotaxis was eliminated. The number of MCPs in most A. tumefaciens strains exceeded 20, and they belonged to classes I–IV, excluding class II. Class I had the largest number of MCPs, and the Ib subtype had two transmembrane domains, whereas the Ib subtype had one transmembrane domain. Among class IV cytoplasmic MCPs, the IVa subtype had more transmembrane domains than the IVb subtype. About 80% of A. tumefaciens MCPs had ligand-binding domains (LBDs), belonging to 15 different domain models, classified into four domain superfamilies: 4HB, Cache, PAS and protoglobin. Cache, the most abundant LBD superfamily in A. tumefaciens, was found to bind to amine ligands. The 4HB superfamily covered all the domain models, namely 4HB_MCP_1, CHASE3, TarH and HBM. PAS and protoglobin superfamilies were only found in cytoplasmic MCPs, which might be related to intracellular redox state induction. Our study explains the chemotaxis system of A. tumefaciens, and serves as a basis for future studies on A. tumefaciens sensing and chemotaxis to exogenous signals.

Chemotaxis not only affects survival under stress and in polluted environments, but also is closely related to host infection. A. tumefaciens possesses two contrasting lifestyles: independent saprophytic or pathogenic. Considering the importance of chemotaxis to A. tumefaciens, it was expected to become a model bacterium in chemotaxis research. In this study, the Che system and MCPs of the model strain, A. tumefaciens C58, were annotated. The effect of the ternary signalling complex on chemotaxis was validated, as an example of the MCP located on the chemotactic operon. Furthermore, the chemotactic operons of other A. tumefaciens strains were compared, and their MCP types and the diversity of the ligand-binding domain (LBD) were systematically investigated.
Fig. 1. Chemotactic pathway in A. tumefaciens C58.

**METHODS**

**Multiple sequence alignment and phylogenetic analyses**

NCBI nucleotide BLAST was used for homologous comparison against the local library of che genes in A. tumefaciens C58. Default parameter values were >90% similarity and <10^{-30} E value. MEGA X software was used to reconstruct 16S rRNA gene phylogenetic trees by the neighbour-joining method [28].

**Protein analysis of MCPs**

**HMMER** [29] was used to interrogate all protein sequences of the A. tumefaciens strains for matches to Pfam PF00015. Protein domains of MCPs were preliminarily predicted by the SMART server (http://smart.embl.de/). Transmembrane domains of MCPs were determined by the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). LBDs of MCPs were determined by comparing 77 existing LBDs via HMMER.

**Construction of A. tumefaciens deletion mutants and complementation mutants**

Using diluted bacterial liquid as the template, the upstream sequence of target genes was amplified by using DgeneU-F and DgeneU-R primers, and the downstream sequence of target genes was amplified by using DgeneD-F and DgeneD-R primers (see Table S2). Here, the gene refers to 0514, 0517, W1 and W2 in Table S2. The target fragment was obtained by overlap PCR using DgeneU-F and DgeneD-R primers. The target fragments were identified via agarose gel electrophoresis (1%) and were recovered using a TaKaRa agarose gel DNA extraction kit. The purified PCR products and suicide vector pEX18Km were digested, linked and ligated, and used to transform E. coli DH5α. After PCR and DNA sequencing, the correctly constructed plasmids were used to transform A. tumefaciens C58. Deletion mutants were selected and validated using a kanamycin-resistance gene as the positive selection marker and a suicide gene, sacB, as the counter-selectable marker.

Using diluted bacterial liquid as the template, target genes with their native promoters (upstream 500 bp) were amplified using Hgene-F and Hgene-R primers (see Table S2). The identified and purified PCR product was inserted into plasmid pCB301. Correctly constructed plasmids were used to transform the corresponding deletion mutation strains of A. tumefaciens. The success of the complementation was verified by detecting kanamycin resistance and PCR sequencing. All the strains, plasmids, culture media and primers used in this study are listed in Tables S1–S3.

**Chemotaxis assays using swim agar plates**

Chemotactic responses to nutrient substances were assayed by performing an agar swim test [30]. The biomass in the mid-exponential growth phase was adjusted to OD_600_0.5. Cell cultures (3 µl) were added to a swim agar plate (0.18% agar) containing AB-sucrose medium. After incubation at 28 °C for 48 h, the colony size was observed and recorded in photos.

**RESULTS AND DISCUSSION**

**Annotation of the chemotaxis system of A. tumefaciens C58**

Bacterial chemotaxis generally involves histidine kinase CheA, coupling protein CheW, response regulator CheY, MCP and flagellar motor proteins. There was only one distinct chemotaxis operon located on the A. tumefaciens C58 circular chromosome (from atu0514 to atu0521), which contained some two-component Che proteins. However, coupling protein CheW was not in the operon, but in other positions on the circular chromosome (CheW1 atu2075 and CheW2 atu2617). There were two homologous methyltransferase CheR proteins; one (atu0518) was found in the operon and the other (atu4805) was on the linear chromosome. In addition, 20 genes were predicted to encode MCPs in the C58 genome;
atu0514 was found in the chemotaxis operon, whereas the others were scattered in the Ti plasmid (atu6132), At plasmid (atu5342), circular chromosome (atu0373, atu0387, atu0526, atu0646, atu0738, atu0872, atu1027, atu1912, atu2173, atu2223, atu2360, atu2618), and linear chromosome (atu3094, atu3330, atu3363, atu3725, atu4736).

CheA (atu0517) was the core component of the chemotaxis operon in A. tumefaciens C58. Among three histidine kinase categories, atu0517 belonged to group I, including the histidine-containing phosphotransferase (HPr) domain, homodimeric domain of histidine kinase (H-kinase_dim, 337–400), histidine kinase-like ATPase domain (HATPase_c, 445–586) and CheA regulatory domain (CheA_reg, 578–719). The chemotaxis signal pathway of A. tumefaciens C58 is shown in Fig. 1. CheA activity can be regulated by methylation and demethylation of methyltransferase CheR (atu0518) and methyltransferase CheB (atu0519), respectively, and activated by removing specific glutamine residues from MCPs of glutamine deamidase CheD (atu0521). CheA phosphorylates CheY1 (atu0516) and CheY2 (atu0520). Phosphorylated CheY interacts with the flagellum motor complex (Flis/Mots) and drives flagellum rotation in response to chemoattractants. Like for other Alpha proteobacteria, phosphatase CheZ was not found in the A. tumefaciens C58 genome [31]. The second gene, atu0515, in the chemotaxis operon was annotated as CheS, which works with CheA to dephosphorylate CheY.

**Chemotaxis of mutants of the ternary signalling complex in A. tumefaciens C58**

The colony size on the swim agar plate is often used to examine chemotaxis behaviour [30]. When bacterial cells are inoculated onto swim agar, they utilize the nutrients around the inoculation area. If bacterial cells show a sensitive chemotactic response, they move outward along the nutrient gradient and form large colonies. If their chemotactic system is damaged, the colonies are small. CheW1 (atu2075) and CheW2 (atu2617) couple CheA dimer to two chemoreceptor trimers of dimers to form a chemoreceptor-CheW-CheA ternary signalling complex. External chemical substances and signal stimuli can be sensed by the ternary signalling complex allowing movement towards favourable environmental conditions and avoidance of harmful chemoattractant repellents. The homologous recombination method with a suicide gene, sacB, as the counter-selectable marker was used to delete an MCP (atu0514), CheW1 (atu2075), CheW2 (atu2617) and CheA (atu0517). Mutant colony sizes were detected on a swim agar plate. As shown in Fig. 2, chemotaxis ability was lost after deleting CheA but recovered to the level of the wild-type by the introduction of an expression plasmid, pCB301, harbouring complete CheA. The colony size of atu0514 was significantly smaller than that of the wild-type, and larger than that of ∆CheA, suggesting that knockout of atu0514 would weaken A. tumefaciens C58 chemotaxis. Colonies of C58Δw1 and C58Δw2 mutants were smaller than those of wild-type C58, but larger than those of ∆CheA. The colony size of the double-mutant (ΔCheW) was almost the same as that of ∆CheA. One CheW could partially compensate the effects of the other CheW on chemotaxis, but in knockouts of both, CheWs chemotaxis was completely lost. In that case, CheA were not bounded to cell poles by CheWs but dispersed in cells, so that the ternary signalling complex could not form, and chemotaxis signal transduction was blocked.

**Characteristics of MCPs in A. tumefaciens C58**

The 20 MCPs predicted in A. tumefaciens C58 were a far higher number than those found in the model microorganism, E. coli (5 MCPs), and its close relative Sinorhizobium meliloti [32]. The large number of MCPs indicates that A. tumefaciens could recognize more chemicals for survival in complex environments. According to the number of heptad repeats, chemoreceptors were classified into seven categories (24, 28, 34, 36, 38, 40, and 44 h) [33]. All the MCPs in A. tumefaciens C58 belonged to the 36 h type. Six MCPs without transmembrane regions accounted for 30% of all the MCPs in A. tumefaciens, and the ratio was between the mean value of MCPs in bacteria (~14%) and archaea (~43%). Some domains in the six soluble chemoreceptor proteins could sense small molecules or redox-sensitive cofactors. For example, two PAS domains, the most abundant domain in soluble MCPs, were located at the N-terminal of the four MCPs [34]. Protoglobin was located at the N-terminal of the other two MCPs, belonging to the haemoglobin family, which could bind with the prosthetic group of haem [35]. Among 14 transmembrane MCPs, atu0387 and atu3330 had only one transmembrane region, 7 MCPs had two transmembrane regions, and 5 MCPs had three transmembrane regions. Nine of the fourteen MCPs were annotated with the periplasmic LBD, which were distributed in three types. Double Cache (atu1912, atu2173, atu0526,
Xu et al., Microbial Genomics 2020;6

Fig. 3. Organization of the chemotaxis operons in A. tumefaciens strains and a phylogenetic tree reconstructed based on published 16S rRNA sequences of A. tumefaciens.

atu2223, atu0373 and atu3725) consisted of two α/β subdomains and a long N-terminal helix. It is the most common extracellular domain in prokaryotes [36]. The single Cache domain of atu0646 was similar to the double Cache domain, both of which belong to the Cache-like superfamily. A single 4HB domain existed in atu0738 and atu0387, comprising two symmetric antiparallel coiled coils.

Comparison of the chemotaxis operon in A. tumefaciens strains

Among the 22 A. tumefaciens strains, the 16S rRNA sequences of 17 were publicly available in the NCBI database; phylogenetic trees were reconstructed based on these 16S rRNA sequences. As shown in Fig. 3, 16 different strains of A. tumefaciens were clustered into one branch, and Agrobacterium radiobacter K84 was on a separate branch. Comparing the che gene cluster of the 17 strains, the distribution of chemotaxis genes appears consistent with that of A. tumefaciens C58 in this order: CheS, CheY1, CheA, CheR, CheB, CheY2 and CheD. However, the chemotaxis genes of A. tumefaciens GW4 were distributed on different scaffolds, i.e. MCP, CheS, CheY1, CheA and CheR on NZ_AWGV01000458.1; CheR, CheB, CheY2 and CheD on NZ_AWGV01000386.1 in reverse arrangement. Without considering the quality of published genome sequences, this distribution might be related to GW4 being isolated from As-contaminated aquifer sediments [37]. No MCP like atu0514 was found in the chemotaxis operon of non-pathogenic A. tumefaciens B2 [38]. All the 17 strains had two CheWs outside the chemotaxis operon. In addition, CheR had other homologous genes outside the chemotaxis operons in the C58, LBA4401, N2/73, LMG215, Ach5 and 5A strains.

Distribution of MCPs in A. tumefaciens strains

MCPs typically include a cytoplasmic signalling domain (SD), transmembrane helices (TM) and a LBD [39]. The SD, the most conservative element, consists of a methylation helices subdomain (MH), signalling subdomain (SSD) and flexible bundles subdomain (FB) between both subdomains. Sites for the interaction of MCPs and Che proteins (CheA, CheW, CheR, CheB and CheD) are located in the SD region. The LBD widely exists in the cytoplasm or periplasm, and recognizes extracellular or intracellular chemical and physical signals. Chemoreceptors for extracellular signals have at least one TM and one periplasmic LBD. Based on the membrane topology, MCPs are divided into four major classes (I–IV) and seven subclasses (Ia, Ib, II, IIIa, IIIc, IVa, IVb) [40].

A total of 465 MCPs were predicted in the 22 isolates of A. tumefaciens. Most strains had 20–25 MCPs, there were 22 MCPs in five strains, 21 MCPs in four strains, and 20 MCPs in four strains (Table 1). A. tumefaciens S2 and A. radiobacter K84 had only 18 MCPs, while A. tumefaciens DSM 30147 had up to 28 MCPs, possibly related to its isolation from saprobioc soil, and need to adapt to a more complex and changeable environment. The 465 MCPs belonged to three classes, excluding class II, which has a cytoplasmic LBD at the N-terminal connected with a cytoplasmic SD via two TMs. However, class II receptors only account for about 3% of present MCPs, far less than other classes. The predominant MCPs of A. tumefaciens were the class I MCPs (200). Typical structures in class I included TMs, a periplasmic LBD located between TMs and a cytoplasmic SD, which were further categorized into two subclasses: Ia (two TMs) and Ib (one TM). Ia subclass MCPs were more common than Ib MCPs. A total of 198 subclass Ia and 2 subclass Ib MCPs were found in A. tumefaciens. The second most common MCPs were the class III MCPs with 1–8 TMs. Among the 144 class III MCPs of A. tumefaciens, 103 MCPs with sensor elements were located within the membrane (IIIm subclass), and the cytosolic LBDs of 41 MCPs were located after the last TMs region (IIic subclass). A total of 121 MCPs belonged to class IV, which were soluble cytoplasmic MCPs without TMs. A large majority belonged to theIVA subclass with LBD regions, and four MCPs without LBD regions belonged to IVb.
Table 1. Classification of MCPs from *A. tumefaciens* strains

|   | I86 | ID1609 | 2E-2-2 | H13 | K599 | K84 | 5A | Ack5 | LBA4404 | LMG125 | LMG140 | B6 | C58 | N273 | P4 | S2 | S33 | CCNWGS0286 | DSM30147 | F2 | GW4 | WRT31 |
|---|-----|--------|--------|-----|------|-----|----|------|---------|---------|---------|----|-----|------|----|----|-----|------------|-----------|----|------|------|
| 1a | 8(I) | 4(I) | 6(I) | 7(I) | 7(I) | 9(I) | 7(I) | 2(I) | 1(I) | 3(I) | 2(I) | 6(I) | 8(I) | 8(I) | 5(I) | 9(I) | 7(I) | 9(I) | 7(I) | 9(I) | 10(I) | 5(I) | 4(I) | 6(I) |
| 1b | 1   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3I1m | 4 | 5 | 3 | 3 | 3 | 3 | 5 | 4 | 5 | 3 | 3 | 4 | 5 | 6 | 6 | 2 | 5 | 4 | 9 | 3 | 7 | 6 |
| 3I1c | 2 | 1 | 2 | 3 | 5 | 4 | 1 | 2 | 2 | 2 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | 2 | 1 | 2 | 3 | 2 | 2 | 1 |
| 3b | 6 | 6 | 6 | 6 | 4 | 2 | 7 | 6 | 5 | 5 | 5 | 5 | 5 | 4 | 5 | 5 | 5 | 6 | 5 | 7 |   |   |   |   |
| 3bb |     | 1 | 1 |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | 1 |     |   |   |
| Total | 22 | 20 | 19 | 22 | 20 | 18 | 20 | 25 | 21 | 21 | 22 | 23 | 20 | 22 | 22 | 18 | 19 | 21 | 28 | 19 | 21 | 23 |

Based on ligand-binding domain (LBD) length, the Ia subclass can be divided into type I (120-215 aa) and type II (215-299 aa); the first line is the strain number (the names are omitted, see the material method for details).
The different subtypes of MCPs in the 22 isolates of *A. tumefaciens* are listed in Table 1. Types Ia(I), IIm and IVa were found in all 22 strains, followed by IIIc and Ib(I) in 20 and 19 strains, respectively; the least frequent were IVb and Ib, which were only found in 4 and 2 strains, respectively. Based on LBD length, the Ia subclass can be divided into type I (120–215 aa) and type II (215–299 aa).

**Analysis of LBDs in A. tumefaciens strains**

Further analyses of the 465 MCPs in the 22 *A. tumefaciens* showed that 100 MCPs did not have LBD regions or could not be matched with existing LBDs in the Pfam database. At least one region on the remaining 365 MCPs was an identifiable LBD, which covered 516 LBDs, but was not evenly distributed. More than 95% of MCPs had only one LBD, and about 2% of MCPs had two LBD segments. The highest number used was five LBDs, such as CDN92820.1 of *A. tumefaciens* contained was five LBDs, such as CDN92820.1 of *A. tumefaciens* had all the domain models of the 4HB superfamily. The single 4HB type included three domain models: 4HB_MCP_1 (55), CHASE3 (73) and TarH (6). There were 11 HBM domain models in the double 4HB type. Many kinds of 4HB superfamily ligands have been found, among which the HBM domain mainly recognizes intermediates of the TCA cycle [43]. The PAS superfamily was also a common LBD in the bacterial signal transduction system. Unlike the Cache and 4HB families, the PAS of *A. tumefaciens* only exists in cytosolic MCPs. The binding sites of haem, FAD or FMN were found in the PAS domain, which might be a function of aerotaxis and redox sensing [44].

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**Author contributions**

N. X. and M. G. conceived the project. N. X. and M. W. completed data collection and data analysis. N. X. and Y. X. completed wet experiments. N. X., M. W., X. Y. and M. G. wrote the manuscript.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.
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