A novel protein of *Erysipelothrix rhusiopathiae* that confers haemolytic activity on *Escherichia coli*

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***Erysipelothrix rhusiopathiae*, the cause of swine erysipelas and human erysipeloid, produces a haemolysin. A recombinant plasmid, pHLY, conferring haemolytic activity on *Escherichia coli* was isolated from a genomic library of *Ery. rhusiopathiae* strain Tama-96. This plasmid was stable in RecA⁻ *E. coli*, but unstable in a RecA⁺ strain. A spontaneous deletion plasmid, pMini-HLY, also conferring haemolytic activity was derived from pHLY. Two ORFs were detected in pHLY. Analysis of pMini-HLY and other deletion clones established that ORF2 was associated with haemolytic activity. The sequence of ORF1 was highly homologous to those of transposases in the IS30 family. The deletion which generated pMini-HLY was between two short direct repeat (DR) sequences flanking the ORF1 sequence, and there were inverted repeat sequences inside the two DR sequences, suggesting an insertion element. No sequence homology to the deduced amino acid sequence of ORF2 was detected in the databases, but its sequence was characteristic of a surface lipoprotein. Western blot analysis, using antiserum against the 16 kDa protein produced from ORF2, found the protein to be commonly distributed in all *Erysipelothrix* species.**

Keywords: *Erysipelothrix rhusiopathiae*, haemolysis, transposase, IS30

INTRODUCTION

Erysipelothrix rhusiopathiae causes erysipelas in swine and a variety of diseases in other animals, as well as erysipeloid, a human skin disease (Wood, 1984). Erysipelas can occur as an acute septicaemia or chronic disease with development of arthritic lesions and endocarditis and continues to be a major cause of economic loss in swine-producing areas of the world. Much of the interest in *Ery. rhusiopathiae* is due to the chronic and progressive character of the arthritic lesions in pigs, which superficially resemble those of human rheumatoid arthritis. However, the pathogenesis of these lesions remains unclear.

Erysipelothrix species form a weak haemolytic zone (α -haemolysis) on blood agar plates. In many pathogenic

bacteria haemolysins are involved in virulence (Stanley *et al.*, 1998). The haemolysin (listeriolysin) in *Listeria monocytogenes*, which is phylogenetically closely related to *Ery. rhusiopathiae*, is essential for intracellular survival (Bielecki *et al.*, 1990). The aim of this work was to identify the gene encoding the haemolysin of *Ery. rhusiopathiae* and to compare this haemolysin to haemolysins in other pathogenic bacteria.

METHODS

Bacterial strains and media. Table 1 lists the *Erysipelothrix* strains used in this study. *Ery. rhusiopathiae* Tama-96 was used as the basis for construction of a genomic library. *Escherichia coli* MC1061 was used as a host strain to maintain recombinant plasmids. *E. coli* HB101 was used as a RecA⁻ strain. Brain Heart Infusion broth (BHI; Difco) supplemented with 0.1% (w/v) Tween 80 (BHIT) and L broth were used to cultivate the *Erysipelothrix* and *E. coli* strains, respectively. When necessary, media were supplemented with 1.5% (w/v) agar or the appropriate antibiotic. To examine the haemolytic activity of bacterial cells, we used Columbia agar (BRL) supplemented with 5% sheep blood (blood agar).

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Abbreviation: DR, direct repeat.

The DDBJ accession number for the sequence described in this paper is AB017185.

Table 1. *Erysipelothrix* strains and their characteristics

All strains were positive for haemolysis, and a 16 kDa protein was detected in Western blots of all cell lysates using anti-Hly.

Strain	Serotype	Southern hybridization (kb)*	Reference
<i>Ery. rhusiopathiae</i>			
Koganei	1a	5.2, 4.8	Seto <i>et al.</i> (1971)
EM7	1a	5.2, 4.5	Takahashi <i>et al.</i> (1992)
Fujisawa	1a	5.2, 4.5	Takahashi <i>et al.</i> (1992)
422/1E1	1b	5.2, 4.5	Takahashi <i>et al.</i> (1992)
Tama-96	2	5.2, 4.5	Zarkasie <i>et al.</i> (1996)
Doggersharde	4	5.2, 4.5	Takahashi <i>et al.</i> (1992)
Pécs 67	5	5.2, 4.5	Takahashi <i>et al.</i> (1992)
Dolphin E-1	6	5.2, 4.5	Takahashi <i>et al.</i> (1992)
Goda	8	6.5, 3.2	Takahashi <i>et al.</i> (1992)
Kaperak	9	5.2, 4.5	Takahashi <i>et al.</i> (1992)
IV12/8	11	5.2, 4.5	Takahashi <i>et al.</i> (1992)
Pécs 9	12	6.5, 3.2	Takahashi <i>et al.</i> (1992)
Pécs 3597	15	5.2, 4.5	Takahashi <i>et al.</i> (1992)
Tanzania	16	6.5, 3.2	Takahashi <i>et al.</i> (1992)
545	17	6.5, 3.2	Takahashi <i>et al.</i> (1992)
2017	19	5.2, 4.5	Takahashi <i>et al.</i> (1992)
Bäno 36	21	5.2, 4.5	Takahashi <i>et al.</i> (1992)
MEW22	N	6.5, 3.2	Takahashi <i>et al.</i> (1992)
<i>Ery. tonsillarum</i>			
Witlling	3	—	Takahashi <i>et al.</i> (1992)
ATCC 43339	7	—	Takahashi <i>et al.</i> (1992)
Lengyel-P	10	—	Takahashi <i>et al.</i> (1992)
Iszap-4	14	—	Takahashi <i>et al.</i> (1992)
2553	20	—	Takahashi <i>et al.</i> (1992)
Bäno 107	22	—	Takahashi <i>et al.</i> (1992)
KS20A	23	—	Takahashi <i>et al.</i> (1992)
<i>Erysipelothrix</i> spp.			
Pécs 56	13	5.2, 4.5	Takahashi <i>et al.</i> (1992)
715	18	6.5, 3.2	Takahashi <i>et al.</i> (1992)

* Fragments detected in Southern blots of *Bam*HI-digested genomic DNA using the *Hind*III fragment of pHLY as a probe.

Plasmid construction. Fig. 1 shows the plasmids used in this study. The vector plasmid pHY300PLK was used to construct a library of genomic DNA of *Ery. rhusiopathiae* strain Tama-96 partially digested with *Sau*3AI. *E. coli* transformants from the library were spread on blood agar plates supplemented with ampicillin ($150 \mu\text{g ml}^{-1}$) to detect colonies from the library showing haemolytic activity. Deletion derivatives shown in Fig. 1 were derived by restriction endonuclease digestion of pHLY.

Expression of haemolytic protein and its detection. A *Bam*HI linker was inserted into the *Spe*I site of pHLY (Fig. 1), and then a *Bam*HI–*Hind*III DNA fragment was cloned into the *Bam*HI and *Hind*III sites of pQE30 (Qiagen) to yield pQE-HLY. The polyhistidine-tagged fusion protein was purified according to the manufacturer's instructions using the QI-Aexpress system and used to immunize rabbits to make rabbit polyclonal antibody against the fusion protein (anti-Hly). Using anti-Hly, Western blotting was performed with *Erysi-*

pelothrix and *E. coli* strains. Antigens were prepared from bacterial cell pellets and culture supernatants. Briefly, 1 ml of the bacterial cell culture was centrifuged, the supernatant was collected, and the bacterial cell pellet was suspended in 200 μl protein lysis buffer (0.125 M Tris/Cl, pH 6.8, 4% SDS, 20% glycerol, 0.042% bromophenol blue, 5% mercaptoethanol). The supernatant was mixed with 50 μl 100% trichloroacetic acid, and after centrifugation precipitated protein was re-suspended in 20 μl lysis buffer. Protein suspensions were heated at 100 °C for 5 min, and then 10 μl of each suspension was loaded on a 20% SDS-PAGE gel.

Haemolysis experiments. The polyhistidine-tagged fusion protein and PBS-washed *E. coli* MC1061 cells carrying pHLY were suspended in 200 μl PBS at concentrations of 1.0 mg ml^{-1} and $1 \times 10^8 \text{ cells ml}^{-1}$, respectively. Then the solutions were incubated with a 1% (v/v) suspension of sheep red blood cells at 37 °C for 1 h. Unlysed cells were removed by centrifugation at 740 g. The amount of lysis was determined by measuring

Fig. 1. Physical map of pHLY and its deletion derivatives. Haemolytic activity on blood agar plates of *E. coli* carrying the plasmids is indicated by + (positive) or - (negative). DNA sequences shown are the regions bounding the deletion in pMini-HLY, with numbers indicating the distance from the *SalI* site. Underlined and italic sequences are DR and inverted repeat sequences, respectively. The hatched box shows the region deleted in pMini-HLY. The open box in ORF2 shows the deduced signal sequence and the open box in pQE-HLY indicates the signal sequence of pQE30. The open boxes in pHLY and pMini-HLY indicate DRs and thick black lines show the regions cloned in deletion plasmids. The arrows show the orientation of the ORFs.

the A_{570} of released haemoglobin spectrophotometrically. *E. coli* O157:H7 EDL933 and MC1061 were used as positive and negative controls, respectively.

Southern hybridization. Total DNA was purified from *Erysipelothrix* species strains as described by Makino *et al.* (1994) and digested with *Bam*HI. Fragments were electrophoresed in an agarose gel, blotted onto nylon membrane (Schleicher & Schuell) and then probed with a *Hind*III DNA fragment (pΔH; Fig. 1) labelled with DIG-dUTP (Roche Diagnostics). Hybridization was performed in hybridization buffer containing 50% (v/v) formamide at 42 °C according to the manufacturer's manual, and the DIG Luminescent Detection kit (Roche Diagnostics) was used for the detection of hybridized DNA.

Surface-labelling experiments. *Ery. rhusiopathiae* strain Tama-96 cells grown in BHIT were washed once with PBS and resuspended in PBS. Anti-Hly was added to the suspension, and the mixture was incubated at 37 °C for 1 h. Then bacterial cells were washed three times with PBS and resuspended in PBS, then incubated with anti-rabbit IgG conjugated to FITC. The cells were washed five times with PBS and examined with a fluorescence microscope. Rabbit polyclonal antibody against the surface protein SpaA (Makino *et al.*, 1998) of *Ery. rhusiopathiae* was used as a positive control for surface labelling.

DNA sequencing and other techniques. The DNA sequences of the cloned fragments were determined using an ABI PRISM 377 (Applied Biosystems) DNA sequencer. Plasmid DNA isolation, agarose gel electrophoresis, SDS-PAGE and transformation of *E. coli* were done by the methods of Maniatis *et*

al. (1982). Database searches with amino acid sequences used the FASTA program in the DDBJ. Vaccination using the ORF2 product and challenge with the Tama-96 strain were performed as described by Makino *et al.* (1998).

RESULTS AND DISCUSSION

Isolation of a plasmid inducing haemolysis and its genetic analysis

E. coli clones from the Tama-96 gene library were spread on blood agar plates to select colonies showing haemolysis (Hly⁺). Two clones were independently isolated, and one of them, pHLY, was used for this study (Fig. 2). Deletion mutants were constructed using the restriction enzyme cleavage sites in pHLY to find the region essential for haemolytic activity. In addition, when *E. coli* MC1061 containing pHLY was spread on a blood agar plate supplemented with ampicillin, the plasmid pMini-HLY was generated spontaneously by deletion of about 1 kb from pHLY. The spontaneous deletion mutant still induced haemolysis (Fig. 1). The region essential for the haemolytic activity of pHLY was located within a 0.5 kb fragment containing the *Pvu*II and *Hind*III sites (Fig. 1).

To characterize the gene essential for haemolysis and the deletion which generated pMini-HLY, the nucleotide sequences of the cloned regions in pHLY and pMini-HLY were determined. Two ORFs were identified in

Fig. 2. Haemolytic activity on blood agar plates of pHY300PLK and pHLY in *E. coli* MC1061.

pHLY (Fig. 1). ORF1 encoded 313 amino acids to give a predicted 37 kDa product and ORF2 encoded 149 amino acids, predicting a 16.2 kDa product. A deletion of 1078 bp had generated pMini-HLY from pHLY (Fig. 1). The results demonstrated that ORF2 was associated with haemolysis (Fig. 1).

To understand the mechanism generating the deletion in pMini-HLY, the stability of pHLY in *E. coli* MC1061 RecA⁺ and HB101 RecA⁻ was examined by subculturing for about 20 generations in L broth supplemented with ampicillin (50 µg ml⁻¹). When plasmids carried in 10 Hly⁺ colonies of each host strain were examined, pHLY was found to be stable in the RecA⁻ host, but eight of ten Hly⁺ colonies from the RecA⁺ host had plasmids with molecular masses similar to pMini-HLY, as determined by agarose gel electrophoresis (data not shown).

Two direct repeat (DR) sequences of 11 bp (ACACCGAATC) were found bracketing the deleted region in pHLY, and pMini-HLY carried only one DR (Fig. 1). The deleted region in the eight deletion plasmids obtained by subculturing the RecA⁺ host strain was identical to that in pMini-HLY from DNA sequence analysis (data not shown). These results suggest that deletion of ORF1 to give pMini-HLY occurred by RecA⁻-dependent homologous recombination between DRs (Fig. 1).

ORF1 is homologous to IS30 transposases

A database search for sequences similar to ORF1 found that the sequence of the C-terminal region from P₁₄₂ had 30.7–35.7% identity to the sequences of the C-terminal region of the transposases of IS4351 (Smith, 1987), IS30 (Dalrymple *et al.*, 1984), IS1161 (Giffard *et al.*, 1993), IS1070 (Vaughan & de Vos, 1995) and IS1139 (Lortie *et al.*, 1994), which are insertion elements of the IS30 family. In addition, at both ends of a 1067 bp region bracketed by two DRs in pHLY, two inverted repeat sequences of 20 bp, which were 50% identical to each

		Lipoprotein consensus sequence																					
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ORF2	1	M	K	K	I	V	A	V	L	F	S	L	V	L	V	-	G	C	S	A	N	20	
OspA	1	M	K	K	Y	L	L	G	I	G	L	I	L	A	L	I	-	A	C	K	Q	N	20
TrsH	1	M	K	K	L	L	T	I	V	I	L	F	T	L	V	L	A	G	C	G	Q	N	21
LytC	1	M	K	K	F	I	A	L	L	F	F	I	L	L	L	S	-	G	C	G	V	N	20
YdhF	1	M	R	R	I	L	S	T	L	V	F	A	I	M	L	A	-	G	C	S	S	N	20

Fig. 3. Alignment of the 20-amino-acid N-terminal segment of the ORF2 product with related sequences. Boxes and black shading indicate residues that are similar or identical, respectively, as determined using SeqVu version 1.1 from the Garvan Institute of Medical Research, Sydney, Australia.

other, were detected (Fig. 1). These findings suggest ORF1 may function as a transposase, and the region deleted from pHLY may be the first insertion element identified in *Ery. rhusiopathiae*. We have not yet confirmed that the element is able to transpose. Since the sequences of the inverted repeats were only 50% identical to each other, it is likely that the frequency of its transposition may be extremely low even if the ORF1 product has a transposase activity (Mahillon & Chandler, 1998).

Characteristics of the haemolysin

Although we were unable to identify sequences with significant similarity to ORF2 in the database, the protein it encoded was predicted by the PROST program (Nakai & Kanehisa, 1991) to be secreted and acylated, with a signal peptidase II cleavage site between G₁₆ and C₁₇ (Fig. 3). Although pΔH-S, in which the first 42 bp of ORF2 were deleted, did not induce haemolytic activity, pQE-HLY, with the same deletion but the remainder of the ORF2 coding sequence fused in-frame to the signal sequence of pQE30, induced haemolysis in *E. coli* (Fig. 1). The 20-amino-acid N-terminal sequence of the protein encoded by ORF2 was very similar to the N-terminal ends of OspA (Dykhuizen *et al.*, 1993; Will *et al.*, 1995), TrsH (Morton *et al.*, 1993), LytC (Kunst *et al.*, 1997; Lazarevic *et al.*, 1992) and YdhF (Kunst *et al.*, 1997), all of which contain the lipoprotein consensus sequence (von Heijne, 1989) (Fig. 3). In addition, computer prediction (Chou & Fasman, 1978) of the amino acid secondary structure suggested that a β-turn may occur after the C₁₇ putative acylation site in an 11-amino-acid peptide which contains S₁₈, N₂₀ and N₂₂, indicating that the ORF2 product may be an efficient processed prolipoprotein (Hayashi & Wu, 1990). Thus, ORF2 might encode a lipoprotein with haemolytic activity. However, haemolysis was not detected when purified polyhistidine-tagged 16 kDa protein or PBS-washed *E. coli* cells containing pHLY were incubated with sheep red blood cells (data not shown). Thus it is possible that the 16 kDa protein might not be a haemolysin, but rather facilitate haemolytic activity.

Using anti-Hly, Western blot analysis was performed to

Fig. 4. Western blot analysis of *E. coli* MC1061 and *Erysipelothrix* strains. (a) Expression of the 16 kDa protein was examined by Western blotting of *E. coli* MC1061 harbouring pHY300PLK (lanes 1 and 3) and pHLy (lanes 2 and 4). Bacterial cell pellets (lanes 1 and 2) and culture supernatants (lanes 3 and 4) and purified fusion protein (lane 5) were used as antigens. (b) Expression of the 16 kDa protein was examined by Western blotting of *Erysipelothrix* strains. Bacterial cell pellets from 23 *Erysipelothrix* strains were used as antigens: 1, Tama-96; 2, Fujisawa; 3, Doggersharde; 4, Pécs 67; 5, Dolphin E-1; 6, Goda; 7, Kaperak; 8, IV12/8; 9, Pécs 9; 10, Pécs 3597; 11, Tanzania; 12, 545; 13, 2017; 14, Băno 36; 15, Witlling; 16, ATCC 43339; 17, Lengyel-P; 18, Iszap-4; 19, 2553; 20, Băno 107; 21, KS20A; 22, Pécs 56; 23, 715.

examine the production of the 16 kDa protein. The expression of a protein which reacted with anti-Hly was examined in *E. coli* carrying pHLy (Fig. 4a). The protein was not detected in *E. coli* carrying the vector pHY300PLK (Fig. 4a, lanes 1 and 3), but was detected in the bacterial cell pellets and the culture supernatant of *E. coli* carrying pHLy (Fig. 4a, lanes 2, 4 and 5). Most of the protein reacting with anti-Hly was detected in the *E. coli* cell lysate, rather than in the culture supernatant (Fig. 4a, lanes 2 and 4). Proteins reacting with anti-Hly were detected in all strains of *Erysipelothrix* species tested, although there were some variations in apparent molecular mass (Fig. 4b; Table 1). Pre-immune serum did not react with *Erysipelothrix* strains and anti-Hly did not react with other pathogenic bacterial species, including *L. monocytogenes*, *Staphylococcus aureus*, *Corynebacterium pseudotuberculosis*, *Streptococcus pneumoniae*, *Streptococcus suis*, *Bacillus anthracis*, *Actinobacillus pleuropneumoniae*, *Vibrio parahaemolyticus*, *Yersinia pseudotuberculosis*, *Aeromonas* sp., *Salmonella choleraesuis* and enterohaemorrhagic *E. coli* (data not shown). Most of the reactive protein was detected in the bacterial cell fraction of *Erysipelothrix* strains, and live *Erysipelothrix* cells could be labelled with anti-Hly using anti-rabbit IgG conjugated to FITC (data not shown). This suggests that the 16 kDa protein is probably a cell surface protein which is commonly

distributed amongst *Erysipelothrix* species. Since a surface protein of *Ery. rhusiopathiae*, SpaA, was shown to induce protective immunity in mice (Makino *et al.*, 1998), we thought that the 16 kDa protein commonly distributed in *Erysipelothrix* species might also induce protective immunity. However, purified 16 kDa protein or live *E. coli* cells carrying pHLy did not induce protective immunity in mice and serum from convalescent mice did not react with the 16 kDa protein (data not shown). In addition, although the *Hind*III fragment of pHLy hybridized to two genomic *Bam*HI fragments of all strains of *Ery. rhusiopathiae* and two other *Erysipelothrix* strains (Fig. 1; Table 1), none of the *Erysipelothrix tonsillarum* strains hybridized to this probe. Thus although *Ery. tonsillarum* produced a 16 kDa protein reactive with antibody against the ORF2 product from *Ery. rhusiopathiae*, the gene had lower levels of sequence similarity, and the IS30-like element was also not detected in *Ery. tonsillarum*. Although we were unable to clarify the function of the 16 kDa protein, our results are suggestive of a role as a surface protein involved in haemolysis in all *Erysipelothrix* species.

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