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The Gene *ail* for the Attachment-Invasion Locus Protein of *Yersinia enterocolitica* Biotype 1A Strains Is Located on the Genome of Novel Prophages

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Abstract

The attachment-invasion locus protein Ail of pathogenic *Yersinia* strains is an important virulence factor for both invasion of eucaryotic cells as well as serum resistance. In other *Yersinia* strains, e.g. those belonging to biotype (BT) 1A of *Yersinia enterocolitica*, *ail* has only occasionally been described. Sequence analysis of 370 BT 1A isolates in our laboratory revealed 41 (11.1%) being *ail*-positive. Most of these isolates were recovered from minced meat and wild boars and belong to 17 MLST allele profiles. A closer look at DNA sequences surrounding *ail* disclosed that the gene of most isolates is embedded in DNA regions encoding phage proteins. The genomes of four prophages belonging to four different phylogenetic clusters were determined and analysed by in silico studies. They have sizes of 34.9 and 50.7 kb and are closely related to each other, but not to known phages. Unlike other regions of the prophages, the integrases and attachment sites of some of them diverge leading to different integration sites in the isolates. In a fifth cluster, *ail* is relocated at a different position on the *Y. enterocolitica* chromosome, but surrounded by prophage-related sequences. In addition, highly pathogenic 1B/O:8 strains contain a DNA segment including *ail* that is similar to the prophage sequences determined in this study.

Keywords: ail; virulence; *Yersinia enterocolitica*; prophage; horizontal gene transfer

1. Introduction

Yersiniosis is *an infectious disease of the gastrointestinal tract* caused by *Yersinia* (*Y.*) *enterocolitica* and, to a lesser extent, by *Y. pseudotuberculosis* [1,2]. It is the third most common bacterial enteritis in Europe. Infections are mainly caused by the consumption of raw or undercooked pork [3–9]. *Y. enterocolitica* comprises six biotypes (BT) and up to 70 serotypes. While the BTs 1B, 2, 3, 4 and 5 possess the 70 kb virulence plasmid pYV encoding a type III secretion system and effector proteins (*Yersinia* outer proteins = YOPs) with in part toxic activity for eucaryotic cells, BT 1A strains are generally devoid of pYV [10]. Moreover, some important chromosomally encoded virulence factors of pathogenic biotypes are only rarely or not existing in BT 1A [11,12]. Two of those are the enterotoxin YstA and the attachment-invasion locus protein (Ail), which is involved in both the invasion of eucaryotic cells as well as in serum resistance [13–15]. The *ail* gene has yet only been detected in some BT 1A strains [16,17]. Because of the lack of these virulence factors, BT 1A strains have been considered to be non-pathogenic for a long time. However, during the last years, there is an increasing number of reports describing BT 1A isolates from clinical cases [18–26]. This biotype obviously comprises at least two phylogenetic lineages, each with different virulence factors, some of which are toxins [17,27]. The question arises, whether horizontal gene transfer may be involved in the heterogeneity of this biotype. In this study, we sequenced 370 BT 1A isolates from different

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sources, of which 41 contain the virulence gene *ail*. A closer analysis of this gene revealed that unlike in the strictly pathogenic biotypes, *ail* of most BT 1A isolates is located on the genome of prophages, which were characterized by in silico analyses.

2. Results

2.1. Forty-One Out of 370 BT 1A Genomes Contain a Prophage-Associated Ail Gene.

Sequence analyses of 370 *Y. enterocolitica* BT 1A isolates revealed that they harbor the gene *ail*. They were isolated between 2013 and 2025 from different sources (mainly minced meat and wild boars) and belong to 17 different MLST allele profiles (Figure 1A). All isolates possess the enterotoxin gene *ystB*, 30, 10 and 3 of them additionally the virulence-associated genes *hreP*, *myfA* and *tccC*, respectively (Table S1). For almost all isolates, plasmid-borne sequences of approximately 3 to 106 kb were predicted.

A closer look at the *ail* gene of these isolates showed that it is up to 99% identical to *ail* of pathogenic biotypes, e.g. the bio/serotype 1B/O:8 strain 8081. In addition, the upstream sequence containing the promoter and ribosome binding site of *ail* are similarly related to their counterpart in other biotypes suggesting that *ail* may be active in BT 1A.

However, the regions encompassing *ail* diverge significantly in the various biotypes. In most BT 1A isolates like in 24-YE00064 studied here, the gene is surrounded by partitioning genes and genes for cell lysis (lysin and holin) and DNA packaging (small and large terminase) typically associated with phage (Table 1). Moreover, genes for phage assembly (capsid and tail), the genetic switch as well as an integrase and excisionase were also identified in most chromosomes suggesting that *ail* is part of a prophage (Table S2).

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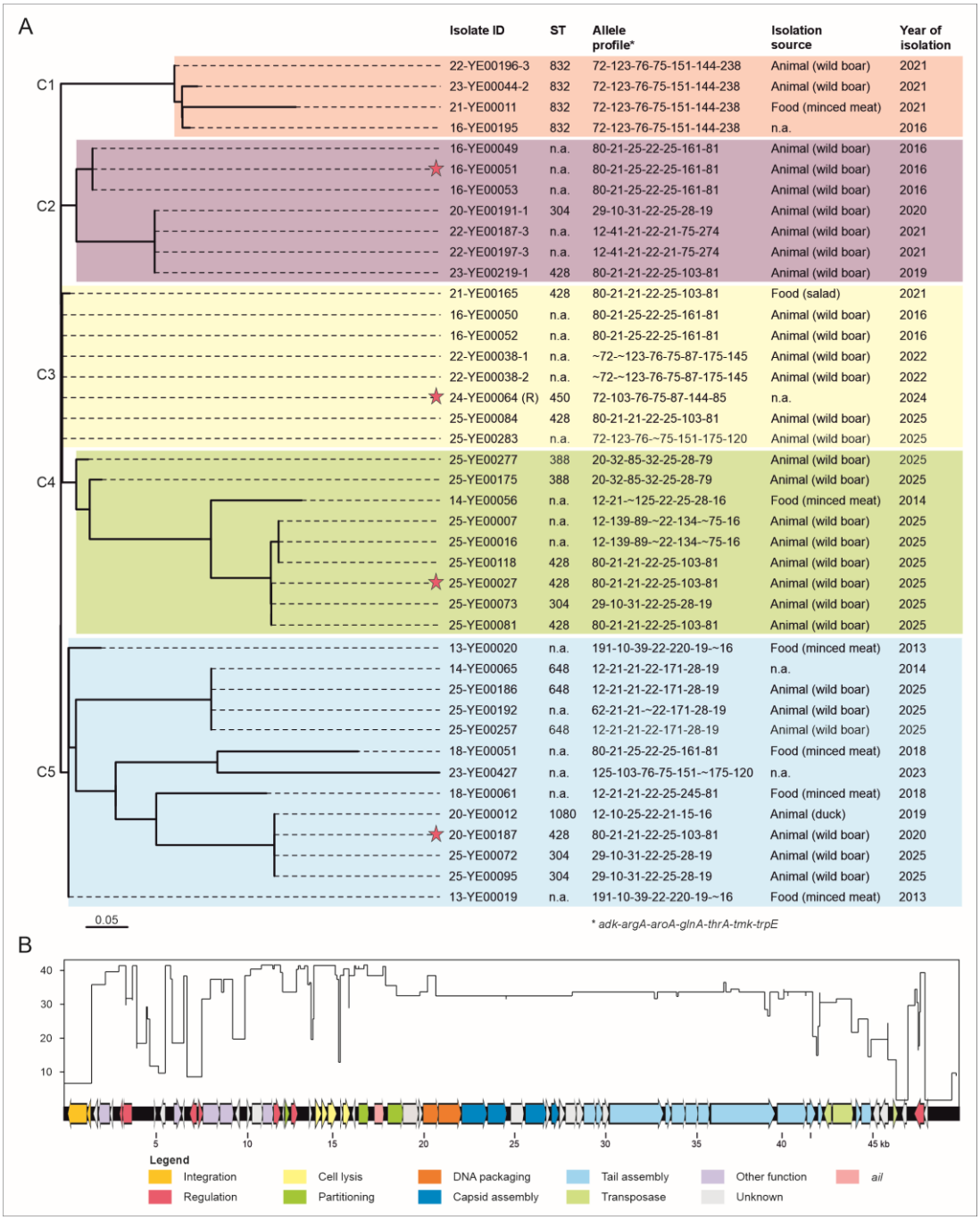

Figure 1. Relationship of prophages containing an *ail* gene. A. Clusters of similar prophage genomes in *Y. enterocolitica* BT 1A isolates, obtained by comparison with the vB_Yen_24-YE00064 prophage (R) of cluster 3. The ST type of their hosts as well as the source and year of isolation are stated. Red stars indicate isolates in which the whole *ail* prophage sequences were determined. B. Conserved DNA regions of the prophage genomes. The plot gives the numbers of related phage sequences identified in the compared WGS dataset of the isolates. The map shows the genome of the prophage vB_Yen_24-YE00064 used as reference for sequence comparison (Table 1).

Table 1. ORF analysis of the prophage vB_Yen_24-YE00064.

Element	Start	Stop	Strand	Predicted function	Accession	E-value
ORF01	1229	147	-	Phage integrase	WP_339099454	0
ORF02	1473	1204	-	Phage excisionase	WP_032819545	9,65E-58
ORF03	1850	1548	-	Unknown	WP_050123456	9,16E-67
ORF04	2496	1873	-	Single-stranded DNA-binding protein	WP_050123460	8,2E-151
ORF05	2676	2506	-	Unknown	WP_219644695	8,92E-32

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ORF06	3206	3033	-	Phage CIII repressor	CNH66013	3,38E-33
ORF07	3745	3230	-	Phage AntA/AntB antirepressor	EKN4711341	3,1E-123
ORF08	4959	5075	+	Unknown	-	-
ORF09	5588	5280	-	Unknown	WP_219644503	9,54E-66
ORF10	6039	6506	+	Phage super-infection exclusion protein B	EKN5118889	1,6E-107
ORF11	6667	6509	-	Unknown	WP_400175511	2,44E-28
ORF12	7338	6916	-	Phage CI-like repressor	WP_219649054	6,25E-98
ORF13	7442	7699	+	Phage Cro/Cl family transcriptional regulator	WP_151432101	1,28E-56
ORF14	7686	8636	+	DNA-binding protein	WP_258018631	0
ORF15	8626	9372	+	Replisome organizer	EKN4711333	0
ORF16	9741	9454	-	Unknown	WP_400175508	1,3E-63
ORF17	10129	10335	+	Unknown	WP_050123484	5,88E-40
ORF18	10421	11020	+	Unknown	EKN4711330	1,3E-143
ORF19	11020	11592	+	Phage NinG rap recombination	WP_400175506	3,3E-138
ORF20	11592	12005	+	Phage antitermination protein Q	WP_219648280	6,23E-97
ORF21	12185	12090	-	Unknown	EHB0983027	0,008902
ORF22	12226	12510	+	Type II toxin-antitoxin system (RelE/ParE family)	EKN5956923	3,7E-59
ORF23	12583	12957	+	Transcriptional regulator	WP_050162972	1,09E-84
tRNA01	13325	13400	+	tRNA-Thr-CGT		
tRNA02	13402	13476	+	tRNA-Gly-TCC		
ORF24	13653	13772	+	Unknown	WP_144405165	9,81E-16
ORF25	13925	14320	+	Phage holin	WP_050123331	3,02E-88
ORF26	14320	14616	+	Phage holin family protein	WP_219647003	1,4E-62
ORF27	14603	15145	+	Phage lysozyme (N-acetylmuramidase) family	HDL7801217	1,6E-125
ORF28	15305	15418	+	Unknown	WP_219644478	1,2E-15
ORF29	15474	15869	+	Phage endopeptidase Rz	EKN4799071	3,51E-87
ORF30	16181	15999	-	Unknown	SRY18578	1,42E-06
ORF31	16338	16895	+	KilA-N domain-containing protein	WP_219644727	9,9E-132
ORF32	17202	17738	+	Attachment invasion locus protein Ail	WP_219647006	4E-126
ORF33	17951	18820	+	Chromosome (plasmid) partitioning protein ParB	CNF12705	0
ORF34	18813	19685	+	Unknown	WP_050130101	0
ORF35	19670	19918	+	Unknown	WP_050130103	9,51E-50
ORF36	19922	20788	+	Phage terminase, small subunit	WP_258018632	0
ORF37	20766	22070	+	Phage terminase, large subunit	WP_050123349	0
ORF38	22075	23487	+	DNA-binding protein	WP_050123375	0
ORF39	23492	24604	+	Phage head morphogenesis protein	ELI7924874	0
ORF40	24795	25547	+	Unknown	WP_151431638	9,3E-180
ORF41	25602	26747	+	Phage major capsid protein	WP_242365527	0
ORF42	26814	27002	+	Unknown	WP_219647076	1,39E-34
ORF43	27014	27496	+	DnaT-like ssDNA-binding protein	WP_050123385	1,4E-114
ORF44	27500	27853	+	Unknown	WP_050123386	6,52E-79
ORF45	27856	28446	+	Unknown	WP_151431635	1E-141
ORF46	28443	28862	+	Unknown	WP_050123389	4,56E-98
ORF47	28880	29542	+	Phage tail protein	WP_050123390	1,4E-159
ORF48	29565	29921	+	Phage tail assembly chaperone	WP_050123392	1,56E-80
ORF49	29924	30235	+	Unknown	WP_373368631	6,9E-70
ORF50	30232	33339	+	Phage tail, tail length tape-measure protein H	WP_219651824	0
ORF51	33412	33753	+	Phage tail tip, assembly protein M	WP_050123394	1,7E-77
ORF52	33762	34514	+	Phage tail tip, assembly protein L	WP_219654916	0
ORF53	34517	35233	+	Phage tail tip, assembly protein K	MFM1259745	8,9E-178
ORF54	35233	35838	+	Phage tail tip, assembly protein I	MFM1259744	9,4E-141
ORF55	35851	39552	+	Phage tail tip, host specificity protein J	MFM1259743	0
ORF56	39619	41256	+	Phage tail fiber protein	WP_289823745	0
ORF57	41256	41783	+	Phage tail fiber assembly protein	MFM1259741	8,3E-124
ORF58	41881	42189	+	Phage tail fiber protein	WP_400175531	8,95E-65
ORF59	42650	42225	-	Transposase	WP_050132355	3,3E-98
ORF60	42708	43871	+	Transposase	WP_400175835	0
ORF61	43997	44314	+	Phage tail fiber protein	MFJ1219555	1,36E-65
ORF62	44321	44884	+	Phage tail fiber assembly protein	WP_050162967	1,7E-135
ORF63	45339	44956	-	Unknown	WP_032820973	1,07E-84
ORF64	45809	45339	-	Unknown	WP_004392760	6,9E-111
ORF65	46078	46362	+	Transposase, IS3/IS911 family	AJI84358	1,41E-55

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ORF66	46856	46572	-	Unknown	WP_050123427	9,42E-62
ORF67	47840	47244	-	Phage antirepressor protein	WP_339099468	7,8E-142
ORF68	48028	47837	-	Unknown	WP_032820969	3,09E-37

2.2. Comparison of the Prophages Indicates Relationships Between Them.

The 41 prophage sequences identified in the investigated isolates by comparison with the vB_Yen-24-YE00064 prophage form five major clusters (Figure 1A), of which the prophages in the clusters C2 to C5 harbor the *ail* gene, whereas prophages in cluster C1 are devoid of *ail*, since here, the gene is located at a different position on the chromosome (see below). An alignment disclosed that most regions of the prophages, particularly those encoding structural proteins, are closely related, whereas e.g. the integrase genes show major differences (Figure 1B). Short read sequencing allowed the prediction of four whole prophage genomes (vB_Yen_16-YE00051, vB_Yen_20-YE00187, vB_Yen_24-YE00064 and vB_Yen_25-YE00027) belonging to four clusters (Figure 1A). They have genome sizes of 34,918 to 50,744 bp and are composed of 55 to 78 Open Reading Frames (ORFs, Table S1). The overall genome organization of the prophages is similar (Figure 2A). As with other temperate phages, ORFs for repressor proteins, cell lysis, DNA packaging, capsid and tail assembly are clustered, even though some ORFs, particularly those encoding structural proteins, are missing in vB_Yen_16-YE00051. The prophages showed no identities to other phages and only some relatedness to two *Y. enterocolitica* BT 1A chromosomes (Y201, CP124238.1 and Y115, CP124259.1).

Three prophages (vB_Yen_16-YE00051, vB_Yen_20-YE00187 and vB_Yen_25-YE00027) have an identical attachment site *att* of 40 bp (Figure 2B). Their integrases are 100% identical (Figure 2C). By contrast, the prophage vB_Yen_24-YE00064 has an *att* site of only 27 bp (Figure 2B). The integrase of this prophage is only approximately 35% identical to those of the other ones (Figure 2C). Thus, it is not surprising that the two groups have different integration sites on the bacterial chromosome. While the prophages vB_Yen_16-YE00051, vB_Yen_20-YE00187 and vB_Yen_25-YE00027 are integrated between two genes for hypothetical proteins, vB_Yen_24-YE00064 is integrated between a gene for an integrase and a YebY family protein.

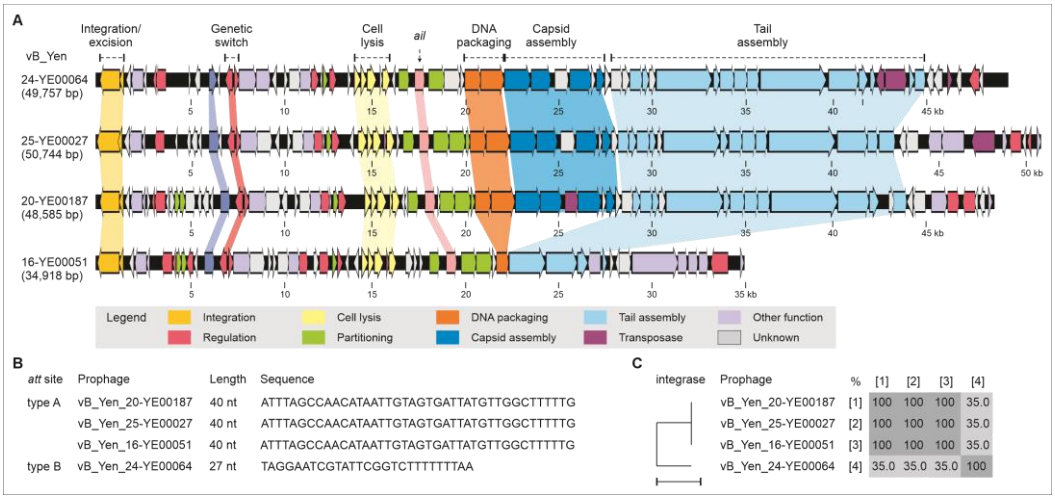

Figure 2. Genome organization and similarities of the integrases and attachment sites of the four analysed prophages. A. Genome maps of the prophages. Sizes of the genomes and predicted functions of assigned ORF are indicated. B. Sequences of the attachment sites of the prophages. C. Similarities of their integrases.

2.3. Relocation of ail in Cluster C1 and Analysis of 1B/O:8 Strains.

Sequence analysis of cluster C1 revealed similar prophage sequences as in isolate 24-YE00064. However, at the position of *ail* in vB_Yen_24-YE00064, there is a gap in the C1 prophage genomes (Figure 3A). In this cluster, *ail* is located approximately 600 kb apart from the vB_Yen_24-YE00064-related prophage sequences. Interestingly enough, the gene and its adjacent sequences are

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surrounded by DNA segments, which are similarly present in the corresponding prophages, as shown for the prophage vB_Yen_23-YE00044.2 (Figure 3B). The homologous upstream and downstream sequences of *ail* have a length of 144 bp and 925 bp, respectively, but are not related to each other. Therefore, it remains open how *ail* was relocated in isolates belonging to cluster C1. Nevertheless, it is noteworthy that even in the highly pathogenic *Y. enterocolitica* 1B/O:8 strains 8081 (AM286415.1), WA (CP009367.1) and Billups-1803-68 (CP173224.1), *ail* is associated with phage genes. Indeed, a stretch of approximately 20 kb of strain 8081 containing *ail* is similar to vB_Yen_24-YE00064 (Figure 3C). This stretch essentially corresponds to ØYE200 identified in 8081 (Thomson et. al, 2006), which, however, has been determined as a smaller prophage (15.5 kb) without *ail*. Besides *ail*, the 20 kb prophage of strain 8081 comprises genes for e.g. an integrase, lysis proteins (holin and lysin) and the terminase large subunit. Moreover, the fact that this DNA segment also contains the 27 bp attachment site of vB_Yen_24-YE00064 upstream of the integrase gene and that is linked to tRNA genes suggests that *ail* was once associated with a similar prophage. It is conspicuous that the DNA segment in strain 8081 harbors several transposase genes which are lacking in vB_Yen_24-YE00064 and which might have been involved in genetic reassortments.

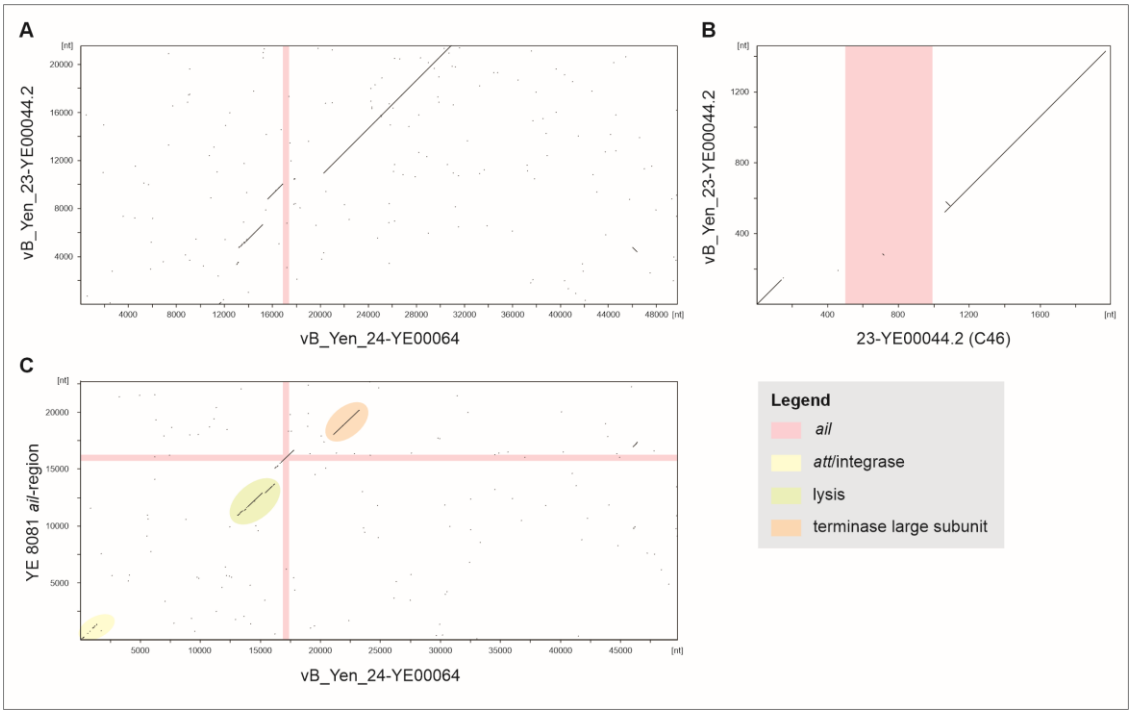

Figure 3. Dot plots of the prophages vB_Yen_24-YE00064, vB_Yen_23-YE0044.2 and the ØYE200 region of strain 8081. (A) The vB_Yen_24-YE00064 *ail*-prophage is similar to the cluster C1 prophage vB_Yen_23-YE0044.2 lacking *ail*. (B) In isolate 23-YE00044.2, *ail* is located on a different contig (C46) than the prophage, but surrounded by prophage-related sequences. The dot plot shows only the small part of vB_Yen_23-YE0044.2, which is similar to the *ail* region. (C) vB_Yen_24-YE00064 is related to the ØYE200 prophage region of strain 8081, which also includes *ail* and an abridged terminase large subunit gene.

3. Discussion

The attachment-invasion locus protein Ail of *Y. enterocolitica* is an important virulence factor, which is produced by all pathogenic biotypes of this species, as well as by *Y. pseudotuberculosis* and *Y. pestis* [28]. It has yet only rarely been described in BT 1A strains of *Y. enterocolitica* and in *Y. enterocolitica*-like species, e.g. *Y. kristensenii,* some of which have been reported to contain an additional *ail*-related gene which may be associated with plasmids or phages [11,29,30]. For that reason, *ail* is routinely used as target for the detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* by RT-PCR (ISO TS 18867:2015). Though, sequencing of 370 BT 1A isolates showed

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that *ail* is more commonly present in this biotype as expected. We identified the gene in 41 (11.1%) out of 370 isolates recovered from food and wild boars in the last 12 years. The *ail*-positive isolates represent a broad range of MLST alleles profiles, even though some types (ST304, ST428, ST832) were prevailing. The question arises, how BT 1A strains may acquire *ail*. This study suggests that it may occur by lysogenic conversion via temperate phages. Indeed, the analysis of *ail*-positive BT 1A isolates showed that in most of them the gene is located on a prophage. The prophages are related to each other, but form five different clusters. Up to now, four prophage genomes containing *ail* could be analysed in detail. The analysis suggests that three of them (vB_Yen_20-YE00187, vB_Yen_24-YE00064 and vB_Yen_25-YE00027) may be active, since all elements required for the formation of a phage particle are obviously present, whereas the prophage vB_Yen_16-YE00051 is presumably defective, because some essential ORFs for capsid and tail proteins are lacking. The fact that genes for structural proteins of the complete prophages are very similar indicates that the corresponding phage particles may have the same morphology. A striking difference, however, pertains to the integration site of them on the *Y. enterocolitica* chromosome. Unlike some other parts of their genomes, genes for the integrase and the attachment sites are in part highly diverse. As a consequence, the prophages are not integrated at the same position in BT 1A strains. Whether these sites also exist in other *Y. enterocolitica* biotypes or even other *Yersinia* species and whether those strains may also acquire *ail* by phage-mediated transfer has still to be studied. It has to be taken into account that for lysogenic conversion, the host range of a temperate phage is of major importance. *Yersinia enterocolitica* BT 1A strains belong to various serotypes, which may determine the host specificity of a phage. It appears, however, that *ail*-prophages are subjected to genetic recombinations. This can be clearly seen in cluster C1 comprising related prophages, in which *ail* including adjacent sequences has been relocated. Similarly, highly pathogenic 1B strains like 8081 contain remnants of *ail*-prophages suggesting recombination events or even a horizontal gene transfer in the past. Regrettably, information on temperate *Y. enterocolitica* phages and their potential to exchange genes is scarce [31,32]. We will therefore now determine the inducibility of the identified *ail*-prophages and phenotypic properties of possibly produced phage particles.

4. Materials and Methods

4.1. Typing of Y. enterocolitica Strains.

Isolates were initially cultivated on Columbia agar supplemented with 5% sheep blood (bioMérieux Deutschland GmbH, Nürtingen, Germany) at 28°C for 16-20 hours for whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometrical identification (MALDI-TOF MS) using the direct transfer method with HCCA matrix on a Biotyper (Bruker Daltonics GmbH & Co. KG, Bremen, Germany). In addition, physiological and biochemical tests using classical tube and plate procedures for species confirmation and biochemical differentiation were conducted as previously described [33]. Unless otherwise indicated, YP cultivation was conducted at aerobic conditions at 28°C for 18-24 hours using lysogeny broth (LB)-based media. For solid media preparation, LB medium was supplemented with 1.8% bacto agar no. 1 (Oxoid Deutschland GmbH, Wesel, Germany) [34,35].

4.2. Genome Sequencing and Bioinformatics Analysis

Whole-genome sequencing (WGS) of *Y. enterocolitica* BT 1A isolates was performed by short-read, paired-end sequencing (2 x150 cycles) on a NextSeq500 benchtop device (Illumina Inc., San Diego, CA, USA). Bacterial genomic DNA was extracted from liquid cultures grown at 37°C for 20-24 hours using the PureLink Genomic DNA Mini Kit (Invitrogen, Ebersberg, Germany) according to the recommendation of the manufacturers. DNA sequencing libraries were prepared using the Nextera XT DNA Sample Preparation Kit (Illumina Inc.) [31,36–38]. Raw sequencing data were subjected to the Aquamis pipeline (Deneke et al., 2021) for quality evaluation, demultiplexing and trimming, while general in silico typing purposes were conducted using BakCharak

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(https://gitlab.com/bfr_bioinformatics/bakcharak; access: Jul-2025) [39]. Prophage detection was conducted using the Phastest tool for initial screening, while manual curation using Accelrys DS Gene (v2.5; Accelrys Inc., San Diego, CA, USA) was performed to determine the complete prophage sequence from attachment sites of the bacteria (*attB*) and prophages (*attP*). Genome annotation of the prophage genomes was conducted using the Bacterial and Viral Bioinformatics Resource Center (BV-BRC). Initial functional prediction of open reading frames (ORFs) was manually curated according to predicted functions of closely related protein sequences derived from blastp searches at NCBI (National Center for Biotechnology Information) [31,36–38]. Phylogenetic relationship of vB_Yen_24-YE00064 (reference) to *Y. enterocolitica* genome datasets encoding Ail were conducted using CSI phylogeny (v1.4; default settings; https://cge.food.dtu.dk/services/CSIPhylogeny/; access: Sep-2025)

4.3. Genome Accession Numbers

Deposition of prophage genomes at NCBI Genbank was conducted using BankIt for the prophages vB_Yen_24-YE00064 (PV779719), vB_Yen_16-YE00051 (SUBMITTED), vB_Yen_20-YE00187 (PX109664) and vB_Yen_25-YE00027 (PX109665).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: in-silico typing features of *Y. enterocolitica* genomes analyzed in this study; Table S2: Annotation of the prophage genomes vB_Yen_24-YE00064, vB_Yen_25-YE00027, vB_Yen_20-YE00187 and vB_Yen_16-YE00051.

Author Contributions: Conceptualization, Stefan Hertwig and Jens Hammerl; Data curation, Jens Hammerl; Formal analysis, Stefan Hertwig and Jens Hammerl; Funding acquisition, Stefan Hertwig and Jens Hammerl; Investigation, Stefan Hertwig and Jens Hammerl; Methodology, Stefan Hertwig and Jens Hammerl; Project administration, Stefan Hertwig; Resources, Stefan Hertwig; Software, Jens Hammerl; Validation, Stefan Hertwig and Jens Hammerl; Visualization, Jens Hammerl; Writing – original draft, Stefan Hertwig and Jens Hammerl; Writing – review & editing, Jens Hammerl.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The original data presented in the study are openly available in Genbank under accession numbers PV779719 (vB_Yen_24-YE00064), SUBMITTED (vB_Yen_16-YE00051), PX109664 (vB_Yen_20-YE00187) and PX109665 (vB_Yen_25-YE00027).

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Conflicts of Interest: The authors declare no conflicts of interest.

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