Original Article

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Genome Analysis of the *Enterococcus faecium* Entfac.YE Prophage

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Abstract

Background: Bacteriophages are viruses that infect bacteria. Bacteriophages are widely distributed in various environments. The prevalence of bacteriophages in water sources, especially wastewaters, is naturally high. These viruses affect evolution of most bacterial species. Bacteriophages are able to integrate their genomes into the chromosomes of their hosts as prophages and hence transfer resistance genes to the bacterial genomes. Enterococci are commensal bacteria that show high resistance to common antibiotics. For example, prevalence of vancomycin-resistant enterococci has increased within the last decades.

Methods: Enterococcal isolates were isolated from clinical samples and morphological, phenotypical, biochemical, and molecular methods were used to identify and confirm their identity. Bacteriophages extracted from water sources were then applied to isolated *Enterococcus faecium (E. faecium)*. In the next step, the bacterial genome was completely sequenced and the existing prophage genome in the bacterial genome was analyzed.

Results: In this study, *E. faecium* EntfacYE was isolated from a clinical sample. The EntfacYE genome was analyzed and 88 prophage genes were identified. The prophage content included four housekeeping genes, 29 genes in the group of genes related to replication and regulation, 25 genes in the group of genes related to structure and packaging, and four genes belonging to the group of genes associated with lysis. Moreover, 26 genes were identified with unknown functions.

Conclusion: In conclusion, genome analysis of prophages can lead to a better understanding of their roles in the rapid evolution of bacteria.

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Keywords: Anti-bacterial agents, Bacteriophages, *Enterococcus faecium*, Genome analysis, Prophages

Introduction

Two important species of commensal enterococci, *Enterococcus faecalis (E. faecalis)* and *Enterococcus faecium (E. faecium)*, are one of the leading causes of medical conditions, causing various hospital infections such as endocarditis and sepsis [1]. Due to the increased antibiotic resistance properties of these bacteria, their infections are often difficult to treat [2]. For example, the prevalence of Vancomycin-Resistant Enterococci (VRE) has increased inducing complexities in hospitalized patients in the last two decades [3]. Since one of the most important health concerns is to find novel solutions to fight these multidrug-resistant bacterial infections, the use of novel strategies seems urgently necessary. In this regard, the use of phages can hopefully be promising [4]. Bacteriophages (Phages) are prokaryotic viruses detected in various environments within their bacterial hosts or in large numbers of free virions [5]. Currently, these bacterial viruses are under the spotlight as appropriate substitutes for the available antibiotics. Phages can effectively infect and kill antibiotic-resistant bacteria regardless of the resistance patterns of these bacteria [6]. In addition, phages have several advantages over other antimicrobial agents with no serious or irreversible side effects [7]. Therefore, the characterization of novel phages and understanding their evolutionary ecology can greatly help scientists improve the process of chemical antimicrobial replacement. Novel genome analyzing methods, including next-generation sequenc-

ing methods, and established genome databases have relatively facilitated the development of phage knowledge. Therefore, the purpose of the current study was to analyze the Entfac.YE prophage.

Materials and Methods

Bacterial strain

E. faecium was isolated from clinical samples in a university teaching hospital in Tehran, Iran, using routine culture methods as well as phenotypic and genotypic methods [8]. The isolate was verified using morphological, biochemical, and molecular techniques such as catalase test, arabinose fermentation, salt tolerance, optochin susceptibility, CAMP, and PYR tests. Enterococcal *tuf* gene was amplified using PCR (Polymerase chain reaction) and partially sequenced using the Sanger sequencing platform (Kawsar Biotech, Iran). The bacterial strain was also used for the isolation of enterococcal phages.

Whole-genome sequencing

After isolation of phages on *E. faecium*, the bacterial strains challenged by the phages were used to extract their genome. Briefly, a small volume of two-layer agar-containing bacteria with phages was removed and dissolved in Saline Magnesium (SM) buffer. This was centrifuged at 4480 *g* for 10 *min*. After centrifugation, the supernatant was filtered through 0.45-*μm* syringe filters and mixed with DNase 1 and RNase A. The mixture was stored at 37°*C* for 30 *min*. Then, the bacterial genome was extracted using the precipitation method with ethanol and propanol. The extracted bacterial genome was completely sequenced using the Illumina Hiseq platform (Novogene, China) (Table 1). SPAdes algorithm was used in the *de novo* technology of genome assembling. Furthermore, the reference assembly method was used for the raw data. The prophage was analyzed using Regulatory Sequence Analysis Tools (RAST) (https://rast.nmpdr.org/) and then DDBJ Nucleotide Sequence Submission System (NSSS) (https://www.ddbj.nig.ac.jp).

Results

Sanger sequencing results of the bacterial *tuf* gene verified the initial characteristics of the isolated bacteria (DDBJ accession numbers: LC580430 and LC580431). The bacterial genome was completely sequenced and information obtained through Novaseq 6000 platform (Illumina, USA) are demonstrated in table 1. The Entfac.YE prophage included 69,990 nucleotides, consisting of 31.13% A, 31.60% T, 18.94% C, and 18.29% G nucleotides. In total, 88 prophage genes were analyzed for their functions (Table 2). The prophage content included four housekeeping genes, 29 genes in the group of replication and regulation, 25 genes in the group of structure and packaging, and four genes in the group of lysis. The functions of other 26 genes were unknown (Figure 1). Bacteriophages isolated in a relative phase of the current study included three lytic members. Two tailed phages included isometric shapes (*Siphoviridae* and *Myoviridae*) and the other one was filamentous (*Inoviridae*).

Discussion

In general, phages isolated from the clinical strain of *E. faecium* using sources of wastewaters were lysogenic phages with the suggestion of further prophages in the bacterial genome. Therefore, whole-genome sequencing of the enterococcal strain was carried out. The selected prophage of *E. faecium* was named Entfac.YE, including 88 genes. Of the identified genes, four genes were in the group of housekeeping genes, 29 genes were in the group of replication and regulation genes, 25 genes were in the group of packaging and structure genes, four genes were in the group of lysis genes, and 26 genes were in no groups due to their unknown functions. In this study, 14 *E. faecium* were identified in 25 enterococcal isolates. In 2019, Karna *et al* identified four *E. faecium* in five enterococcal isolates [9]. Although *E. faecium* is a large-intestine symbiotic bacterium of humans and animals,

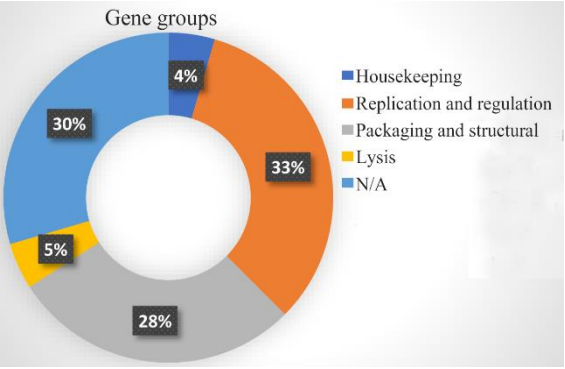

Figure 1. Analysis of the prophage gene groups.

Table 1. Information of the Illumina Hiseq platform

Platform type	Illumina Novaseq 6000
Read length	Paired-end 150 *bp*
Recommended sequencing depth	≥100×for bacterial genomes
Standard data analysis	- Data quality control: filtering reads containing adapter or with low quality - Alignment with reference genome, statistics of sequencing depth and coverage - SNP/InDel calling, annotation and statistics - CNV calling, annotation and statistics - SV calling, annotation and statistics

Table 2. An overview of the genes from Entfac.YE prophage

CDS	Protein	Gg	*bp*	DDBJ	NCBI	Cv.	Id.
1	Tape measure protein	PS	3848	LC606177	WP_002350712	99	100
2	Phage tail tape measure protein	PS	3320	LC606192	WP_010776531	99	100
3	YhgE/Pip domain-containing protein	H	2705	LC603693	WP_002296556	99	100
4	Phage/plasmid primase P4 family domain-containing protein	RR	2312	LC606170	WP_012197635	99	100
5	Phage tail tape measure protein	PS	2309	LC602689	WP_002303051	99	100
6	Hypothetical protein	N/A	2003	LC602243	HAQ8643042	95	99.8
7	Terminase large subunit	PS	1727	LC603684	WP_002303033	99	100
8	Terminase large subunit	PS	1694	LC606188	WP_002286533	99	100
9	Terminase large subunit	PS	1391	LC606195	MBA1326607	94	100
10	Phage major capsid protein	PS	1364	LC602693	WP_002303037	99	100
11	Hypothetical protein	N/A	1319	LC606183	EOG13061	99	100
12	Hypothetical protein	N/A	1295	LC606163	HAQ9917245	99	100
13	Phage portal protein	PS	1229	LC603683	WP_002303034	99	99.7
14	Site-specific integrase	RR	1226	LC603694	HAP7047036	99	99.75
15	Phage portal protein	PS	1220	LC606182	WP_002350718	99	100
16	Phage major capsid protein	PS	1193	LC606180	WP_002350716	99	100
17	Virulence-associated protein E	N/A	1190	LC606216	EOH59192	91	95.6
18	Site-specific integrase	RR	1178	LC606176	WP_002288969	99	100
19	Phage portal protein	PS	1178	LC606189	WP_002317387	99	100
20	Phage portal protein	PS	1175	LC606197	WP_002296486	99	100
21	Tyrosine-type recombinase/integrase	RR	1142	LC606186	WP_010729348	99	100
22	Site-specific integrase	RR	1142	LC606193	WP_002287705	99	100
23	Site-specific integrase	RR	1139	LC603692	WP_002286587	99	100
24	Site-specific recombinase, phage integrase family	RR	1136	LC606165	EJX50302	96	100
25	Site-specific integrase	RR	1127	LC606187	WP_002288357	99	100
26	Site-specific integrase	RR	1121	LC606158	WP_033647077	99	100
27	Site-specific integrase	RR	1034	LC606210	HAQ1208098	95	99.7
28	YqaJ viral recombinase family protein	RR	941	LC606160	WP_033646797	99	100
29	Recombinase RecT	RR	890	LC606161	WP_002301573	99	100
30	Hypothetical protein	N/A	875	LC606191	WP_129984688	99	99.6
31	BppU family phage baseplate upper protein	PS	854	LC606214	WP_192183965	99	100
32	Hypothetical protein	N/A	800	LC606162	WP_048946585	99	100
33	Tyrosine-type recombinase/integrase	RR	785	LC606215	WP_074399919	94	98.7
34	Phage antirepressor	RR	776	LC603691	WP_002290310	99	100
35	Helix-turn-helix domain-containing protein	RR	749	LC603689	WP_130017038	99	100
36	Phage antirepressor	RR	746	LC606211	WP_047641521	99	100
37	Hypothetical protein	N/A	713	LC603687	AGS74848	99	100
38	Hypothetical protein	N/A	698	LC603690	KXH16430	99	100
39	AAA family ATPase	H	686	LC606169	WP_012197638	99	100
40	Clp protease ClpP	PS	686	LC606190	WP_002286527	99	100
41	Clp protease ClpP	PS	677	LC606181	WP_002350717	99	100
42	Site-specific integrase	RR	638	LC606202	WP_148905844	97	100
43	Tail protein	PS	626	LC602690	WP_002303047	99	99.5
44	Hypothetical protein	N/A	599	LC606224	PWT87042	36	55.5
45	Recombinase family protein	RR	581	LC600466	WP_002294320	99	100
46	HK97 family phage prohead protease	PS	569	LC602694	WP_002349220	99	100
47	Phi13 family phage major tail protein	PS	560	LC606178	WP_002332434	99	100
48	Hypothetical protein	N/A	554	LC606174	ELB35873	98	100
49	Hypothetical protein	N/A	518	LC606164	HAQ9543613	97	100
50	Hypothetical protein	N/A	485	LC606204	KKJ73631	95	99.3
51	Hypothetical protein	N/A	479	LC606223	WP_168472540	66	96.2
52	Tyrosine-type recombinase/integrase	RR	473	LC606173	WP_002290382	99	100
53	Phage terminase-like protein, large subunit	PS	473	LC606185	AGS76423	99	100
54	Phage terminase small subunit P27 family	PS	473	LC606194	WP_002296488	99	100

Contd. Table 2. An overview of the genes from Entfac.YE prophage

CDS	Protein	Gg	*bp*	DDBJ	NCBI	Cv.	Id.
55	P27 family phage terminase small subunit	PS	452	LC603685	HAQ5377436	99	99.3
56	Hypothetical protein	N/A	449	LC606213	WP_002341785	99	100
57	Hypothetical protein	N/A	425	LC606221	MBA1326600	69	100
58	ImmA/IrrE family metallo-endopeptidase	RR	422	LC606166	WP_002350678	99	100
59	Hypothetical protein	N/A	419	LC606168	EGP5556540.1	99	100
60	Helix-turn-helix transcriptional regulator	RR	410	LC606159	WP_002297387	99	100
61	Hypothetical protein	N/A	395	LC606179	WP_002350714	99	100
62	HNH endonuclease	L	380	LC603686	WP_002304435	99	100
63	Helix-turn-helix transcriptional regulator	RR	374	LC606167	WP_002315392	99	100
64	Hypothetical protein	N/A	338	LC602691	WP_002303043	99	100
65	VRR-NUC domain-containing protein	H	317	LC606171	WP_002350665	99	99.0
66	MazG-like family protein	RR	314	LC606172	WP_012197627	99	100
67	Hypothetical protein	N/A	308	LC606218	WP_180753898	99	100
68	MazG-like family protein	RR	302	LC603688	WP_002303017	99	100
69	Hypothetical protein	N/A	299	LC606196	KKJ73220	99	100
70	Phage gp6-like head-tail connector protein	PS	281	LC602692	WP_002303039	99	100
71	Glucosaminidase domain-containing protein	H	278	LC606175	WP_196975365	88	100
72	Phage gp6-like head-tail connector protein	PS	248	LC606198	HAQ4209649	79	87.8
73	Hemolysin XhlA family protein	L	242	LC606200	WP_002332427	98	100
74	Hemolysin XhlA family protein	L	242	LC606207	WP_002299182	98	98.7
75	Hypothetical protein	N/A	239	LC606205	WP_002296498	98	100
76	Hypothetical protein	N/A	230	LC606203	RBS38180	98	100
77	Hypothetical protein	N/A	224	LC606157	WP_002304837	98	98.6
78	Phage holin	RR	224	LC606222	WP_002286683	98	100
79	Tyrosine-type recombinase/integrase	RR	221	LC606220	WP_148779155	83	95.1
80	Phage holin	RR	197	LC606199	WP_002302122	98	100
81	Phage holin	RR	197	LC606206	WP_005876842	98	100
82	Ribbon-helix-helix domain-containing protein	RR	158	LC606184	WP_010729417	98	100
83	XkdX family protein	L	137	LC606201	WP_002332428	97	100
84	Hypothetical protein	N/A	128	LC606208	HAP9761800	97	100
85	Hypothetical protein	N/A	125	LC606219	PZM70292	69	93.1
86	Phage gp6-like head-tail connector protein	PS	119	LC606209	WP_002296484	97	100
87	Hypothetical protein	N/A	95	LC606212	KKJ66783	81	96.3
88	Phage baseplate upper protein	PS	89	LC606217	HAQ6822550	90	100

CDS: Coding sequence; Gg: Gene group; *bp*: base pair; DDBJ: DNA Data Bank of Japan; NCBI: NCBI reference sequence; Cv: Coverage; Id: Iidentity; N/A: Not applicable; L: Lysis; RR: Replication and regulation; PS: Packaging and structural; H: Housekeeping.

it is listed by the World Health Organization as a global priority for multidrug-resistant pathogens [10]. In the present study, *E. faecium* isolates were resistant to vancomycin, erythromycin, clindamycin, ceftriaxone, and cefoxitin. Based on the published studies, 70% of isolated *E. faecium* strains from Tehran hospitals were resistant to vancomycin and erythromycin [11]. In a similar study by Rahbar *et al* on clinical samples in Tehran, antibiotic resistance patterns of enterococcal isolates included 79% resistance to erythromycin and 51% to vancomycin [12]. In this study, the prophage was identified in the *E. faecium* genome as reported in other studies [13].

In the present study, two recombinase family protein genes were reported. This protein catalyzes sensitive DNA exchange reactions between short target sequences (30-40 nucleotides). In 2010, Lopes *et al* reported the gene in the phage genome [14]. Other genes identified in this study were hypothetical protein genes. The exact functions of these genes are unknown. In total, 25 hypothetical protein genes were reported in Entfac.YE prophage. Similar genes were identified in the EFRM31 phage genome by Mazaheri *et al* [15]. Another gene was the tail tape measure protein, which determines the tail length and facilitates DNA transfer to the cell cytoplasm during cell infection. In the current study, two copies of this gene were identified. This gene was reported in 2016 in phage TP901-1 as well [16]. In the present study, two copies of the tail protein gene were reported. This gene encodes proteins linked to the

phage head. Three copies of gp6-like head-tail connector protein gene were identified in Entfac.YE prophage, encoding proteins that connect the phage head and tail. This gene was also identified in phages of *Staphylococcus aureus (S. aureus)* SA12 [17]. Two copies of the major capsid protein gene were identified in Entfac.YE prophage. As the gene name imparts, this gene encodes a capsid protein. In 2010, this gene was identified in the EFRM31 phage genome [15]. In the present study, HK97 family phage prohead protease gene was reported. The major functions of this gene were breaking down scaffold proteins and processing capsid proteins. The mechanism of action of this gene was investigated in 2013 by Duda *et al* [18]. Four copies of the portal protein gene were reported in the present study. The portal protein formed a channel for viral DNA to pass bilaterally. Another gene, the terminase large subunit gene, is involved in DNA transfer and packaging termination. Four copies of this gene were identified in Entfac.YE prophage. These genes were also reported in the EFRM31 phage genome [15]. A similar gene, P27 family phage terminase small subunit gene, is responsible for binding to several identifying elements at the start of packaging. This gene has also been identified in *S. aureus* phages [19]. A copy of HNH endonuclease gene was reported in the present study. The possible biological role of this gene is stimulating homologous recombination by nicking DNA, which enhances gene conversion. This gene was also reported in a study by Mazaheri *et al* [15]. Two copies of MazG-like family protein gene were identified in Entfac.YE prophage which are involved in regulating the survival of bacterial cells under stress conditions. The function and structure of this gene in *Deinococcus radiodurans* were investigated by Goncalves *et al* [20].

A copy of helix-turn-helix domain-containing protein gene was identified in the present study. This protein can bind to DNA. In this study, two copies of anti-repressor gene were reported. This gene prevents suppressor proteins from binding to their operators. This gene was identified in *Salmonella* phages as well [21]. Nine copies of the site-specific integrase gene were reported in Entfac.YE prophage to rearrange DNA fragments. This gene was identified in the phage genome of TP901-1 [22]. A copy of YhgE/Pip domain-containing gene was identified in the phage genome. In the present study, a copy of helix-turn-helix transcriptional regulator gene, involved in transcription regulation, was characterized. Another gene, the recombinase RecT gene is involved in DNA binding and metabolic processing. A copy of this gene was reported in the phage genome. Functions of this gene in the phage genome of *Bacillus subtilis* were investigated in previous studies [23]. A copy of ImmA/IrrE family metallo-endopeptidase gene was detected in Entfac.YE prophage. The ImmA protein, also encoded by transposons, has been shown to be essential for the breakdown of ImmR. The AAA family ATPase genes, identified in the present study, have a variety of roles such as cell cycle regulation, proteolysis and protein breakdown, and intracellular transport. The mechanisms of action of these genes were also investigated before [24]. The function of the primase P4 family domain-containing gene includes nucleotide binding. The molecular function of the *VRR-NUC* gene includes domain-containing hydrolase activity that affects ester bonds. Based on previous studies, the exact function of this gene in phages is unknown [25]. The tyrosine-type recombinase/integrase gene is involved in DNA binding and recombination, of which four copies were identified in the present study.

A domain version of the glucosaminidase domain-containing protein was reported in this study, which is responsible for the structural determination of peptides and glycans during vegetative growth. A copy of the phi13 family phage major tail gene was identified in Entfac.YE prophage and its molecular function was also investigated before [26]. Two copies of ClpP protease gene were detected in the present study. This gene includes ATP-dependent peptidase and serine endopeptidase activities [27]. Naturally, the ribbon-helix-helix domain-containing protein gene is involved in transcriptional regulation, a copy of which was identified in this study. Three copies of holin genes were identified in Entfac.YE prophage. Holins are a diverse group of small proteins produced by dsDNA phages to stimulate and control the destruction of the host cell walls at the end of the lytic cycle. Holins have been suggested as the clock proteins of phage infections [28]. Two copies of the hemolysin XhlA family protein gene were identified in this study. XhlA is a cell surface-associated hemolysin that breaks down two common types of insect immune cells (Granulocytes and plasmatocytes) as well as rabbit and horse red blood cells. A copy of XkdX family protein gene reported in this study is detected in phage genomes close to choline and endolysin genes. The BppU family phage baseplate upper gene detected in the present study has been identified in the *S. aureus* phage genome. The virulence-associated protein E gene has also been identified in *Streptococcus* spp. [29]. In the current study, a copy of the baseplate upper protein gene was reported. Based on the complete genomic analysis of an enterococcal phage by Mazaheri *et al*, genes similar to those of the present study were found, including genes of hypothetical protein, HNH endonuclease, tape measure protein, terminase small subunit, portal protein, prohead protease, major capsid protein, and major tail protein [15]. In a study by Tan *et al* in 2007, genes similar to those of this study were reported, including terminase large subunit and portal protein genes [30]. In the present study, 25 genes of hypothetical proteins were analyzed. In a study by O'Flaherty *et al* on the phage genome, 63 hypothetical protein genes were identified. Furthermore, they identified one AAA family ATPase, one endonuclease and one P27 family gene as well as

one holin and one major capsid protein gene [31].

Conclusion

Bacteriophages are naturally prevalent in the environment, especially in wastewaters. Nowadays, clinical bacterial isolates generally show resistance to antibiotics, which has created many problems for health professionals and patients. Prophages can be considered as mobile genetic elements in the transfer of antibiotic resistance genes to genomes of their host bacteria. Furthermore, genome analysis of prophages can help researchers better understand their roles in bacterial ecology and evolution.

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Conflict of Interest

The authors declare no conflict of interest.

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