Veterinary Microbiology 239 (2019) 108488

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

A putative transcription regulator involved in the virulence attenuation of an acriflavine-resistant vaccine strain of *Erysipelothrix rhusiopathiae*, the causative agent of swine erysipelas

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ARTICLE INFO

Keywords:
Acriflavine
Attenuation mechanism
Erysipelothrix rhusiopathiae
Live vaccine
Swine erysipelas

ABSTRACT

Acriflavine, an acridine dye that causes frameshift mutations, has been used to attenuate various veterinary pathogens for the development of live vaccines. *Erysipelothrix rhusiopathiae* Koganei 65-0.15 strain (Koganei) (serovar 1a) is the acriflavine-resistant live vaccine currently used in Japan for the control of swine erysipelas. To investigate the attenuation mechanisms of the Koganei strain, we analyzed the draft genome sequence of the Koganei strain against the reference genome sequence of the *E. rhusiopathiae* Fujisawa strain (serovar 1a). The sequence analysis revealed a high degree of sequence similarity between the two strains and identified a total of 98 sequence differences within 80 protein-coding sequences. Among them, insertions/deletions (indels) were identified in 9 genes, of which 7 resulted in frameshift and premature termination. To investigate whether these mutations resulted in the attenuation of the Koganei strain, we focused on the indel mutation identified in ERH_0661, an XRE family transcriptional regulator. We introduced the mutation into ERH_0661 of the Fujisawa strain and restored the mutation of the Koganei strain. Animal experiments using the recombinant strains showed that mice survived inoculation with 10^3 colony forming units (CFUs) (equivalent to approximately 100 50% lethal doses [LD50] of the wild-type Fujisawa) of the recombinant Fujisawa strain, and the mice became ill after inoculation with 10^8 CFUs of the recombinant Koganei strain. These results suggest that the transcriptional regulator ERH_0661 is involved in the virulence of *E. rhusiopathiae* and that the ERH_0661 mutation is partially responsible for the attenuation of the Koganei strain.

In the livestock industry, many current vaccines are attenuated live and inactivated vaccines (Meeusen et al., 2007). Attenuated live vaccines are mostly developed using empirical approaches based on serial passages or chemical mutagenesis (Minor, 2015). Blind passage of pathogens results in the accumulation of multiple attenuating mutations, which leaves the molecular mechanisms of attenuation unknown; thus, there is a risk of back-mutation and the reemergence of virulent strains (Meeusen et al., 2007; Minor, 2015).

Acriflavine is an acridine dye that is bacteriostatic against many gram-positive bacteria (Wainwright, 2001). Acriflavine is known to cause frameshift mutations (Drake, 1964) and has also been used for virulence attenuation and the development of live vaccines against various veterinary pathogens, including *Streptococcus suis* (patent: WO2014004361A1), *Streptococcus equi* (patent: US 4788059) and bovine viral diarrhea virus (Rockborn et al., 1974).

In Japan and China, the acriflavine-resistant live vaccine *Erysipelothrix rhusiopathiae* has been used for the control of swine erysipelas, an infection characterized by acute septicemia or chronic endocarditis and polyarthritis (Wood and Henderson, 2006). The Japanese live vaccine *E. rhusiopathiae* Koganei 65-0.15 strain (Koganei) (serovar 1a) was produced from randomly selected strains by 65 passages on agar plates containing 0.15% acriflavine (Seto et al., 1971). The vaccine has played a substantial role in preventing the disease; however, a possible association between the incidence of chronic forms of the disease and vaccination with the Koganei strain has been suggested (Imada et al., 2004). Recently, using a SNP-based PCR assay that can differentiate the vaccine strain from *E. rhusiopathiae* wild-type strains, we determined that 65.2% of field strains (101/155) analyzed, which were isolated from pigs affected with chronic erysipelas in farms where the vaccine had been used, were Koganei strains (Shiraiwa et al.,

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https://doi.org/10.1016/j.vetmic.2019.108488
Received 9 September 2019; Received in revised form 24 October 2019; Accepted 27 October 2019

2017). A high rate of detection of the acriflavine-resistant vaccine strains among the *E. rhusiopathiae* field isolates has also been indicated in reports from China (Zou et al., 2015; Zhu et al., 2016). Thus, there is a strong possibility that the acriflavine-resistant vaccine strains reverted to virulent strains *in vivo* and caused disease. Although the vaccines are still extensively used, little or no studies have characterized the genetic basis of virulence attenuation or reversion to virulence. In this study, we analyzed the mechanism of virulence attenuation of the acriflavine-resistant Koganei vaccine strain.

Whole-genome sequencing of the Koganei strain was performed as described previously (Ogawa et al., 2017). Briefly, 100-bp paired-end reads, which were generated with the Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA) and deposited in the NCBI SRA (Sequence Read Archive) database under accession number DRX032134, were mapped against the reference genome sequence of the Fujisawa strain (serovar 1a) (GenBank accession no. AP012027) with Burrows–Wheeler Aligner (Li and Durbin, 2009) under default parameters. SNPs from the alignments were identified using SAMtools (version 0.1.19) (Li et al., 2009). In this analysis, unlike in the cases of previous studies (Ogawa et al., 2017; Shiraiwa et al., 2015), no SNP filtering was applied to the data by setting the SAMtools mpileup max per-file depth to 8000 to identify all insertions and deletions (indels) that may have been generated by the mutagenic activity of acriflavine.

We obtained 73.7 million reads, with 100% of reads mapped to the reference genome, resulting in 4,034-fold sequence depths. Sequence analysis revealed a high degree of sequence similarity between the two strains and a total of 98 sequence differences, of which 28 were not identified in previous studies (Ogawa et al., 2017; Shiraiwa et al., 2015) that applied filtering criteria. Of the 28 sequence differences, 9 were indels, and the sequences were confirmed by PCR and Sanger sequencing. Among the indels identified, 7 caused a frameshift of protein-coding sequences (CDSs) (Table 1), 2 of which occurred in putative transcription regulator genes, ERH_0661 (XRE family) and ERH_0745 (GntR family).

Transcriptional regulators are often involved in virulence in a variety of bacteria (Vasil and Darwin, 2013; Grove, 2013). To investigate whether the transcriptional regulators were involved in the virulence of *E. rhusiopathiae*, we introduced the ERH_0661 mutation into the Fujisawa strain and restored the mutation of the Koganei strain. The Fujisawa and Koganei strains were complemented with sequences derived from the Koganei strain and the Fujisawa strain, respectively (see Fig. S1 in Supplemental material). Briefly, DNA fragments were PCR-amplified with primers 661F:SalI (5′-CCGTCGACTTTGAAGGCAT TATCAAAGAAC-3′) and 661R:EcoRI (5′-CCGAATTCACGATCCTCCATA TATGAAACT-3′) from each strain and then cloned into the shuttle vector plasmid pMAD (Arnaud et al., 2004). The *E. rhusiopathiae*strains were transformed with the recombinant plasmids as previously described (Shimoji et al., 2002), and the sequences were confirmed by PCR and subsequent Sanger sequencing.

The virulence of the recombinant *E. rhusiopathiae*strains was assayed in mouse experiments, which were approved by the Animal Ethics Committee of the National Institute of Animal Health (NIAH), Tsukuba, Ibaraki, Japan. Groups of 6-week-old female ddY mice (Japan SLC, Inc., Hamamatsu, Shizuoka, Japan) were inoculated with *E. rhusiopathiae*strains and observed for clinical signs for 14 days. Table 2 summarizes the results of the virulence assay. All mice (n = 9) inoculated with 10^3 colony forming units (CFUs) of the Fujisawa strain died within 3 days after challenge infection. In contrast, all mice (n = 3) inoculated with 10^3 CFUs and one of the three mice inoculated with 10^5 CFUs of the recombinant Fujisawa strain (Fujisawa/del*0661*) survived for 14 days without clinical symptoms. For the challenge with 10^8 CFUs of the Fujisawa/del*0661* strain, all mice (n = 4) died within 5 days after infection. To compare the virulence of the Koganei strain with the virulence of the recombinant Koganei strain with the restored mutation (Koganei/intact*0661*), mice were inoculated with 10^8 CFUs of the strains. All mice (n = 10) inoculated with the Koganei strain remained healthy without signs of infection, whereas all mice (n = 10) inoculated with the Koganei/intact*0661* strain showed poor overall conditions, anorexia, depression, ruffled fur, or a hunched posture but recovered by 10 days after infection.

In this study, we revealed that a putative transcriptional regulator of the XRE family, ERH_0661, was involved in virulence of *E. rhusiopathiae* and that the mutation of the gene is partially responsible for the attenuation of the acriflavine-resistant Koganei vaccine strain. This is the first report to show a link between a transcriptional regulator and the virulence of *E. rhusiopathiae*.

Recent advancements in sequencing technologies have enabled the identification of mutations in genomes; however, distinguishing indels from errors in next-generation sequencing (NGS) is very difficult (Kim et al., 2017; Nielsen et al., 2011); therefore, analyses of NGS data must first include a filtering step to remove errors. In the present analysis, we analyzed the draft genome sequence of Koganei without applying the filtering step to the data.

Frameshift mutations caused by the mutagenic activity of acriflavine are derived from indels (Drake, 1964). In this study, we found 7 novel frameshift mutations that were caused by indels and were not detected in our previous study that applied filtering criteria (Ogawa et al., 2017; Shiraiwa et al., 2015). The frameshift mutation identified in ERH_0661 is a 1-bp deletion in an adenine homopolymer that results in the premature termination of the gene. It has been reported that frameshift intermediates in homopolymer runs are efficiently removed by mismatch repair proteins in *Saccharomyces cerevisiae* (Greene and Jinks-Robertson, 1997). *E. rhusiopathiae* possesses the mismatch repair system (*mutL*, *mutS*, and *pcrA*) (Ogawa et al., 2011). Taken together, it is possible that the Koganei strain can revert to virulence by repairing the mutation *in vivo* and regaining virulence.

In this study, we observed that in addition to the mutation identified in ERH_0661, the Koganei strain has another indel mutation in the transcriptional regulator ERH_0745 (GntR family). Previously, we observed that Koganei has nonsynonymous SNP mutations in genes ERH_0075 (collagen-binding protein) and ERH_0543 (MarR family transcriptional regulator) (Ogawa et al., 2017). Surface proteins of gram-positive pathogens play important roles in virulence (Fischetti, 2006), and it has been reported that members of the XRE and MarR families are involved in the virulence of pathogenic bacteria (Hu et al., 2019; Grove, 2013). Taken together, it is possible that all or some of these genes are also involved in the virulence of *E. rhusiopathiae*, which may explain why although a mutation in ERH_0661 resulted in the

Table 1
Indel mutations identified in the *E. rhusiopathiae* Koganei strain.

Locus tag	Predicted function and/or description	DNA sequence length (nt)	Mutation
ERH_0084	Flavin reductase FMN-binding domain-containing protein	525	Insertion (408_409 insTTTA)
ERH_0411	ABC transporter, permease protein	855	Insertion (9_10insTGTCGATGGGAGTT)
ERH_0661	Transcriptional regulator, XRE family	954	Deletion (597delA)
ERH_0745	Transcriptional regulator, GntR family	705	Deletion (403delG)
ERH_1067	RNA methyltransferase	708	Insertion (duplication) (145dupA)
ERH_1107	Alpha/beta hydrolase domain-containing protein	1014	Insertion (duplication) (259_260dupCA)
ERH_1560	Stomatin-like protein	888	Insertion (duplication) (89dupC)

Table 2
Virulence of the *E. rhusiopathiae* strains in mice.

Strain tested	Description	Inoculation dose (CFUs)	No. of survivors/total	Clinical scores
Fujisawa	Wild-type strain, serovar 1a	10^3	0/9	9
Fujisawa/del*0661*	Fujisawa strain with a deletion mutation in the ERH_0661 gene	10^8	0/4	4
		10^5	1/3	2
		10^3	3/3[a]	0[a]
Koganei	Acriflavine-resistant vaccine strain, serovar 1a	10^8	10/10	0
Koganei/intact*0661*	Koganei strain with an intact ERH_0661 gene	10^8	10/10	10[b]

[a] Significant difference ($p < 0.01$ by Fischer's exact test) compared to the result obtained with inoculation of 10^3 CFUs of the Fujisawa strain.
[b] Significant difference ($p < 0.01$ by Chi-square test) compared to the result obtained with inoculation of the Koganei strain. Mice were observed daily for any clinical signs (anorexia, depression, ruffled fur, or hunched posture) throughout the entire experimental period (14 days). Mice that showed at least one of the clinical signs were scored +1, and total scores were calculated by adding the scores for mouse groups.

attenuation of the Fujisawa strain, the restoration of the ERH_0661 mutation did not recover the virulence of the Koganei strain to the level of the Fujisawa strain. To fully understand the attenuation mechanisms of the Koganei strain and the pathogenicity of *E. rhusiopathiae*, further analyses of these genes are necessary.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

This study was supported by the National Institute of Animal Health and NARO.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108488.

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