Activity of a Holin-Endolysin System in the Insecticidal Pathogenicity Island of *Yersinia enterocolitica*

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ABSTRACT *Yersinia enterocolitica* is a pathogen that causes gastroenteritis in humans. Because of its low-temperature-dependent insecticidal activity, it can oscillate between invertebrates and mammals as host organisms. The insecticidal activity of strain W22703 is associated with a pathogenicity island of 19 kb (Tc-PAI_{Ye}), which carries regulators and genes encoding the toxin complex (Tc). The island also harbors four phage-related and highly conserved genes of unknown functions, which are polycistronically transcribed. Two open reading frames showed significant homologies to holins and endolysins and exhibited lytic activity in *Escherichia coli* cells upon overexpression. When a set of *Yersinia* strains was tested in an equivalent manner, highly diverse susceptibilities to lysis were observed, and some strains were resistant to lysis. If cell lysis occurred (as demonstrated by membrane staining), it was more pronounced when two accessory elements of the cassette coding for an i-spanin and an o-spanin were included in the overexpression construct. The pore-forming function of the putative holin, HolY, was demonstrated by complementation of the lysis defect of a phage λ S holin mutant. In experiments performed with membrane preparations, ElyY exhibited high specificity for W22703 peptidoglycan, with a cleavage activity resembling that of lysozyme. Although the functionality of the lysis cassette from Tc-PAI_{Ye} was demonstrated in this study, its biological role remains to be elucidated.

IMPORTANCE The knowledge of how pathogens survive in the environment is pivotal for our understanding of bacterial virulence. The insecticidal and nematocidal activity of *Yersinia* spp., by which the bacteria gain access to nutrients and thus improve their environmental fitness, is conferred by the toxin complex (Tc) encoded on a highly conserved pathogenicity island termed Tc-PAI_{Ye}. While the regulators and the toxin subunits of the island had been characterized in some detail, the role of phage-related genes within the island remained to be elucidated. Here, we demonstrate that this cassette encodes a holin, an endolysin, and two spanins that, at least upon overexpression, lyse *Yersinia* strains.

KEYWORDS *Yersinia enterocolitica*, dual-lysis cassette, endolysin, pathogenicity islands

Yersinia enterocolitica is a foodborne pathogen that is widely spread in aquatic and terrestrial reservoirs as well as in dairy products. It is the major cause of yersiniosis, which is one of the five primary bacterial gastrointestinal diseases in humans (1). The major animal source of pathogenic *Y. enterocolitica* is swine (2). Because *Y. enterocolitica* is a psychrotolerant pathogen, it is of major concern in infection containment. The *Yersinia* genus, belonging to the *Enterobacteriaceae* family, comprises three human

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pathogens (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*), and thus far, 14 environmental species considered harmless to humans have been identified, including *Y. ruckeri*, a salmonid pathogen, and *Y. intermedia*, an intermediate between *Y. pseudotuberculosis* and *Y. enterocolitica*, based on its genetic and phenotypic properties. The species *Y. enterocolitica* comprises six biovars that differ in their biochemical reactions (3). Biovar 1A strains are considered avirulent because they lack *Yersinia* virulence plasmid pYV (4), whereas biovar 1B strains, mainly found in North America, carry a pathogenicity island responsible for iron acquisition that confers high virulence toward humans and mice (5). Biovar 2 to 5 strains, predominately isolated in Europe and Japan, comprise a weakly pathogenic group unable to kill mice (6). Both insecticidal and nematocidal activities were experimentally demonstrated in biovar 2 strain W22703 (7, 8), which demonstrated a multiphasic life cycle of this pathogen that permitted it to oscillate between invertebrates and vertebrates (9–11). A key determinant that enables *Y. enterocolitica* to kill *Caenorhabditis elegans* and insect larvae is the Tc-PAI_{Y_e} pathogenicity island, which encodes the toxin complex (Tc) that was first characterized in *Photobacterium luminescens* and acts on actin and Rho-GTPases through ADP-ribosylation (12). The Tc-PAI_{Y_e} is highly conserved in the genomes of the three pathogenic *Yersinia* species, excluding *Y. enterocolitica* biovars 1A and 1B, as well as in the genomes of *Y. mollaretii* and *Y. similis*, whereas it is absent in the genomes of all other (environmental) *Yersinia* species (13). The expression of the *tc* genes *tcaA*, *tcaB*, *tcaC*, and *tccC* is strictly temperature dependent in *Y. enterocolitica* strain W22703, because they are silenced at 37°C and activated at lower temperatures, with maximal activation at 10°C (8). At low temperatures, the TcaR2 regulator outcompetes its antagonist, the H-NS/YmoA complex, and activates the *tc* genes. At 37°C, *tcaR2* transcription is reduced, TcaR2 is subjected to proteolysis, and the amount of functional YmoA increases, allowing it to interact with DNA-binding protein H-NS and inhibit transcription of the *tc* genes (14, 15).

The *tcaA*, *tcaB*, and *tcaC* gene cluster on Tc-PAI_{Y_e} is separated from *tccC* by two small phage-related genes putatively encoding a holin and endolysin (HE) and by two open reading frames (ORFs) of unknown function. This cassette of four ORFs has a total length of approximately 1.3 kb and is conserved in all examples of Tc-PAI_{Y_e} identified to date. The origin of the cassette is unclear, and no other phage-related genes have been detected in the neighborhood of Tc-PAI_{Y_e}. In the chromosome of *Y. pestis* Angola, the cassette is flanked by a transposon, which suggests the acquisition of the phage-related genes by a common ancestor of the pathogenic *Yersinia* strains, possibly as a relic of a prophage.

In this study, we characterized the lysis cassette of Tc-PAI_{Y_e} in detail.

RESULTS

Phage-related genes of Tc-PAI_{Y_e} encode a putative lysis cassette. Four ORFs related to phage genes are located between *tcaC* and *tccC* within the Tc-PAI_{Y_e} insecticidal island of *Y. enterocolitica* strain W22703 (8). These ORFs are proposed to constitute a lysis cassette, as two of the ORFs code for a holin-endolysin (HE) cassette according to the results of homology searches and the prediction of transmembrane helices. The holin, termed HolY, consists of 103 amino acids. The 11.3-kDa protein is predicted to form three transmembrane helices and therefore belongs to the class I holins represented by the λ S protein (16). Its sequence was compared with sequences in databases using protein BLAST (17). Well-conserved homologs (70% to 87% identity) were encoded by genes located on the insecticidal islands of several *Yersinia* species (13). Less closely related homologs (51% to 60% identity) comprised holins present in human and insect pathogens (Fig. 1A). Holin TcdE from *Clostridium difficile* (18), the phage λ holin, and a holin encoded by *Serratia entomophila* plasmid pADAP, which confers insecticidal activity (19), exhibited low or no significant sequence homology to HolY. The putative endolysin of Tc-PAI_{Y_e}, ElyY, comprises 133 amino acids and is predicted to have a modular organization with an N-terminal, enzymatically active domain and a C-terminal cell wall-binding domain (CBD). The most closely related

FIG 1 Comparison of proteins with homology to the holin and endolysin sequences of *Y. enterocolitica* strain W22703. (A) Alignment of 15 representative holin sequences grouped according to their identity with HolY of strain W22703. A hydrophobicity prediction performed using TMPred revealed three possible transmembrane domains (indicated by cylinders and rectangles). *E. cloacae*, *Enterobacter cloacae*; *C. violaceum*, *Chromobacterium violaceum*; *X. kansasae*, *Xenorhabdus kansasae*. (B) Alignment of 17 representative sequences of endolysins and related proteins. Asterisks indicate positions with identical fully conserved residue amino acids; colons indicate conservation among groups with strongly similar properties. Multiple-sequence alignment was performed using Clustal Omega (54, 55). The accession numbers of all proteins are listed in Table S2. *E. amylovora*, *Erwinia amylovora*; *P. asymbiotica*, *Photobacterium asymbiotica*; *P. mirabilis*, *Proteus mirabilis*; *P. stuartii*, *Providencia stuartii*; *S. boydii*, *Shigella boydii*.

homologs (74% to 90% identity) were detected in *Yersinia* genomes (Fig. 1B). In *Yersinia* strains with Tc-PAI_{Yer} the endolysin gene is located immediately downstream of the holin gene. An endolysin from *Y. enterocolitica* strain 8081 also belongs to this group (64% identity), but its gene (YE1672) is neither part of one of the four prophages in this strain (20) nor flanked by a holin gene. Homologous endolysins (53% to 63% identity) were also found in a number of species in addition to members of the *Yersinia* group (Fig. 1B). In accordance with its homology to VanY from *Escherichia coli*, ElyY is a putative L-alanyl-D-glutamate endopeptidase or a D-alanyl-D-alanine carboxypeptidase. Homologs of ElyY are absent in *P. luminescens* or *S. entomophila*.

The two ORFs, which overlap by 62 bp and are located downstream of the HE genes of strain W22703, are present in all the insecticidal islands of yersiniae (13). BLAST analysis revealed that the first ORF encodes a putatively exported protein of 119 amino acids and N-terminal transmembrane domain (TMD) and that the second ORF encodes a putative membrane protein of 76 amino acids. They may therefore be considered to encode an i-spanin and an o-spanin (21, 22) that are part of the lysis cassette. We termed these two accessory elements yersinial Rz and Rz1 (*yRz* and *yRz1*) in accordance with the Rz and Rz1 nonhomologous genes of phage lambda (23). Significant (>33%) homologies of these two genes beyond *Yersinia* strains were not observed.

Transcriptional organization of the lysis gene cassette. The transcriptional activity of the promoter of the lysis cassette is very low (unpublished data) and below a threshold value defined recently (15). We revealed the transcriptional organization of this lysis cassette using reverse transcriptase PCR with RNA isolated from strain W22703 grown at 15°C to an optical density at 600 nm (OD₆₀₀) of 0.85. cDNA of 12 regions spanning approximately 200 to 800 bp was analyzed (Fig. 2A). All but two PCRs performed with cDNA as the template revealed a DNA fragment with a length corresponding to that of the PCR fragments amplified from genomic DNA (Fig. 2B). The

FIG 2 Transcriptional organization of the lysis cassette in *Y. enterocolitica* strain W22703. (A) Holin/endolysin-encoding genes *holY* and *elyY* are followed by two hypothetical genes; *tcaC* and *tccC* flanking the cassette are also shown. The whole fragment is a part of the 19-kb Tc-PAI_{yc} pathogenicity island. The arrows indicate promoters according to reverse transcriptase PCR, promoter predictions, or reporter fusion experiments (14, 15). (B) Cells were grown at 15°C, and mRNA was extracted at an OD₆₀₀ of 0.85. cDNA was amplified with primers listed in Table S1. Reverse transcriptase PCR was performed with primer pairs specific for regions 1 to 12 (indicated in panel A). All PCR products were separated using 2% agarose gel electrophoresis. PCR was performed with genomic DNA and DNase-treated RNA samples as the templates serving as controls. Primers for cDNA amplification had been tested in a similar manner (data not shown). Each PCR was performed at least twice.

transcription of all genes was shown, and fragments overlapping the genes of the lysis cassette indicate that the transcription of the *holY*, *elyY*, *yRz*, and *yRz1* genes is controlled by a single promoter. This finding was confirmed by quantitative real-time PCR (qRT-PCR) experiments performed with plasmid pBAD-HE⁺ (including the HE genes together with *yRz* and *yRz1* [HE⁺]) (see Fig. S1 in the supplemental material). No amplification was observed using primers whose products would point to a polycistronic mRNA of *tcaC* and *holY* or of *yRz1* and *tccC*. These data illustrate that the lysis cassette of strain W22703 constitutes an independent transcriptional unit of these four genes.

Lysis of *E. coli* upon HE gene overexpression. To test the biological function of the lysis determinant, the HE genes, alone and together with *yRz* and *yRz1* (HE⁺), were overexpressed using plasmids pBAD33-HE and pBAD33-HE⁺ in heterologous host *E. coli* strain TOP10. The OD₆₀₀ decreased within 1 h after addition of 0.02% arabinose (Fig. 3). No significant difference was observed in the growth properties in the presence of the two accessory elements (HE⁺). As a control, we showed that logarithmic growth of *E. coli* cultures was not affected by arabinose or the nonrecombinant vector. Overexpres-

FIG 3 Growth of *E. coli* TOP10 in the presence of overexpressed HE. *E. coli* cells were transformed with pBAD33, pBAD33-HE, and pBAD33-HE⁺. An overnight culture was diluted 1:1,000 in LB medium. Following incubation at 37°C with shaking, each of the three cultures was split when the two cultures reached an OD₆₀₀ of 0.25, and arabinose was added to reach a final concentration of 0.02% or was not added (control). Cells were then incubated under the same conditions for 5 h. Time point zero was set immediately after dilution. Each experiment was independently performed thrice, and the standard deviations (SDs) are shown.

sion of the lysis genes following addition of arabinose was shown by the determination of the relative gene expression levels of *holY*, *elyY*, and *spanin* genes via qRT-PCR (Fig. S1A). Taken together, these data demonstrate the biological functionality of the lysis cassette from the Tc-PAI_{Y_e}.

Overexpression of the lysis cassette in a diverse range of Gram-negative hosts.

To investigate the specificity of the lysis genes, pBAD33-HE and pBAD33-HE⁺ were tested in the representatives of *Y. enterocolitica* biovars 1 to 5, *Y. pseudotuberculosis*, several apathogenic *Yersinia* species, *Salmonella enterica* serovar Typhimurium, and *P. luminescens* (Fig. 4). In biovar 2 strain W22703, arabinose-induced HE and HE⁺ overexpression resulted in growth curves at 37°C similar to those seen in the *E. coli* experiment.

In the biovar 1 to 3 and 5 strains, overexpression of the HE⁺ lysis cassette had an attenuating effect on the growth of *Y. enterocolitica* biovar 1B, 2, 3, and 5 strains at 37°C (Fig. 4A) but exhibited only a very weak effect on biovars 1A and 4 strains (data not shown). When the construct lacking *yRz* and *yRz1* (HE) was used, we also observed lysis that, however, was less pronounced than in the presence of HE⁺ as exemplified by biovar 2.

Growth retardation upon HE⁺ induction was also observed in *Y. pseudotuberculosis*, *Y. ruckeri*, *Y. kristensenii*, and *S. Typhimurium* but not in *Y. intermedia*, *Y. frederiksenii* (Fig. 4B), and *Y. aldovae* (data not shown). Interestingly, *P. luminescens* strain TT01 was resistant to the activity of overexpressed HE (data not shown). Nonrecombinant plasmid pBAD33, used as a control, exhibited no effect on the growth behavior of all strains tested in medium with or without arabinose. Because Tc-PAI_{Y_e} is absent in all strains shown in Fig. 4B as well as in the biovar 1A and 1B strains, no correlation was observed between the presence of the insecticidal island and the lytic capacities of the HE cassette. The addition of arabinose stimulated the growth of strains, indicating that the effect of the lysis cassette on cell viability is stronger than is suggested by the graphs. The successful overexpression of the lysis genes in several strains was demonstrated by qRT-PCR (Fig. S1A to C).

To test whether the decrease of the OD₆₀₀ value is a result of cell lysis, the CFU levels of W22703 and W22703 pBAD33-HE were determined. Figure S2 shows a decrease in CFU at 3 h after the addition of arabinose by more than 1 order of magnitude, thus confirming that HE overexpression had killed a majority of the population. W22703 and

FIG 4 Overexpression of *holY* and *elyY*. (A) Plasmids pBAD33 and pBAD33-HE⁺ were transformed in *Y. enterocolitica* biovar 1A and 1B and biovar 2 to 5 strains, and plasmid pBAD33-HE was transformed in the biovar 2 strain. Overnight cultures were diluted 1:500 and grown at 37°C with shaking. Induction with 0.2% (wt/vol) arabinose was performed at an OD₆₀₀ of 0.2. (B) *Y. pseudotuberculosis*, *Y. ruckeri*, *Y. intermedia*, *Y. kristensenii*, *Y. frederiksenii*, and *S. Typhimurium* were similarly tested. Gray, strains with plasmid pBAD33; black, strains with pBAD-HE⁺. Cultivation was performed in the absence (solid lines) or presence (dashed lines) of arabinose. SDs of results from three independently performed experiments are shown.

W22703 pBAD33-HE cells grown at 37°C with and without arabinose were also subjected to a viability test by which cells with intact membranes were stained green and those with compromised membrane integrity were stained red. Fluorescence microscopy revealed that most W22703 cells were alive, whereas most arabinose-induced W22703 pBAD33-HE culture cells were stained red, clearly suggesting membrane damage and cell death upon HE overexpression (Fig. 5). W22703/pBAD33-HE exhibited a low number of dead cells in the absence of arabinose, probably due to the leakiness of the pBAD33 system.

In contrast to the induction of a prophage, overexpression of the lysis cassette allowed growth resumption, and the strains reached stationary phase after several hours of incubation (data not shown). To investigate whether or not the metabolism of arabinose is responsible for this phenomenon, an *araA* deletion mutant of strain W22703 equipped with pBAD-HE was tested. In the presence of arabinose, we measured a higher maximal OD₆₀₀ of strain W22703 than of W22703 Δ *araA*, unable to utilize arabinose (data not shown), whereas equal growth of the two strains resumed after approximately 7 h. These data suggest that the all-or-none induction of P_{BAD} (24), resulting in a selection for recombinant plasmid loss, is responsible for growth resumption.

FIG 5 Live/dead staining of W22703 cultures without and with overexpression of the HE genes. Strains W22703 and W22703 pBAD33-HE were grown at 37°C until an OD₆₀₀ of 0.2 was achieved. The latter culture was split, 0.2% arabinose was added to one sample, and the two cultures were grown for 1 h. Cells were harvested, washed thrice with phosphate-buffered saline (PBS) buffer, and adjusted to equal densities. The samples were exposed to a mixture of SYTO 9 and propidium iodide (PI), which stains nucleic acids, using a Live/Dead bacterial viability kit (Invitrogen, Karlsruhe, Germany; catalog no. L7007) according to the manufacturer's instructions. SYTO 9 stains live cells (green fluorescence), whereas PI penetrates only cells with damaged membranes, thus reducing the SYTO 9 stain (red fluorescence) as monitored with a fluorescence microscope at 40-fold magnification.

Holin activity of HolY. To define the function of the putative holin, HolY, from *Y. enterocolitica* strain W22703, strain *E. coli* MC1061/*lcl*₈₅₇*Sam7* with a nonsense mutation of the λ S holin was used for a complementation experiment. Thermal induction of this strain, which is equipped with pRG-*holY*RBS, which carries *holY* with its own ribosomal binding site (RBS), by a shift from 30°C to 42°C for 15 min resulted in nearly complete lysis after approximately 20 min at 37°C (Fig. 6), clearly confirming the holin activity of HolY. A similar effect was observed in the presence of the pJN5 control plasmid, a pBR322 derivative carrying the gene encoding holin S¹⁰⁵, although the lysis was incomplete. No such reduction in OD₆₀₀ was observed when the *E. coli* λ lysogen was tested using pBR322 as a control. Because every holin tested previously, irrespective of

FIG 6 Characterization of HolY. *E. coli* strain MC1061/*lcl*₈₅₇*Sam7* was transformed with pRG-*holY*RBS and pJN5, which were able to complement the mutated phage I holin gene. MC1061/*lcl*₈₅₇*Sam7* with plasmid pBR322 was used as the control.

FIG 7 (A) Activity of purified ElyY compared with that of lysozyme. A W22703 culture was grown to the exponential phase, and growth was blocked via the addition of kanamycin. Aliquots were then treated with purified endolysin or lysozyme. Buffer and EDTA were used as controls. Each experiment was repeated twice, and SDs are depicted. (B) Zymograms testing ElyY against membrane preparations of *E. coli* (left) and *Y. enterocolitica* W22703 (right). Following gel electrophoresis, proteins were renatured at 37°C for 24 h. Gels were stained with methylene blue. Transparent bands indicate enzymatic activities toward the peptidoglycan of the bacteria. Each experiment was performed thrice; representative zymograms are shown.

its class, was found to complement λ S null mutants (25), this experiment clearly demonstrated that *holY* indeed encodes a holin.

Enzymatic activity and substrate specificity of ElyY. To analyze the activity of exogenously applied endolysin, the protein was overproduced and purified using pET28b and was added to W22703 cells in a buffer with kanamycin that inhibited cell growth. Measurement of the OD₆₀₀ over 225 min revealed a 30% reduction in the cell density compared with 35% in the control sample similarly treated with lysozyme (Fig. 7A). Cells resuspended in buffer and with kanamycin, or additionally with EDTA as a further control, exhibited an OD₆₀₀ of 0.7 at the end of the experiment, corresponding to a 7% decrease in the cell density. Thus, endolysin displayed a lytic activity toward W22703 cells that was comparable to that of lysozyme.

Using purified enzyme from the aforementioned experiment and lyophilized cells of W22703, *Y. intermedia*, *Y. ruckeri*, *Y. mollaretii*, *Y. frederiksenii*, *E. coli* OP50, and *S. Typhimurium*, the substrate specificity of ElyY was tested via zymography. Bovine serum albumin (BSA) and lysozyme were used as controls. A representative zymogram with cell membrane fractions of *E. coli* and *Y. enterocolitica* W22703 is shown in Fig. 7B. The highest (100%) activity of ElyY was observed against W22703 cells and the lowest (5%) against *E. coli* cells; degradation rates of 32% to 95% were determined for the other lyophilized cells (Table 1). These data describe the high substrate specificity of ElyY with respect to yersinial murein. The absolute enzymatic activity of lysozyme was higher than that of endolysin (Fig. 7B); however, zymograms of this enzyme revealed a distinct activity pattern ranging from 32% activity against *Y. ruckeri* cells to 100% against *Y. intermedia* cells (Table 1).

TABLE 1 Substrate specificity of ElyY from strain W22703

Strain	% lysozyme activity	% endolysin activity
<i>Y. enterocolitica</i> W22703	85	100
<i>Y. frederiksenii</i>	69	77
<i>Y. ruckeri</i>	32	32
<i>Y. mollaretii</i>	74	82
<i>Y. intermedia</i>	100	95
<i>E. coli</i> OP50	64	5
<i>S. Typhimurium</i> 14028	94	89

DISCUSSION

There are three mechanisms that contribute to phage lysis of Gram-negative bacterial host cells. In the canonical phage L model, the S holin harmlessly accumulates in the cytoplasm until it suddenly triggers the formation of micron scale holes that allow its release into the periplasm to degrade peptidoglycan (26). The second mechanism is exemplified by bacteriophage P1 or 21, which possesses a signal-arrest-release (SAR) domain endolysin that accumulates as an inactive enzyme in the periplasm and refolds to an active form when the proton motive force collapses in the presence of a pinholin (27) (28–30). Recently, disruption of the outer membrane by a spanin complex, which comprises a small outer membrane lipoprotein (o-spanin) and an integral cytoplasmic membrane protein (i-spanin), was identified as a third step in cell lysis (21). These spanins are encoded by λ Rz and Rz1 gene equivalents that have been identified among most phages of Gram-negative bacteria and that were grouped into the three classes of embedded, overlapped, and separated genes (21, 22). The overlapping yRz and yRz1 gene pair of *Y. enterocolitica* investigated in this study corresponds to the spanin concept, because yRz is a protein with a TMD and yRz1 is a putative lipoprotein. When the two pBAD33 constructs for HE overexpression with and without yRz and yRz1 (pBAD33-HE⁺ and pBAD33-HE) were tested in *Y. enterocolitica* strains (Fig. 4A), we observed that the lytic effect on the cell culture was more pronounced in the presence of the two accessory genes. Although this effect was demonstrated under the artificial condition of arabinose-induced overexpression, the distinct growth phenotypes of W22703 with pBAD33-HE⁺ or pBAD33-HE are in line with the assumption that the two spanins are required or that they at least significantly contribute to cell lysis. This assumption is supported by the observation of a stronger lysis of *E. coli* cells after HE⁺ overexpression than after HE overexpression in the presence of cations under conditions of mild shaking (see Fig. S3 in the supplemental material).

Endolysins constitute classes of highly specific amidases, glycosidases, muramidases, and endopeptidases that are composed of a N-terminal catalytic domain and a C-terminal CBD (31). The endolysin ElyY investigated in this study is a putative endopeptidase that lacks an SAR domain. Endolysins are known for their high substrate specificity (33), and we observed that ElyY has the strongest preference for W22703 murein and the weakest preference for peptidoglycan of *E. coli* (Table 1).

Cryptic prophages, e.g., prophage elements that are inactive with respect to cell lysis, phage production, and plaque formation, help bacteria to cope with adverse environments (34). For example, they contribute to resistance against serum-mediated killing (35) or antibiotics, to biofilm formation (34, 36), and to the virulence properties of Shiga toxin-producing *E. coli* (37). Some endolysin/holin pairs that are not colocalized with other prophage genes are carried in locations adjacent to those of genes encoding toxins or extracellular enzymes, such as a colicin in *Klebsiella oxytoca*, heat-labile enterotoxins IIA/B of *E. coli*, and a chitinase in *S. Typhi* (38). Another example is a gene encoding an *N*-acetyl- β -D-muramidase similar to phage endolysins that is located close to the gene encoding toxin CdtB of *S. Typhi*, and it was demonstrated that this endolysin, possibly in conjunction with a holin located elsewhere in the genome, is responsible for typhoid toxin secretion. Consequently, it was proposed that this endolysin represents an evolutionarily recent adaptation of phage-associated enzymes that perform a different function involved in protein secretion (38). For *Clostridium difficile*

strain R20291, the holin-like TcdE protein has been demonstrated to facilitate the release of toxins A and B to the extracellular environment (18, 39, 40), whereas such an observation was not made upon *tcdE* inactivation in strain 630 (41). This discrepancy is possibly due to the larger amount of TcdA and TcdB produced by strain R20291 than by strain 630. It is also interesting that *Y. pestis* strain Angola encodes a truncated holin of 44 amino acids with a single transmembrane domain (TMD) only, suggesting a loss of function in line with the apathogenic behavior of *Y. pestis* strains toward fleas (42). The potential influence of the HE cassette from Tc-PAI_{ye} on the phenotype of *Y. enterocolitica* strain W22703 is unknown, and its possible biological role remains to be elucidated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains used in this study are indicated in Table 2. All cultures were grown in lysogeny broth (LB) medium (containing 10 g/liter tryptone, 5 g/liter yeast extract, and 5 g/liter NaCl) or in NZY medium (containing 10 g/liter N-Z-Amine, 5 g/liter yeast extract, and 8 g/liter NaCl; pH 7.0) or on LB agar (LB supplemented with 1.5% agar). *E. coli* was grown at 37°C and *Yersinia* spp. at 30°C or as indicated. If necessary, kanamycin (50 µg/ml), streptomycin (50 µg/ml), chloramphenicol (20 µg/ml), tetracycline (12 µg/ml), nalidixic acid (20 µg/ml), IPTG (isopropyl-β-D-thiogalactopyranoside; 0.1 mM), or arabinose (as indicated) was added to the medium. For zymography, cultures were grown to an OD₆₀₀ of 1.0. The cells were autoclaved, sedimented, and then shock frozen in liquid nitrogen. Following lyophilization, cells were stored at -20°C.

General molecular techniques. DNA manipulation and chromosomal DNA isolation were performed according to standard procedures (49) or in accordance with the manufacturer's protocol. PCR was performed using Thermoprime *Taq* polymerase (ABgene, Hamburg, Germany) via the following protocol: 95°C for 2 min; 30 cycles of 95°C for 10 s (annealing temperature depending on oligonucleotides for 30 s) and 72°C for 20 to 180 s (depending on the expected fragment length); and final extension at 72°C for 10 min. Chromosomal DNA (100 ng) was used as the template.

For qRT-PCR performed on a Bio-Rad CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA), cDNA was generated using 1 µg of DNA-free RNA samples and qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Resulting cDNA samples were diluted 5-fold, and 5 µl of a dilution was applied in each qRT-PCR. Amplicon formation was quantified by monitoring the fluorescence of SYBR green dye (Quanta Biosciences, Gaithersburg, MD). The oligonucleotide primer pairs (see Table S1 in the supplemental material) were designed using Primer3 software, and primer efficiencies were determined in experiments performed with serial dilutions of selected cDNA samples. The 16S rRNA gene was used as the housekeeping control gene for normalization and for calculating the relative levels of gene expression by the threshold cycle (ΔΔC_t) method.

Conjugational transfer was performed using the mobilizing *E. coli* SM10 strain as the donor for matings (43, 50). RNA was isolated according to the modified single-step method of Chomczynski and Sacchi (51). Briefly, a 1-ml culture of *Y. enterocolitica* strain W22703 was grown to an OD₆₀₀ of approximately 0.85 and centrifuged, and the pellet was resuspended in 1 ml of TRIzol (Invitrogen, Karlsruhe, Germany). The cells were disrupted using a Ribolyser system (Hybaid, Heidelberg, Germany) as described previously (52). Following chloroform extraction, nucleic acids were precipitated, washed, and resuspended in 0.5% SDS. DNase treatment was performed using RQ1 DNase I (Promega, Mannheim, Germany) according to the manufacturer's instruction.

Recombinant plasmids and strains. For HE and HE⁺ overexpression, a 772-bp fragment comprising the HE genes was cloned into pBAD33 via *SacI* and *XbaI* using oligonucleotides *toxIF46* and *toxIR52*, resulting in plasmid pBAD-HE. A 1,310-bp fragment comprising the HE cassette plus *yRz* and *yRz1* was cloned in the same manner using primers *toxIF46* and *toxIR53*, giving rise to pBAD-HE⁺. The construct pBAD33-*elyY*-*yRz1* was generated using *SacI* and *XbaI*, pET28-*elyY* was generated using *NcoI* and *XhoI*, and pRG-*holYRBS* was generated using *XbaI* and *HindIII*. The in-frame deletion of *araA* was performed using suicide plasmid pKNG101, into which two fragments flanking *araA* were cloned. Strain W22703 Δ*araA* was then generated via a previously described procedure (8). The deletion was confirmed by sequencing. All oligonucleotides used for these constructs are listed in Table S1.

Overproduction and purification of ElyY. ElyY-His₆ was overproduced and purified as follows. An overnight culture of *E. coli* BL21(DE3) harboring pET28b-*elyY* was diluted 1:100 into 400 ml of LB medium and incubated at 30°C and 180 rpm. At an OD₆₀₀ of 0.6, production of ElyY was induced by the addition of IPTG. After incubation for 4 h, the cells were harvested, and the pellet was resuspended in 4 ml of native lysis buffer (300 mM NaCl, 50 mM NaH₂PO₄, 10 mM imidazole; pH 8.0) in the presence of 1 mM phenylmethanesulfonyl fluoride. The cells were lysed using a French press (SLM Aminco Instruments, Rochester, NY), and the lysate was incubated with 40 µl of DNase I (1 U/µl) and 40 µl of lysozyme (10 mg/ml) for 1 h at 4°C. The cell debris was removed by centrifugation at 4°C (performed at 13,200 rpm 3 times for 10 min each time). Then, 20 µl of the supernatant containing soluble proteins was mixed with 5 µl of 5× Laemmli buffer and applied to sodium dodecyl sulfate (SDS)-polyacrylamide gels, and the separated proteins were stained with Coomassie blue to verify ElyY overproduction. ElyY-His₆ was purified using a nickel-nitrilotriacetic acid (Ni-NTA) Fast Start kit (Qiagen, Hilden, Germany). The fusion protein was bound to the column and then washed and eluted according to the manufacturer's protocol. Protein concentrations were determined using RotiQuant solution (Carl Roth GmbH, Karlsruhe, Germany).

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Genotype/relevant features ^a	Reference or source
Strains		
<i>E. coli</i> B121(DE3)	F [−] <i>ompT</i> <i>hsdS</i> _B (f ₈ [−] m ₈ [−]) <i>gal</i> <i>lon</i> <i>dcm</i> <i>rne</i> 131 λ (DE3)[<i>lacI</i> <i>lacUV5</i> -T7 gene 1 <i>ind1</i> <i>sam7</i> <i>nin5</i>]	Invitrogen, Karlsruhe, Germany
<i>E. coli</i> TOP10	F [−] <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ϕ 80 <i>lacZ</i> AM15 Δ <i>lacX74</i> <i>recA1</i> <i>araD</i> 139 Δ (<i>ara</i> <i>leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen, Karlsruhe, Germany
<i>E. coli</i> LMG194	F [−] Δ <i>lacX74</i> <i>galE</i> <i>thi</i> <i>rpsL</i> Δ <i>phoA</i> (PvuII) Δ <i>ara</i> 714 <i>leu</i> ::Tn10	Invitrogen, Karlsruhe, Germany
<i>E. coli</i> SM10	<i>lacY</i> <i>tonA</i> <i>recA</i> <i>Mu</i> _C ⁺ <i>thi</i> <i>leu</i> <i>supE</i> RP4-2-Tc::Mu Km ^r λ <i>pir</i>	43
<i>E. coli</i> MC1061/ <i>lcl</i> ₈₅₅ - <i>Sam7</i>	Lysogenic derivative carrying λ cl857 <i>Sam7</i>	CGBM collection, 44, 45
<i>Y. enterocolitica</i> W22703	Nal ^r Res [−] Mod [−] pYW [−]	46
<i>Y. enterocolitica</i> W22703 Δ <i>araA</i>	Nonpolar deletion mutant of <i>araA</i> encoding L-arabinose isomerase	This study
<i>Y. enterocolitica</i> 8081v	Wild type, Nal ^r	V. L. Miller, St. Louis, MO
<i>Y. enterocolitica</i> SZ4331/97	Biovar 1A, O:14, Tc-PAI _{ve} negative	Institut für Hygiene und Umwelt, Hamburg, Germany
<i>Y. enterocolitica</i> SZ5108/01	Biovar 1B, O:8, Tc-PAI _{ve} negative	Institut für Hygiene und Umwelt, Hamburg, Germany
<i>Y. enterocolitica</i> H692/94	Biovar 2, O:9, Tc-PAI _{ve} positive	Institut für Hygiene und Umwelt, Hamburg, Germany
<i>Y. enterocolitica</i> H230/89	Biovar 3, O:5,27, Tc-PAI _{ve} positive	Institut für Hygiene und Umwelt, Hamburg, Germany
<i>Y. enterocolitica</i> H31/80	Biovar 4, O:3	Institut für Hygiene und Umwelt, Hamburg, Germany
<i>Y. enterocolitica</i> 519-36/88	Biovar 5, O:2a, 2b, 3	Institut für Hygiene und Umwelt, Hamburg, Germany
<i>Y. pseudotuberculosis</i>	CIP 55.85	Collection Institute Pasteur
<i>Y. ruckeri</i>	CIP 80.20	Collection Institute Pasteur
<i>Y. aldovae</i>	CIP 103162	Collection Institute Pasteur
<i>Y. intermedia</i>	CIP 80.28	Collection Institute Pasteur
<i>Y. kristensenii</i>	CIP 80.30	Collection Institute Pasteur
<i>Y. frederiksenii</i>	CIP 80.29	Collection Institute Pasteur
<i>Y. mollaretii</i>	CIP 103324	Collection Institute Pasteur
<i>P. luminescens</i>	TT01	Collection Institute Pasteur
<i>S. Typhimurium</i>	Wild-type strain ATCC 14028	ATCC
Plasmids		
pKNG101	Conditionally replicating vector; R6K origin, <i>mobRK2</i> transfer origin, sucrose-inducible <i>sacB</i> , Str ^r	47
pBAD33	Arabinose-inducible promoter, Cam ^r	24
pBAD33-HE	A 772-bp fragment comprising the <i>holin</i> and <i>endolysin</i> genes was cloned into pBAD33 via <i>SacI</i> and <i>XbaI</i>	This study
pBAD33-HE ⁺	A 1,310-bp fragment comprising the HE cassette and <i>yRz</i> and <i>yRz1</i> cloned into pBAD33 via <i>SacI</i> and <i>XbaI</i>	This study
pBAD33-ely ⁺ -yRz1	<i>elyY</i> , <i>yRz</i> , and <i>yRz1</i> cloned into pBAD33 via <i>SacI</i> and <i>XbaI</i>	This study
pBR322	Amp ^r Tet ^r	48
pJN5	pBR322 derivative carrying S ¹⁰⁵ under the control of the late transcription regulatory elements of phage λ	44
pRG32	pBRQ(Δ <i>rbs</i>) with <i>tcdE</i> Met1 of <i>C. difficile</i> with its own RBS	39
pRG-hoYRBS	Derivative of pRG32; exchange of <i>C. difficile</i> <i>holin</i> gene with <i>hoY</i> , including its RBS, via <i>XbaI</i> and <i>HindIII</i>	This study
pET28b	Expression vector; T7 promoter, N- and C-terminal polyhistidine tag, Kan ^r pBR322 <i>origin</i>	Merck, Darmstadt, Germany
pET28b-elyY	Plasmid for endolysin gene overexpression; <i>elyY</i> cloned into IPTG-inducible pET28b via <i>NcoI</i> and <i>XhoI</i> ; C-terminal polyhistidine tag	This study

^aAmp^r, ampicillin resistance; Cam^r, chloramphenicol resistance; Km^r, kanamycin resistance; Nal^r, nalidixic acid resistance; Str, streptomycin resistance; Tet^r, tetracycline resistance.

based on Bradford's method (53), and the purity of eluted fractions was analyzed using 12.5% SDS polyacrylamide gel.

Measurement of ElyY activity. W22703 was cultivated in lysogeny broth (LB) medium at 30°C until the culture reached an OD₆₀₀ of 1.0. Aliquots of 1 ml were centrifuged, and the cell pellets were washed in 100 mM Tris and 150 mM NaCl (pH 7.5). Cells were resuspended in 1 ml of this buffer containing 50 mM EDTA and 50 µg/ml endolysin. Cell growth was then inhibited by the use of 50 µg/ml kanamycin, and the samples were incubated at 37°C.

The functionality of ElyY was determined by zymography as follows. Lyophilized cells (0.2% [wt/vol]) were enclosed in the 15% separation gel of an SDS-polyacrylamide gel that was preceded by a 4% stacking gel. Then, 20 µl of purified endolysin (1 mg/ml) was mixed with 5 µl of 5× Laemmli buffer, incubated at 100°C for three min, and separated by gel electrophoresis. BSA and lysozyme were used as controls and treated in the same manner. The gel was washed thrice in distilled water for 10 min. Following equilibration in denaturing buffer (10 mM Tris, Triton X-100, pH 7.0) for 5 min, the zymograms were incubated at 37°C for 24 h. After washing, the gel was stained with 0.1% methylene blue and 0.1% KOH for 3 h and then destained in distilled water. Semiquantitative densitometry of the scanned zymograms was performed using Kodak ID Image Analysis software. For each band, values corresponding to regions of equal size were determined and were then calculated as integrals of the background in percentages.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00180-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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