Identification of the Chromosomal Region Essential for Serovar-Specific Antigen and Virulence of Serovar 1 and 2 Strains of *Erysipelothrix rhusiopathiae*

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ABSTRACT *Erysipelothrix rhusiopathiae* causes swine erysipelas, an infection characterized by acute septicemia or chronic endocarditis and polyarthritides. Among 17 *E. rhusiopathiae* serovars, determined based on heat-stable peptidoglycan antigens, serovars 1 and 2 are most commonly associated with the disease; however, the molecular basis for the association between these serovars and virulence is unknown. To search for the genetic region defining serovar 1a (Fujisawa) strain antigenicity, we examined the 15-kb chromosomal region encompassing a putative pathway for polysaccharide biosynthesis, which was previously identified in the *E. rhusiopathiae* Fujisawa strain. Six transposon mutants of Fujisawa strain possessing a mutation in this region lost antigenic reactivity with serovar 1a-specific rabbit serum. Sequence analysis of this region in wild-type strains of serovars 1a, 1b, and 2 and serovar N, which lacks serovar-specific antigens, revealed that gene organization was similar among the strains and that serovar 2 strains showed variation. Serovar N strains displayed the same gene organization as the serovar 1a, 1b, or 2 strain and possessed certain mutations in this region. In two of the analyzed serovar N strains, restoration of the mutations via complementation with sequences derived from serovar 1a and 2 strains recovered antigenic reactivity with 1a- and 2-specific rabbit serum, respectively. Several gene mutations in this region resulted in altered capsule expression and attenuation of virulence in mice. These results indicate a functional connection between the biosynthetic pathways for the capsular polysaccharide and peptidoglycan antigens used for serotyping, which may explain variation in virulence among strains of different serovars.

KEYWORDS capsule, erysipelas, erysipeloid, *Erysipelothrix rhusiopathiae*, evolution, *Firmicutes*, genome reduction, peptidoglycan, serovars, virulence

Firmicutes is a bacterial phylum whose members largely have low genome G+C content and a typical Gram-positive cell wall. Unlike the two classes in this phylum, *Bacilli* and *Clostridia*, which have the typical, rigid Gram-positive cell wall, another group in this phylum, *Erysipelotrichia*, expresses an unusual type of peptidoglycan (1–5). Since multiple independent pathway losses appear to be a hallmark of *Erysipelotrichia* evolution (6, 7), loss of the genes for cell wall biosynthesis might be responsible for the unique and complex structure of the *Erysipelotrichia* cell wall.

Erysipelothrix rhusiopathiae is an intracellular bacterium that represents the class *Erysipelotrichia* (8). *Erysipelotrichia* stands at a unique phylogenetic position in the phylum *Firmicutes*, being phylogenetically close to *Mollicutes* (mycoplasmas) (6, 7), which has previously been considered a distinct class within *Firmicutes* and was reassigned to a separate phylum, *Tenericutes* (8). The *E. rhusiopathiae* genome represents evolutionary traits of both *Firmicutes* and *Mollicutes* (6, 7). Like mycoplasma

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species, *E. rhusiopathiae* shows genome reduction and depends on its hosts for many nutrients, which reflects the intracellular lifestyle of the organism, specifically adaptation to life under nutrient-rich conditions (6, 7). The cell wall of *E. rhusiopathiae* is atypical in structure and/or composition; it lacks many orthologous genes for the biosynthesis of wall teichoic acids (WTAs), lipoteichoic acids (LTAs), and the *dltABCD* operon (6), all of which are mostly conserved in other Gram-positive bacteria. In *E. rhusiopathiae*, *tagO*, which encodes an enzyme in the WTA biosynthetic pathway, is most likely fused with *mraY* (6), a gene essential for peptidoglycan biosynthesis. Some of the biosynthetic pathways of *E. rhusiopathiae* cell wall components, including WTA and/or LTA, have also been suggested to overlap those of the polysaccharide capsule (6, 9), which is the most important virulence factor of this organism (10). A capsule-defective *E. rhusiopathiae* mutant was shown to display an unambiguous morphological aberration, suggesting that the genes encoding capsular polysaccharides (CPS) and cell wall components share essential functions and contribute to cell wall integrity (9). Thus, an unusual type of peptidoglycan in *E. rhusiopathiae* appears to be an evolutionary characteristic of the class *Erysipelotrichia*.

E. rhusiopathiae is ubiquitous in nature and causes a variety of diseases in many species of mammals and birds. It is best known as the causative agent of swine erysipelas, which results in various pathologies, including septicemia, arthritis, endocarditis, and/or urticaria (11). In the genus *Erysipelothrix* strains, heat-stable peptidoglycan antigens are used for serological classification using a double agar-gel precipitation test with type-specific rabbit antisera (12). Serovars are closely related to clinical forms; among the serovars assigned to the species *E. rhusiopathiae* (serovars 1a, 1b, 2, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, 21, 23, and N; serovar N lacks serovar-specific antigens), serovars 1a, 1b, and 2 are frequently isolated from diseased pigs (13–18). However, it has not been clarified why these serovar strains, especially serovar 1a strains, are pathogenic to pigs, and the genetic and antigenic relationship between these serovars and serovar N has not been clarified (11).

In this study, using a transposon mutant library constructed from the highly virulent Fujisawa strain (serovar 1a), we searched for mutants demonstrating the loss of antigenic reactivity with serovar 1a-specific rabbit serum and then examined capsule expression and virulence of the mutants.

RESULTS

Identification of the putative chromosomal locus defining serovar 1a-specific antigenicity. We previously constructed a transposon mutant library from the highly virulent Fujisawa strain (serovar 1a) (9). We next constructed approximately 700 distinct mutants and obtained a total of nine mutants with a single transposon insertion within the genes of the putative pathway for polysaccharide biosynthesis (ERH_1439 through ERH_1444), which was identified in the *E. rhusiopathiae* Fujisawa strain (serovar 1a) but found to be missing from the *E. rhusiopathiae* ATCC 19414 strain (serovar 2) (6), and neighboring genes. We screened the nine mutants for loss of precipitin reactivity with serovar 1a-specific rabbit serum and found that six mutants lost reactivity (Table 1), suggesting that the chromosomal locus is responsible for determining serovar 1a antigenicity. These mutants did not show antigenic reactivity with 26 serovar-specific rabbit sera (data not shown), indicating their transition from serovar 1a strains to untypeable strains. We constructed in-frame deletion mutants and determined the range of the genetic region defining serovar-1a-specific antigenicity. Transposon or deletion mutants of the genes from ERH_1441 to ERH_1448, with the single exception of ERH_1443, which encodes 61 amino acids, lost reactivity with 1a-specific serum (Fig. 1).

Sequence analysis. Sequences of the corresponding region in other serovar strains, including serovar 1b, 2, and N strains, were compared to those of the Fujisawa strain (Fig. 2; see also Table S1 in the supplemental material). In the genetic region, the gene content and organization were the same between the Fujisawa and Mie 02-47 strains (serovar N). The Mie 02-47 strain had a nucleotide sequence identical to that of the Fujisawa strain, with the exception of a nonsynonymous single-nucleotide polymor-

TABLE 1 *E. rhusiopathiae* strains used in this study

Strain	Serovar	Description	Accession no.
Fujisawa	1a	Wild-type strain	AP012027
ME-7	1a	Wild type, serovar 1a reference strain	
422/1E	1b	Wild type, serovar 1b reference strain	LC380396
Tochigi-20	2	Wild-type strain	LC380397
Tochigi-20/R	N	Tochigi-20 strain transformed with a gene fragment from Ishikawa 02-26	
Nagano 11-2	2	Wild-type strain	LC380398
SE-9	2	Wild-type strain	LC380399
ATCC19414	2	<i>E. rhusiopathiae</i> type strain	ACLK02000002
Mie 02-47	N	Wild-type strain	LC380400
Mie 02-47/C	1a	Mie 02-47 strain complemented with a gene fragment from Fujisawa	
MEW22	N	Wild type, serovar N reference strain	LC380401
Nagano 11-1	N	Wild-type strain	LC380403
Ishikawa 02-26	N	Wild-type strain	LC380404
Yamanashi 04-44	N	Wild-type strain	LC380402
Ishikawa 02-26/C	2	Ishikawa 02-26 strain complemented with a gene fragment from ATCC19414	
Fujisawa derivatives			
Δ1439	1a	In-frame deletion of ERH_1439 (19_375del)	
T1440	1a	Transposon insertion in ERH_1440 (124_125ins)	
Δ1440	1a	In-frame deletion of ERH_1440 (19_1167del)	
Δ1441	Untypeable	In-frame deletion of ERH_1441 (19_1080del)	
T1442	Untypeable	Transposon insertion in ERH_1442 (427_428ins)	
Δ1442	Untypeable	In-frame deletion of ERH_1442 (19_1020del)	
Δ1443	1a	In-frame deletion of ERH_1443 (19_168del)	
T1444	Untypeable	Transposon insertion in ERH_1444 (338_339ins)	
T1445	Untypeable	Transposon insertion in ERH_1445 (574_575ins)	
T1446	Untypeable	Transposon insertion in ERH_1446 (388_389ins)	
T1447	Untypeable	Transposon insertion in ERH_1447 (902_903ins)	
T1448	Untypeable	Transposon insertion in ERH_1448 (461_462ins)	
Δ1449	1a	In-frame deletion of ERH_1449 (13_972del)	
T1450	1a	Transposon insertion in ERH_1450 (1522_1523ins)	
Δ1450	1a	In-frame deletion of ERH_1450 (31_1773del)	
T1451	1a	Transposon insertion in ERH_1451 (738_739ins)	

phism at 586G→A in ERH_1447, resulting in a change from glycine to arginine. The gene content and organization were also almost completely identical between the 422/1E strain (serovars 1b) and the other two serovar N strains, MEW 22 and Yamanashi 04-44. In the ATCC 19414 strain (serovar 2), genetic organization in the 5' half of the region was similar to that in Fujisawa; however, many of the genes in the 3' half of the region were missing, and some were replaced with other genes. Sequence comparison of the genetic region between ATCC 19414 and Ishikawa 02-26 (serovar N) revealed that the sequences were almost completely identical to each other; however, Ishikawa 02-26 had a 10-base deletion (471_480del), which results in a truncation in a gene corresponding to HMPREF0357_10926 of ATCC 19414 or ERH_1447 of Fujisawa. Sequence comparison between the serovar 2 strains Tochigi-20, Nagano 11-1, and

FIG 1 Agar-gel precipitation tests for the serotyping of the *E. rhusiopathiae* strains. Center wells contained serovar 1a-specific antiserum raised against heat-stable antigens of ME-7, a serovar-1a reference strain.

FIG 2 Schematic representations of the chromosomal region defining the antigenicity of serovar 1a of *E. rhusiopathiae* and corresponding regions in serovar 1b, 2, and N strains. Identical genes are indicated by the same numbers or letters. Small arrows indicate the orientation and corresponding locations of the primer pairs (Table S2) used for sequencing. The number in parentheses after the strain name indicates the serovar.

Nagano 11-2 showed high nucleotide sequence similarity in this region. The Nagano 11-1 (serovar N) and Nagano 11-2 (serovar 2) strains, both of which were isolated from the same farm, had complete nucleotide sequence homology, with the exception of a nonsynonymous single-nucleotide polymorphism at 966T→G in HMPREF0357_10929 in Nagano 11-1, resulting in a premature stop codon in the gene.

Primers for reverse transcription-PCR (RT-PCR) were designed to create overlapping PCR products encompassing the genomic region defining serovar 1a antigenicity (Fig. 3A). RT-PCR analysis confirmed that all of the primer sets amplified a single band with the expected length and sequence, except for the genomic region between ERH_1451 and ERH_1450 (Fig. 3B), suggesting that all genes from ERH_1450 to ERH_1438 are transcribed as a polycistronic mRNA and that the chromosomal region forms an operon.

Effects of restoration and introduction of an ERH_1447 mutation. To examine whether the chromosomal region is indeed responsible for serovar 1a-specific antigenicity, we substituted a single nucleotide from the Mie 02-47 strain (serovar N) for another identified in ERH_1447 of Fujisawa. Transformation of the Mie 02-47 strain with a genomic fragment from Fujisawa recovered the antigenic reactivity of the Mie 02-47/C strain with serovar 1a-specific rabbit serum (Fig. 4A). Furthermore, we confirmed that the introduction of a truncated ERH_1447 identified in Ishikawa 02-26 (serovar N) into the Tochigi-20 (serovar 2) strain resulted in the loss of antigenic reactivity with serovar 2-specific rabbit serum (Fig. 4B, Tochigi-20/R). Additionally, complementation of the Ishikawa 02-26 strain with the genetic region from the ATCC 19414 strain (serovar 2) allowed antigenic reactivity with serovar 2-specific rabbit serum (Fig. 4B, Ishikawa 02-26/C). Taken together, these results show that the genetic region is responsible for the antigenicity of not only serovar 1a but also serovar 2 of *E. rhusiopathiae*.

Virulence analysis of mutants in mice. To investigate whether the genetic region is involved in the virulence of *E. rhusiopathiae*, we inoculated mice subcutaneously (s.c.) with mutant strains and the parent Fujisawa strain and observed the mice for 14 days. Forty-four to 100% of the mice inoculated with the deletion mutant Δ 1441, Δ 1449, or

FIG 3 (A and B) Primer locations (A) and RT-PCR analysis (B) of the chromosomal region defining the antigenicity of serovar 1a of the *E. rhusiopathiae* Fujisawa strain. (A) Small arrows indicate the orientation and corresponding locations of the primer pairs (Table S2) used in the RT-PCR assay. (B) Numbers above the lanes correspond to the Arabic numerals of the primer names. All lanes labeled with *a* indicate PCR analysis with cDNA. All lanes labeled with *b* indicate corresponding control reactions with RNA extracts not subjected to reverse transcription. All the lanes labeled *c* indicate control reactions with mixtures that do not contain template. Lane 1*a* contains a control reaction with the primers 1F and 1R, which were both designed to amplify an intragenic region of the 23S rRNA gene. A molecular size marker (M; 1-kb plus DNA ladder; Invitrogen) is shown to the left.

Δ1450 survived for 14 days without clinical symptoms, and a delay in the time to death was observed in the mice inoculated with Δ1441, Δ1442, and T1444 (Table 2), indicating that the chromosomal region including the genes defining serovar 1a antigenicity, i.e., ERH_1441, ERH_1442, and ERH_1444, is involved in the virulence of the organism.

FIG 4 Agar-gel precipitation tests for *E. rhusiopathiae* strain serotyping. (A) Center well contains serovar 1a-specific antiserum raised against heat-stable antigens of ME-7 (a serovar-1a reference strain). (B) Center well contains serovar 2-specific antiserum raised against heat-stable antigens of R32E11 (a serovar 2 reference strain).

Expression of CPS antigens. CPS antigen expression was examined with the CPS-specific monoclonal antibody (MAb) ER21. In a Western blot analysis, different banding patterns were observed for the mutants $\Delta 1441$, $\Delta 1442$, and T1444, and reactivity was lost or very faint in $\Delta 1449$ and $\Delta 1450$ (Fig. 5), suggesting that attenuation is attributable to the loss of capsular expression and/or changes in cell wall structure (9).

Morphology of the mutants. Light microscopic examination of the mutant cells revealed that many of the $\Delta 1441$ and $\Delta 1450$ mutant cells grew in long chains, whereas no clear difference was observed between the Fujisawa strain and the other mutants (Fig. 6).

DISCUSSION

Most *E. rhusiopathiae* clinical isolates from diseased pigs belong to serovar 1a, 1b, or 2 (11). Serovars are determined based on heat-stable peptidoglycan antigens; however, the serovar-specific antigen has not been clarified, and a molecular explanation for the association between these serovars and virulence remains unknown (11).

In this study, we identified the chromosomal region responsible for serovar 1a-specific antigenicity and the serovar-specific virulence of this organism. We confirmed that the region is also responsible for the antigenicity of serovar 2 strains and likely serovar 1b strains. By performing a sequence comparison of the chromosomal region, we observed that serovar N strains displayed the same gene organization as serovar 1a, 1b, and 2 strains and had certain mutations in that region. We confirmed that the

TABLE 2 Virulence of *E. rhusiopathiae* Fujisawa derivatives in mice

Strain	Median time to death ^a (days) (no. of survivors/total no.)
Fujisawa	3 (0/9)
$\Delta 1439$	3 (0/9)
$\Delta 1440$	4 (0/9)
$\Delta 1441$	8 (4/9)*
$\Delta 1442$	6 (0/9)*
$\Delta 1443$	4 (0/9)
T1444	5 (0/9)*
T1445	3 (0/9)
T1446	3 (0/9)
T1447	4 (0/9)
T1448	4 (0/9)
$\Delta 1449$	>14 (9/9)*
$\Delta 1450$	>14 (9/9)*

^aAn asterisk indicates difference (*, $P < 0.001$) compared to values for the Fujisawa-inoculated group.

FIG 5 Western blot detection of CPS antigens. The positions of the protein molecular mass standards (kDa) are shown to the left.

introduction of a mutation in this genetic region resulted in the transition of a serovar 1a strain (Fujisawa) or 2 strain (Tochige-20) into an untypeable strain. Furthermore, in serovar N strains (Mie 02-47 and Ishikawa 02-26), restoration of the mutation identified in this region recovered antigenicity with anti-serovar 1a or 2 serum. Serovar N strains lack a type-specific antigen and fail to induce precipitating antibodies against their heat-stable extracts (11, 18). Our study suggests that untypeable strains, which demonstrate no precipitation lines with antisera raised against known serovars, are the same as serovar N strains. Untypeable strains have been frequently isolated from diseased pigs (13, 14, 16–19), and therefore it is highly possible that some, if not all, of these untypeable strains originated from clinically important serovar 1a, 1b, or 2 strains, with mutations in certain genes responsible for serovar antigenicity. However, the results of this study do not exclude the possibility that other genetic regions also are involved in serovar-specific antigenicity and that other serovars can change to serovar N.

We confirmed that the genetic region responsible for serovar 1a-specific antigenicity lies between ERH_1448 and ERH_1441, which is a part of an operon consisting of the genes extending from ERH_1450 to ERH_1438. Among the genes in this operon, ERH_1441, ERH_1442, ERH_1444, ERH_1449, and ERH_1450 were confirmed to be involved in the virulence of the organism. Unexpectedly, the Δ 1443 strain produced a

FIG 6 Light microscopy images of *E. rhusiopathiae* Fujisawa derivatives.

precipitation line with anti-serovar 1a serum and was found to be virulent in mice. Unlike the other attenuated mutant strains, the $\Delta 1443$ strain showed a Western blot banding pattern similar to that of the Fujisawa strain. ERH_1443 encodes only 61 amino acids that have no substantial sequence identity to any proteins in the searched database; thus, it is possible that this small open reading frame does not actually code for a protein.

Some of the genes in the 5' half of the chromosomal region responsible for the production of serovar 1a-specific antigen in the Fujisawa strain are shared with serovar 1b (422/1E strain) and serovar 2 (ATCC 19414, SE-9, and Tochigi-20) strains; however, some genes in the 3' half of the region were missing and/or replaced with other genes. Serovar 2 can be subdivided into 2a and 2b; however, their differentiation is difficult, and therefore they are not usually subtyped (14). Thus, as observed between serovars 1a and 1b, the sequence variation in the 3' side of the region within the serovar 2 strains may be explained by antigenic variation among them.

Importantly, in the 3' end of the genetic region, serovar 1a (Fujisawa), 1b (422/1E), and 2 strains (SE-9 and Tochigi-20) possess a *tagD* gene (ERH_1439), which encodes a glycerol-3-phosphate cytidyltransferase and is involved in the biosynthesis of WTA in Gram-positive bacteria. In *E. rhusiopathiae*, the genes that may encode enzymes involved in the WTA biosynthetic pathway are dispersed over the chromosome (6), and a putative *tagO* gene (ERH_0529) is most likely fused with the gene encoding *mraY* (6), a gene essential for peptidoglycan biosynthesis. We observed that many cells of the mutants for the genes ERH_1441 and ERH_1450, both of which encode EpsG, a putative glycosyltransferase, grew in long chains. Gram-positive WTAs have been suggested to be involved in bacterial elongation and cell division (20). Taken together, we hypothesize that the genetic region identified in this study also is involved in the biosynthesis of the unusual peptidoglycan of the organism.

In this study, we observed that in the ATCC 19414 strain, strain-specific genes (HMPREF0357_10931 through HMPREF0357_10937) lie adjacent to the sequences encoding transposases in the downstream region of the chromosomal locus. The G+C contents of ATCC 19414-specific genes and the chromosomal region (ERH_1438 through ERH_1453) in the Fujisawa strain differ from those in other parts of the genome. Taken together, it appears that the chromosomal region identified in this study of serovars 1 and 2 consists of laterally transferred sequences. Based on large-scale whole-genome sequencing data, Forde et al. (21) reported that *E. rhusiopathiae* strains belonging to phylogenetically distinct clades displayed the same serovars. It is possible that the appearance of the same serovar among strains in phylogenetically distinct clades is attributable to the acquisition of foreign genes by horizontal gene transfer, resulting in serovar switching.

In conclusion, we identified a chromosomal locus responsible for the production of serovar-specific antigens in *E. rhusiopathiae* serovar 1 and 2 strains, which are often isolated from diseased pigs. It was determined that this chromosomal region is required to express and maintain the molecular integrity of the capsule, which is the most important virulence factor of the organism. Assuming that sequence variation in this chromosomal region also accounts for the antigenic differences among serovars, it will be of interest to examine whether a new molecular typing scheme for this organism can be developed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type *E. rhusiopathiae* strain Fujisawa (serotype 1a), which was originally isolated from a septicemic pig, was used to generate a transposon mutant library. *E. rhusiopathiae* strains used in this study and the genetically deleted loci or transposon insertion sites in the mutants are shown in Table 1. The serovar N strains used were confirmed not to induce precipitating antibodies against their heat-stable extracts in rabbits (11, 22). *E. rhusiopathiae* strains were grown at 37°C in brain heart infusion broth (Becton, Dickinson and Company Baltimore, MD) supplemented with 0.1% Tween 80 and 0.3% Tris (pH 8.0) (BHI-T80). The *Escherichia coli* strains used were JM109 (Toyobo, Tokyo, Japan) and DH5 α (Toyobo). Cultivation of the *E. coli* strains was performed as described previously (23).

DNA methods. Genomic DNA of the *E. rhusiopathiae* strains was prepared as described previously (24). Plasmid DNA was isolated from *E. coli* using a plasmid Miniprep kit (Promega, Madison, WI) according to the manufacturer's protocol.

PCR was performed using KOD FX DNA polymerase (Toyobo, Osaka, Japan) and a Bio-Rad T-100 thermal cycler (Bio-Rad, CA, USA). The PCR conditions are described in each section below. Amplified DNA fragments were directly sequenced with an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA) using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

RT-PCR was performed as previously described (9). Briefly, total RNA obtained from the Fujisawa strain was converted to cDNA with a reverse transcriptase. The cDNA was amplified with primer sets (see Table S2 in the supplemental material) designed to create overlapping PCR products using PrimeSTAR GXL DNA polymerase (TaKaRa, Shiga, Japan). Corresponding controls lacking reverse transcriptase or template RNA were included for each reaction to confirm the absence of genomic DNA. For confirmation, the amplified DNA fragments were directly sequenced as described above.

Transposon mutants of the Fujisawa strain were generated by a *mariner*-based transposition system with pMC plasmids as previously described (9, 25). Briefly, pMC plasmids were introduced by electroporation into the Fujisawa strain, and transformants were selected at 30°C on BHI-T80 plates supplemented with 1 µg/ml erythromycin. Individual colonies were grown in BHI-T80 with erythromycin at 30°C. The cultures were diluted and plated on BHI-T80 containing erythromycin at 30°C and then shifted to 40°C to eliminate thermosensitive pMC plasmids. Erythromycin-resistant clones resulting from insertion of the transposon into the chromosome were picked and passaged on BHI-T80 plates supplemented with erythromycin. The transposon insertion site was determined by sequencing the transposon-flanking DNA regions of transformants that contained a single transposon insertion by arbitrary primers (25, 26).

Recombination of *E. rhusiopathiae* strains was performed as previously described (27), except that the shuttle vector plasmid pMAD (28) was used. For complementation analysis, the Mie 02-47 strain (serovar N) was transformed with a 2,940-bp fragment, which was PCR amplified from the genome of the Fujisawa strain (serovar 1a) with the primers ERH_1445_BgIII_F and ERH_1448_Sall_R (Table S2). The nucleotide sequence in the genomic fragment differs by one base between Fujisawa and Mie 02-47 at 586 in the gene corresponding to ERH_1447. The Ishikawa 02-26 strain (serovar N) was transformed with a 3,220-bp fragment, which was PCR amplified from the genome of the ATCC 19414 strain (serovar 2) with the primers 10928_BgIII_F and ERH_1448_Sall_R (Table S2). The Ishikawa 02-26 strain has a 10-base deletion in the gene corresponding to ERH_1447. To introduce a mutation, a 3,210-bp fragment, which was PCR amplified from the genome of the Ishikawa 02-26 strain (serovar N) with the same primers, 10928_BgIII_F and ERH_1448_Sall_R, was used to transform Tochigi-20 (serovar 2). PCR amplification was performed as described above with the following conditions: initial denaturation of 94°C for 2 min and 3 amplification steps (35 cycles) consisting of 98°C for 10 s, 55°C for 30 s, and 68°C for 3 min.

Sequence information. The sequences of the chromosomal region (ERH_1438 through ERH_1451) of the Fujisawa strain (serovar 1a) (accession no. [AP012027](#)) and the corresponding genomic region of the ATCC 19414 strain (serovar 2) (accession no. [ACLK02000002](#)) were retrieved from GenBank. For the serovar 2 strains SE-9 and Tochigi-20, draft genome sequences were generated using the Illumina HiSeq platform. For other strains, the chromosomal region and/or corresponding region were divided into three genetic regions, and each region was PCR amplified with the following primer sets: seq1F and seq1R or seq1R', seq2F and seq2R, and seq3F and seq3R (Table S2) (Fig. 2). PCR was performed with the following conditions: initial denaturation at 94°C for 2 min and 3 amplification steps (35 cycles) consisting of 98°C for 10 s, 60°C for 30 s, and 68°C for 10 min. The sequences were assembled by the software ATGC ver.7 (Genetyx Corporation, Tokyo, Japan), and protein-coding sequences (CDSs) were identified using IMCGE (*in silico* molecular cloning genomics edition) software (29). The functions of each CDS were determined according to the results from a BLASTP search against the NCBI RefSeq database (30).

Phenotype analyses of *E. rhusiopathiae* strains. (i) Serovars. Transposon and gene deletion mutants were tested in agar-gel precipitation tests using autoclaved cell extracts and rabbit antisera against formalin-killed cells of all 26 reference strains as previously described (14). The reference strains are listed elsewhere (31).

(ii) Virulence. Animal experiments in this study were performed according to the regulations and guidelines of the Animal Ethics Committee of the National Institute of Animal Health, Tsukuba, Ibaraki, Japan.

Groups of nine female 4-week-old ddY mice (purchased from Japan SLC, Inc., Hamamatsu, Japan) were s.c. inoculated with 1.0×10^2 CFU (approximately 10 times the 50% lethal dose [LD₅₀] of the Fujisawa strain) of *E. rhusiopathiae* Fujisawa mutant derivatives. The mice were observed for clinical symptoms and mortality for 14 days. Mortality was recorded daily, and the median time to death was determined by Kaplan-Meier analysis followed by the log-rank test by using Prism 5.0 software (Graph Pad, La Jolla, CA).

(iii) Expression of CPS. The expression of CPS antigens was examined as previously described (9). Briefly, *E. rhusiopathiae* strains were cultured in 10 ml of BHI-T80 at 37°C overnight. Bacterial cells were harvested by centrifugation, washed with 20 mM Tris-HCl (pH 7.6), suspended in 0.5 ml of 20 mM Tris (pH 7.6) containing 0.5% Triton X-100, and incubated at 37°C for 1 h with rotation. After incubation, the bacterial cells were removed by centrifugation, and the supernatants containing crude capsular antigens were used for capsular antigen detection by immunoblot analysis with the CPS-specific MAb ER21 (9, 32).

(iv) Cell morphology. *E. rhusiopathiae* strains were cultured at 37°C in BHI-T80 overnight, and cell morphology was examined after Gram staining under a 100× objective lens with a Leica DM1000 microscope. Images were captured with LAS EZ software.

Accession number(s). The nucleotide sequences analyzed in this paper have been deposited in the DDBJ/GenBank/EMBL databases and are listed in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00324-18>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

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The authors have no conflicts of interest to declare associated with the manuscript.

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