Screening for a stable region in the T7 phage genome and repurposing Riboflavin as an anti-phage molecule

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Abstract
Over the past century, the emergence of antibiotic-resistant bacterial strains has heightened interest in bacteriophages. Advances in genomic engineering techniques have expanded the potential applications of phages across various fields. However, the long-term effects of phage therapy on the human body remain poorly understood. As a result of this study, a target constant region that is responsible for coding of the overcome classical restriction (Ocr) protein on T7 phage that provides protection from restriction enzymes was detected. A molecule called Riboflavin that interacts with this region could be used in the clearance of T7 phages. In addition, another docking study revealed that Ocr has an antitranscriptive effect on the Human Early B-cell Factor 1 (EBF1) DNA-binding domain, which affects the cellular processes. Confirmation of these findings by further clinical and *in vitro* studies will provide new insights into phage-human cell interactions and new phage-clearing treatments.

Key words: Bacteriophage, Drug repurposing, EBF1, Protein modelling, Riboflavin, T7, Overcome classical restriction, Ocr

INTRODUCTION
Bacteriophages are classified as viruses that can infect bacteria and are the most prevalent life form on Earth [1]. They play a significant role in altering global genetic diversity and are disseminated in environments with enlarged scale diversity. Phages are substantial components of the gut microbiome, and they have various genes that cause selective pressure on their bacterial hosts [2, 3]. As reviewed by De Smet et al. [4], phages that infect *Pseudomonas* spp. influence host cell transcription, RNA degradation, cellular motility, metabolism, CRISPR-mediated immunity to phages, and phage DNA silencing. Interestingly, in addition to influencing intracellular molecular interactions during infection, phages also affect their host at the population level. Indeed, phage infection substantially affects both the evolution and ecology of the bacterial host, mainly through phage–host co-evolution, lysogenic conversion, and transduction [5].

Our bodies are subjected to a high number of phages during daily activities, and per gram of faeces contains several billion bacteriophage particles [2]. Because of the absence of specific receptors for phages on eukaryotic cells, these viruses were considered to be neutral to animals and humans for a long time. However, recent studies in mammals, including humans, have provided clear evidence that phages can interact with eukaryotic cells, significantly influencing

the functions of tissues, organs, and systems [6]. They are also visible in the blood and serum of samples showing symptomatic and asymptomatic cases [7, 8, 9]. Phage administration via oral feeding and gastric lavage causes its rapid migration into the vascular system [10]. It permeates into the lung, liver, kidney, spleen, urinary tract, and brain by crossing the blood-brain barrier [11, 12]. The gut entitles reservation of the highest number of phages that they excess the whole parts of the body using various mechanisms [13, 14]. In the most observable cases leaky gut leads to cellular damage and inflammation that enable phages to penetrate through epithelial layers and transfer into the blood stream [15-19]. Significantly, Sweere et al. [20] documented that a bacteriophage associated with *Pseudomonas aeruginosa* (Pa) instigates antiviral immunity in humans and impedes the clearance of bacterial infection. These findings suggest that the interaction involving the immune cell uptake of phage-infected Pa resulted in the production of phage RNA, indicating the potential ability of a natural, unmodified bacteriophage to generate mRNA within human cells [20].

There has been a great interest in phages as an alternative treatment method against bacteria with antibiotic resistance. Some successful case reports have reported positive effects of these treatments [21, 22]. However, some failed studies have been announced, depending on incomplete coverage and insufficient phage cocktail [23-25]. In addition, other uses are gaining interest for foodborne infections and crop protection [26-28].

The virulent T7 bacteriophage is among the initial seven phages discovered in *Escherichia coli* in 1945 [29]. T7 is a nonenveloped, short-tailed icosahedral bacteriophage specific to *Escherichia coli,* characterized by a genomic DNA content of approximately 40 kilobase pairs (kbp) [30]. T7 phages have been well studied from many aspects, and their engineered variants have been employed in new technologies, such as a phage surface display system for the treatment and diagnosis of infectious diseases (bacteria, viruses, parasites) and tumour diseases [31]. However, research focused on its interactions with human cell transcription factors and clearance of this phage from the human body via antiphage molecules has remained unknown.

In this study, T7 phage sequences' unvarying regions with less mutation than other parts of the genome on 82 various genome sequences available in the NCBI database were investigated. This study aims to show stable regions in the viral genome, to perform prediction on protein structure and docking analysis to find an effective molecule interacting with proteins of T7 phage to prevent its dissemination, and to understand its possible mode of interaction with a human transcription factor.

MATERIALS AND METHODS

Homology genome blast and genome information

A total of 82 complete genome sequences of the T7 bacteriophage were retrieved from the NCBI database as of December 14, 2023. Only the complete genomes with high coverage were included in the dataset. The list of T7 phage genomes is given in supplementary data (S1 data, S2 data).

Phylogenetic analysis

To analyze the obtained T7 phage genomes, sequence alignment was performed using Multiple Sequence Comparison by MAUVE from the MegAlignPro subunit of DNAstar software (Burland 2000). The sequences were analyzed, and common regions of all genomes detected using MAUVE from pairwise alignment results were obtained with ClustaI W of MegAlignPro, DNASTAR software [32] (Supplementary data S3).

Nucleotide and amino acid sequence alignment and analysis

Each unvarying genomic region was excised from whole sequences and subjected to the protein similarity program of the NCBI database using BlastX. This obtained FASTA sequence was converted to protein sequence using the ExPASy proteomics server (https://web.expasy.org/translate/) [33] and then loaded to the I-TASSER (Iterative Threading ASSEmbly Refinement) server of Michigan University, US (https://zhanglab.ccmb.med.umich.edu/I-TASSER) for protein prediction [34].

Homology modelling and protein prediction

Corresponding homology models predicted by the ITASSER server System for the target protein were downloaded from the Protein Data Bank (PDB) (www.rcsb.org) (Table 1). Alignment of the protein sequences and subsequent homology modeling were performed using the ExPASy proteomics server to study the protein sequence and further structural details [33].

TABLE 1 Homology modelling of the Ocr protein using ITASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER) considering the specific sequence of T7. *The highest pdb hit was 1s7zA.

Rank	PDB Hit	Identity 1	Identity 2	Coverage	Norm. Z-score
1*	1s7zA	0.869	0.49	1.000	0.886
2	3jrqA	0.581	3.75	0.082	0.955
3	5a63C	0.576	3.25	0.068	0.875
4	4ofzA	0.575	3.92	0.047	0.932
5	7kprA	0.573	3.90	0.071	0.943
6	8t6uA	0.572	3.42	0.013	0.841
7	2w1pA	0.571	3.80	0.035	0.966
8	6uebA	0.565	3.46	0.060	0.932
9	5kbwA	0.565	2.28	0.041	0.716
10	4glwA	0.565	3.42	0.063	0.875

Ligand retrieval

The structure of Riboflavin (Vitamin B_2) was retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). This structure was used for docking calculations. The selected 3D structure of the ligand was retrieved from the PubChem Compound database in SDF format followed by conversion to PDB format. The ligand parameters were analyzed using the PRODRG online server (http://prodrg2.dyndns.org/cgi-bin/prodrg.cgi) [35]. The shape complementarity principle was applied with clustering RMSD 4.0 for docking calculations.

Molecular docking studies

Homology modelling and protein prediction analysis have directed us to test the gene 0.3 protein of phage T7, also known as Ocr (overcome classical restriction) T7, with our ligand. The flexible docking study was conducted using AutoDock v 4.0 [36]. The interaction analysis of protein-ligand complexes and their amino acid positions with bond distances were calculated and visualized using PyMol. Molecular docking simulation results were also confirmed again by the protein dock server SWISSDOCK (http://www.swissdock.ch/docking) within protein receptors and ligand interactions [37].

Later, Pymol software was used to gain insight into their all-binding preferences within the active site of these receptors.

Protein-protein interaction between Ocr from T7 phage and EBF1 (a transcription factor)

To investigate the protein-protein interaction between the Ocr protein from T7 and EBF1 (Human Early B-cell Factor 1 DNA-binding domain), this data served as the basis for modelling the corresponding Ocr protein from T7, which exhibited similarity with the active sites (A subunit) of 1S7Z (https://www.rcsb.org/structure/1S7Z) [38] and 3LYR (https://www.rcsb.org/structure/3lyr) [39] as templates. Protein-protein interactions were analysed using the HADDOCK server [40].

The active site/interface residues of Ocr (1S7Z) and EBF1 (3LYR) can be found in the Supplementary data S4. The interacting residues were visualized using HADDOCK and PyMol [41].

RESULTS

Phylogenetic tree

The maximum likelihood phylogenetic tree shows main clades containing several clusters, and the viral genome sequences show genetic diversity according to MAUVE and Clustal W analyses, respectively (Supplementary data S5). Genetic diversity among sequences of T7 phage genomes clearly indicated that various types of T7 phage are present in different locations around the world.

Nucleotide and amino acid sequence alignment and analysis

Our results showed high mutational changes in whole genomes, except for the region between 746 and 1190 bp, which is a constantly unvarying part in whole sequences. MAUVE results have reciprocally confirmed the Clustal W analyses.

Homology modeling and protein prediction

The excised uniform regions of sequences subjected to alignment for protein similarity indicated that the 444-bp region included the overcome classical restriction (Ocr) protein with constantly unvarying sequences. These stabile sequences were selected as a template for further protein structural predictions (Fig. 1). The results of the submitted sequence converted to amino acid sequence using the ExPASy proteomics server (https://web.expasy.org/translate) were structurally very close to 1S7Z (Structure of Ocr from Bacteriophage T7) (www.rcsb.org/structure/1s7z) [38] as a target protein according to I-TASSER analysis, and its ligand was determined as Riboflavin (Vitamin B_2)($C_{17}H_{20}N_4O_6$), a small molecule showing interaction with this stabile sequences (Supplementary data S6).

MAMSNMTYNNVFDHAYEMLKENIRYDDIRDT
DDLHDAIHMAADNAVPHYYADIFSVMASEGID
LEFEDSGLMPDTKDVIRILQARIYE

Fig. 1 The results of submitted sequence converted to amino acid sequence using the ExPASy proteomics server (https://web.expasy.org/translate) for structural analysis by the I-TASSER server.

Molecular docking studies

For docking analysis of riboflavin with 1S7Z, the ligand structure of riboflavin retrieved from the PubChem database was analysed (Fig. 2, 3, 4). Docking of 1S7Z with the target molecule riboflavin was studied with respect to the following parameters: (a) interacting amino acids (b) ligand and protein atoms involved in hydrogen bonding (Supplementary data S6). The results of the SWISSDOCK server confirmed our results obtained using AutoDock software calculations. Furthermore, the binding possibilities of ligand on protein surface have been confirmed with the results of SWISDOCK (Fig. 2, 3, 4).

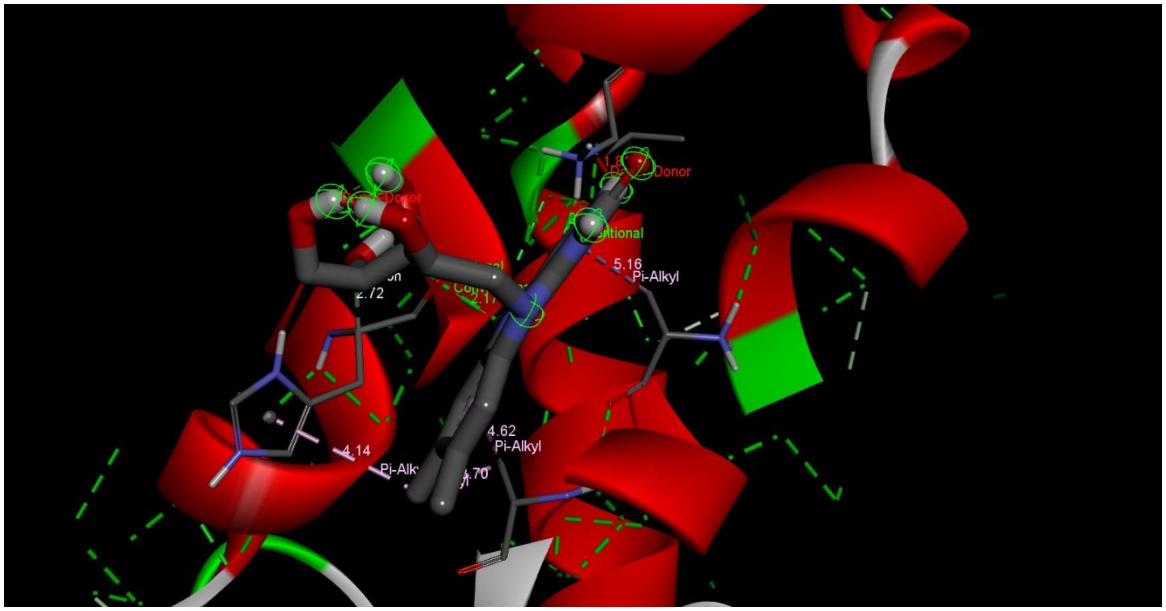

Fig. 2 The Ocr protein (red) interaction with riboflavin (gray).

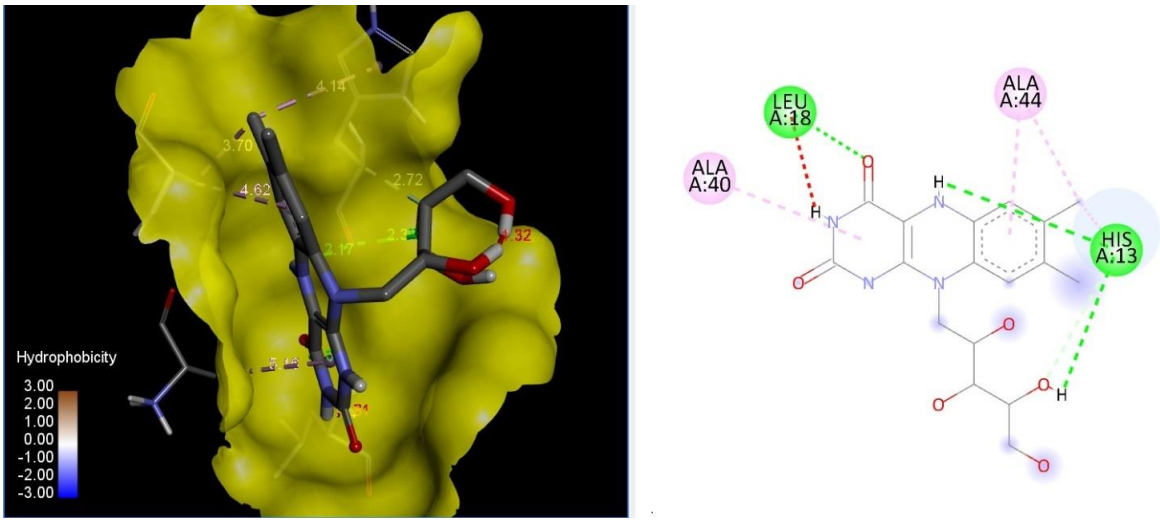

Fig. 3 Riboflavin docking coordinates in 3D with Ocr on the left, Riboflavin docking in 2D with Ocr on the right.

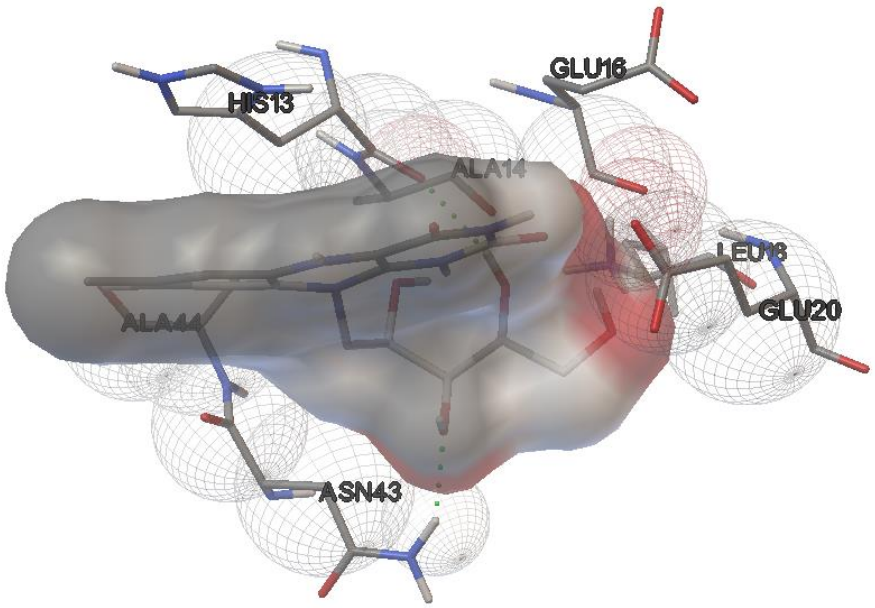

Fig. 4 The interaction at the highest binding site of Riboflavin to Ocr shows the amino acids binding in 3D.

Protein-protein interaction between Ocr from T7 phage and EBF1 (a transcription factor)

The parameters mentioned in the material and methods were used to model the Ocr and EBF1 interaction using the HADDOCK server, and the scores obtained according to the parameters are given below (Fig. 5 and Table 2). The results indicated the HADDOCK score -42.7 +/- 13.4 which shows high binding possibility within two selected proteins (Table 2) (Supplementary data S7).

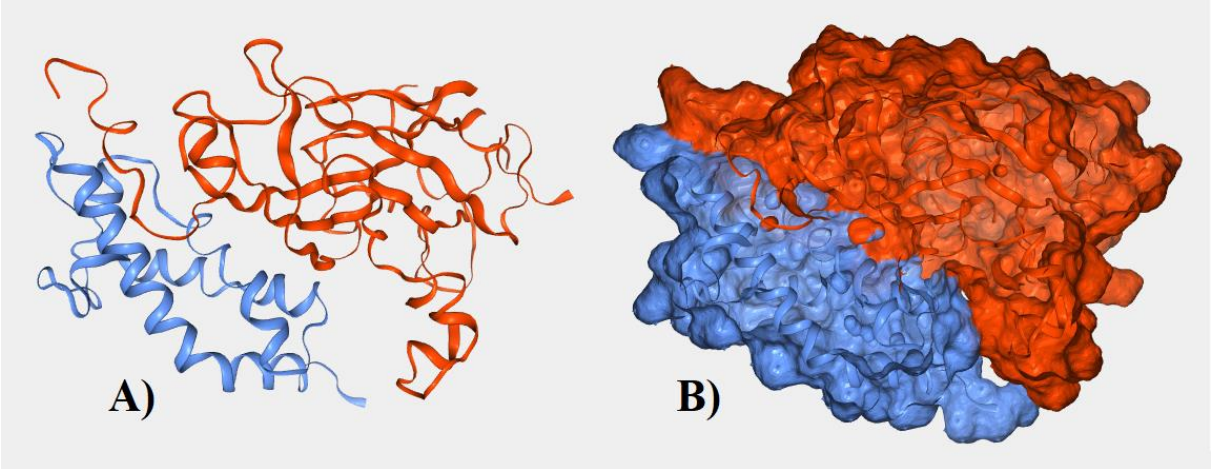

Fig. 5 Protein-protein interaction between Ocr (blue) and EBF1 protein (red). **(A)** Structural motifs, **(B)** Surface-covered structural motifs.

TABLE 2 Parametric values for the docking assay of Ocr and EBF1 protein.

Docking Parameters	Values
HADDOCK score	-42.7 +/- 13.4
Cluster size	12
RMSD from the overall lowest-energy structure	19.4 +/- 0.4
Van der Waals energy	-65.6 +/- 8.9
Electrostatic energy	-306.9 +/- 67.1
Desolvation energy	-2.8 +/- 4.0
Restraints violation energy	870.9 +/- 25.9
Buried Surface Area	2136.9 +/- 128.5
Z-Score	-2.4

DISCUSSION

The data demonstrated the presence of a genetically specific and conserved region within the entire T7 phage sequences (Supplementary data S1). The following alignment of all sequences using MAUVE, uniformity across all 82 distinct sequences were observed. The analysis identified common sequences exhibiting no discernible mutational differentiations. MAUVE represents the most effective method for conducting genome comparisons and can be suggested for identifying syntenic regions between two or more genomes.

Since some sequences were engineered sequences, they were considered as variants, and were included in the phylogenetic map (Supplementary data S5). The initial 444 base pairs emerge as the sole segment displaying a uniform sequence, distinct from the remaining portion of the phage genome. The identified invariant segment within the phage genome is responsible for overcome classical restriction (Ocr) protein and exhibits distinctive properties that could be used in further immunological studies.

In the sight of "*Baysal's hypothesis*" it can be suggested that in the genome, it is possible to find an invariant region in the genome which could be related to the evolution of the living organism. Therefore, these regions could be considered as a target point for the purpose of targeted drug-design. In accordance with this concept, the results of this study confirmed with our previous studies that the targeted region could be selected in the genomes [42-45]. Our studies indicated that effective and low-labour cost drug production could be possible on any targeted genomic region if various invariant sequences are available.

The conserved region of Bacteriophage T7 which was found through MAUVE analysis in this study, has evolved through co-evolution to modulate its host is overcome classical restriction (Ocr) protein that mimics B-Form DNA. Ocr, the gene 0.3 protein of phage T7 [46], functions as a competitive inhibitor of type I DNA restriction enzymes. Its mechanism involves preventing these enzymes from binding to their DNA targets. Notably, Ocr is one of the few well-characterized anti-restriction proteins [47-51, 38].

The binding affinity between Ocr and type I restriction enzymes is estimated to be 50-fold greater than that for DNA with a K_A of $\sim 2 \times 10^{10}\ M^{-1}$ [47, 50]. The host DNA exhibits resistance to cleavage, primarily because the target sequences are methylated by the modification methyltransferase activity of the R/M system. In the absence of the Ocr protein, the T7 genome becomes vulnerable to cleavage by the restriction endonuclease activity of the type I R/M system. This susceptibility arises due to the presence of target sequences in the T7 genome that lack the requisite pattern of methylation. This high-affinity binding effectively disables the cellular defence mechanism provided by the type I DNA restriction/modification (R/M) system, thereby facilitating phage propagation throughout the bacterial population [52-54]. In addition, the findings of Dunn et al. [55] showing the abundance of negatively charged

amino acids on Ocr is confirmed by its high-affinity for binding with EBF1 in this study (Fig. 5, Table 2).

The ligand that binds to Ocr found in this study is Riboflavin (vitamin B_2), which is an essential dietary component for humans (Fig. 2, 3, 4). Riboflavin is prevalent in aerobic organisms and is abundant in various food sources, including milk, beer, eggs, yeast, and leafy vegetables. It is a non-toxic vitamin crucial for the synthesis of two major coenzymes, namely flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These coenzymes play pivotal roles in energy metabolism, cellular respiration, antibody production, and the regulation of normal growth and development [56, 57].

The potential therapeutic role of riboflavin and its analogues in addressing various infectious diseases has been extensively deliberated previously. This includes their direct anti-infective effects, such as the inhibition of bacterial lipopolysaccharide transcription, reduction of nitric oxide (NO) levels or expression of inducible nitric oxide synthase (iNOS), and regulation of the balance of reactive oxygen species. Additionally, indirect effects have been proposed, such as the modulation of immune responses [57-59].

When aqueous solutions containing riboflavin are exposed to sunlight, riboflavin undergoes conversion into lumichrome under neutral conditions and into lumiflavin in alkaline solutions [60, 61]. Lumichrome is also recognized as a metabolic breakdown product of riboflavin within the human body [62]. It is known that vitamin B_2 is present in blood components, and it is possible to measure it [63, 64]. Riboflavin modifies nucleic acids upon exposure to light [65-67]. Light-activated riboflavin oxidizes guanine residues in nucleic acids, thereby impeding the replication of the pathogen's genome [68]. In addition, Corbin [69] found that riboflavin as an endogenous photosensitizer can inactivate high levels of a broad range of viruses and bacteria in platelet concentrates, fresh frozen plasma, and in red blood cells, preserving the activity and functionality of the components. Comparison of these previous results with the anti-Ocr effect of riboflavin found in this study suggests that this proven antiviral property of riboflavin and lumichrome substances may also be effective in T7 phages.

Riboflavin and Lumichrome production in bacteria have been known for a long time [70, 71]. However, there have been no studies on its antiphage effects. Since Liu et al. [72] reported that *Escherichia coli* O157:H7 senses microbiota-produced riboflavin to increase its virulence in the gut, it can be concluded that the bacteria could facilitate riboflavin for anti-phage activity. Considering the promoting feature of riboflavin in the advantage of *E. coli* infections, it can also be predicted that a riboflavin-free diet can be advised for patients.

Interactions between phage preparations and the human immune system have emerged as an intriguing area of investigation in recent years. However, only a few studies have described the antibacteriophage activity of human sera in patients undergoing phage treatment and in healthy volunteers [73-75]. Data derived from animal experiments suggest that phages have the capacity to provoke the production of antibodies capable of neutralizing their antibacterial activity [76, 77]. Bacteriophages serve as antigens and interact with the immune system. Recent elucidation has provided insights into the immune responses provoked by the phages and their potential effects [78, 20]. Similar proteins to Ocr in other phages can be responsible for these responses, further research is needed.

It is hypothesized that the human body continually absorbs phages from the gut, facilitating their transportation across cellular structures and throughout the body. It is noteworthy that bacteriophages possess the capability to circumvent the blood-brain barrier. In a study involving human samples, it was observed that individuals with a higher abundance of *Caudovirales* exhibited enhanced performance in executive processes and verbal memory. Conversely, elevated levels of *Microviridae* have been linked to a more pronounced decline in the brain's executive abilities [79]. These findings elucidate the direct interaction of phages with cells and organs within the human body, suggesting a potential contribution to human health

and immunity. However, phages are foreign and immunogenic particles capable of eliciting humoral immune responses and triggering the production of antiphage antibodies [80, 81]. In addition, it is noteworthy that patients showing symptoms linked to AIDS or cardiovascular diseases characteristically represent higher amount of phage DNA compared with healthy adult human plasma [82, 83], which may be a result of imbalanced/suppressed immunity. There may also be a link between the development of Parkinson's disease and bacteriophages as a possible factor [18, 84]. Ocr and similar proteins in other phages can be a key point for better understanding the phage-human cell interaction.

Complementary with this, we focused on Ocr and EBF1 interaction. EBF1 (The early B-cell factor) transcription factors are central regulators of development in several organs and tissues. The process of eukaryotic transcription is highly regulated and requires fine-tuned machineries in which transcription factors play a pivotal role as DNA binders [39]. The binding of the EBF transcription factor with Ocr from T7, as found in this study (Fig. 5), could result in cellular dysregulations and stalling in RNA transcription. These cellular dysregulations are noticeable for their immune suppressive effect. Also, it is known that as we get older, the immune system weakens, and because phages cannot be suppressed in many areas, including the brain, the body's organs could be damaged. It can be concluded that to prevent this case, a riboflavin-based diet or injection of riboflavin/lumichrome into blood is possible. Planned animal and clinical studies are expected to solidify this suggestion into a well-characterized process that can be safely and readily applied to reduce phages.

Notably, phages appear to exhibit a dual relationship with their human host, with both beneficial and potentially harmful effects [6]. Since the interest on the usage of bacteriophages in medicine, food protection, and agriculture increases, it is highly valuable to know how to remove them from the human body by further studies.

In the present study, as a model organism, T7 phage has been considered to determine a possible anti-phage compound from its genome sequences to test the validity of Baysals' hypothesis. Riboflavin showed promising results on the Ocr protein of T7 phage by docking studies. Also, by protein-protein interactions, the effect of Ocr on a human transcription factor were predicted.

In the present study, as a model organism, T7 phage was considered for determining a possible anti-phage compound from its genome sequences by testing the validity of Baysals' hypothesis. Riboflavin showed promising anti-phage results on the Ocr protein of T7 phage by docking studies. Also, by protein-protein interactions, the effect of Ocr on a human transcription factor were predicted.

CONCLUSION

Over the last century, the potential failure of antibiotics via resistant bacterial varieties has increased the interest in bacteriophages. With the help of genomic engineering techniques, new ways of using the phages in different areas are also developed. However, its long-term effects on the human body remain unclear. The methods of its clearance from the human body must be addressed clearly before they are approved for widespread use.

As a result of this study, a target constant region that is responsible for coding of the overcome classical restriction (Ocr) protein on T7 phage that provides protection from restriction enzymes was detected. A molecule called riboflavin that interacts with this target protein is predicted to be used in the clearance of T7 phage. In addition, another docking study revealed that Ocr has anti-transcriptive effect on the Human Early B-cell Factor 1 (EBF1) DNA-binding domain, which affects the cellular processes. Confirmation of these findings by further clinical and *in vitro* studies could inform us on phage-human interactions and new phage-clearing treatments.

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AUTHOR CONTRIBUTION
RSS: conceptualization, resources, funding acquisition, project administration, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review and editing.

CONFLICT OF INTEREST DISCLOSURE
The author declares no competing financial interests or personal relationships that could have influenced the work reported in this article.

DATA AVAILABILITY STATEMENT
The sequence data of this study are openly available in the NCBI database. The data supporting the findings of this study are available in the supplementary material of this article.

CODE AVAILABILITY
Not applicable.

ETHICAL APPROVAL
Not applicable.

CONSENT TO PARTICIPATE
Not applicable.

CONSENT FOR PUBLICATION
Not applicable.

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