XRE Family Transcriptional Regulator XtrSs Modulates Streptococcus Suis Fitness of Hydrogen Peroxide Stress

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XRE family transcriptional regulator XtrSs modulates *Streptococcus suis* fitness of hydrogen peroxide stress

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Abstract: *Streptococcus suis* is an important emerging zoonosis that causes economic losses in the pig industry and severe threats to public health. Transcriptional regulators play essential roles in bacterial adaptation to host environments. In this study, we identified a novel XRE family transcriptional regulator in *S. suis* CZ130302, XtrSs, involved in the bacterial fitness to hydrogen peroxide stress. Based on electrophoretic mobility shift and β-galactosidase activity assays, we found that XtrSs autoregulated its own transcription and repressed the expression of its downstream gene *psePs,* a surface protein with unknown function in *S. suis*, by binding to a palindromic sequence from the promoter region. Furthermore, we proved that the deletion of the *psePs* gene attenuated bacterial antioxidant response. Phylogenetic analysis revealed that XtrSs and PsePs naturally co-existed as a combination in most *S. suis* genomes. Collectively, we demonstrated the binding characteristics of XtrSs in *S. suis* and provided a new insight that XtrSs played a critical role in modulating *psePs* to the hydrogen peroxide resistance of *S. suis*.

Keywords: *Streptococcus suis,* XtrSs, transcriptional regulator, hydrogen peroxide stress

Introduction

Gram-positive bacterium *Streptococcus suis* is recognized to be one of the most

important swine pathogens, causing huge economic losses especially in countries with large-scale swine production (Segura 2009; Goyette-Desjardins et al. 2014). In addition, *S. suis* is also considered as an emerging zoonotic pathogen of humans，responsible for streptococcal toxic shock syndrome (STSS) in human (Eisenberg et al. 2015). Currently, there were 33 serotypes identified based on the antigenicity of capsular polysaccharides (CPS) in *S. suis* (Votsch et al. 2018). Besides, 27 novel cps loci (NCL) in total have been identified (designated as NCL1-26 and serotype Chz) from non-typeable isolates. The Chz variant serotypes strain was first reported in China and subsequently isolated in Canada, indicating a global epidemic trend (Pan et al. 2015; Zheng et al. 2017). One of the serotype Chz strains, CZ130302, was isolated from an acute meningitis piglet and showed higher pathogenicity in mouse models compared to the virulent serotype 2 strains P1/7 (Pan et al. 2015).

Streptococcus suis is highly invasive and infects host through the respiratory tract, digestive tract and wound. Once the host mucosal barrier is breached, *S. suis* can reach and survive in the bloodstream, and eventually invade multiple organs and tissues (Votsch et al. 2018; Herold et al. 2019). To respond to various environment and host immune systems, *S suis* can modulate the expression of specific genes by complicated bacterial regulatory networks, including quorum sensing systems, stand-alone regulators, regulatory RNAs, and two-component regulatory systems (TCSs), among which stand-alone regulators are the most intensively studied (Patenge et al. 2013).

XRE family transcriptional regulator as a member of stand-alone regulators is widely distributed throughout the eukaryotic and prokaryotic organisms (Ulrich et al. 2005; Lu et al. 2019; Trouillon et al. 2021). The XRE family transcriptional regulator has a DNA-binding structural domain which contains the typical HTH (helix-turn-helix) that can bind to the promoter of the target genes and modulate their transcription. Only one XRE family regulator in *S. suis*, SrtR, has been reported and it is responsible for oxidative stress response and high-temperature stress (Hu et al. 2018). However, the target genes regulated by the XRE family regulators in response to oxidative stress have not yet been elucidated. Here, we found a novel XRE family transcriptional

regulator of *S. suis*, XtrSs (XRE family transcriptional regulator of *S. suis*), playing an important role in hydrogen peroxide resistance by binding the promoter of its upstream gene *psePs* (SEC10/PgrA surface exclusion domain-containing protein of *S. suis*) and repressed the expression of *psePs*. Interestingly, *psePs* was confirmed to involve in the bacterial peroxide resistance for the first time. Additionally, we found that XtrSs and PsePs combination widely exists in *S. suis*. Therefore, these data expanded our understanding of the relationship between the regulators and antioxidative stress in *S. suis.*

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

All strains in this study were listed in Table S1. The novel serotype Chz strain CZ130302 was isolated from a diseased piglet in Changzhou, China. The *S. suis* strains were cultured in Todd-Hewitt broth (THB; Oxoid, Basingstoke, UK) or on THB agar plates supplemented with 5% sheep blood (Maojie, Nanjing, China) at 37°C in the presence of 5% CO_2 as described. Spectinomycin (100 μg/mL) or 10% sucrose was added to screen for the mutant strain and the complemented strain. The *E. coli* DH5α strains were grown in LB broth (Difco Laboratories, Franklin Lakes, NJ, USA) or on LB agar plates at 37°C. If necessary, kanamycin (25 μg/mL) was added. All chemicals and enzymes for molecular cloning were purchased Sigma (Beijing, China) and New England Biolabs (Beijing, China), respectively, unless otherwise stated.

Mutant construction

To investigate the function of genes, a series of mutant strains were created via natural DNA transformation. As described in previous study (Zhu et al. 2019)， the peptide ComS13-21 (GNWGKWTDG) was used to stimulate the natural transformation of the *S. suis* CZ130302. Firstly, to construct gene replacement marked mutants, the upstream and downstream fragments of target genes and sacB-spc cassette was individually amplified. Subsequently, the three fagments were ligated by fusion PCR and then transformed into the target bacteria by natural transformation, and spectinomycin was used to screen tmarked mutants. Secondly, the fusion homology

fragment without the sacB-spc cassette was transferred to the primarily marked mutant for the second transformation, and then sucrose was used to screen strains without the sacB-spc cassette. The primers used in this work were listed in Table S2.

Identification of Growth Characteristics

CZ130302, Δ*xtrSs* and CΔ*xtrSs* in the mid-exponential phase (OD_{600} 0.6~0.8) were collected respectively by centrifugation and washed three times with phosphate-buffered saline (PBS). The original OD_{600} of all cultures of the different strains was adjusted to 0.005. Each of the cultures was incubated in a 37°C shaking incubator at 180 rpm. A growth curve was constructed through the measurement of the OD_{600} each hour until the growth process entered the decline phase at least three independent experiments

RNA isolation and qRT-PCR analysis

To analyze the transcription level of the *xtrSs*, *psePs* and COV91_RS12780 genes in THB cultured cells, *S. suis* strains CZ130302, Δ*xtrSs*, CΔ*xtrSs* in OD_{600} 0.6~0.8 were collected. And the wild-type strain CZ130302 was collected in different OD_{600}. Total RNA isolation was performed using the TRIzol method (Vazyme, China) by the manufacturer's instructions. The HiScriptII first-strand cDNA synthesis kit (Vazyme, China) was used to synthesize cDNA using the isolated RNA (1 μg) as a template. Real-time quantitative PCR (RT-qPCR) was performed to validate the transcript concentrations of the selected genes using ChamQ Universal SYBR qPCR master mix (Vazyme, China) with the QuantStudio 6 Flex real-time PCR system. The housekeeping gene GAPDH was used as an internal control, and the relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method. The website (Integrated DNA Technologies; https://www.idtdna.com/scitools/Applications/RealTimePCR/) was used to set the primers of selected genes (Table S2).

Subcellular proteins extractions

The method for extracting secreted proteins of *S. suis* is the same as previously reported (Sayed et al. 2012), with minor modifications. In short, 40 ml of bacterial culture medium was cultured to logarithmic phase (OD_{600} 0.6~0.8) and centrifuged at 10,000 rpm for 15 min. Each supernatant was collected and filtered twice using a

0.22-μm-pore filter, then precipitated on ice with 15% trichloroacetic acid for 30 min. Wash the precipitates with 10 mL cold acetone and repeat. After drying and volatilizing acetone, secreted proteins are obtained.

Western blot analysis

To find out the effect of *xtrSs* on psePs protein level and polymorphisms. Western blot was performed. The proteins were separated by SDS-PAGE and then transferred to the PVDF membrane (Bio-RAD), which was blocked with 5% (W/V) skimmed milk at 37℃ for 2 h. The polyclonal antibody (anti-*psePs*, anti-*xtrSs*) was diluted with milk at 1:1000 and incubated at 37℃ for 2 hours. The anti-rabbit IgG–HRP (1:2000 dilution, Boster, China) was used as the secondary antibody. After washing for 3 times with PBS-Tween 20 buffer, the proteins were visualized using the High-sig ECL western blotting kit (Tanon, China).

Protein purification and antisera preparation

The fragments of target protein were amplified by PCR from purified DNA of *S. suis* CZ130302. A linker sequence (GGGGSGGGGSGGGGS) was used to ligate between the two same amino acid sequences of XtrSs to gain an XtrSs homodimer. The fragments were then cloned into pET28a(+) by *Bam*HI and *ScalI* digestion and ligation to construct plasmids, $XtrSs^{dimer}$-pET28a(+) and PsePs-pET28a(+). To express the recombinant proteins, the plasmids were transformed into *E. coli* BL21(DE3) cells. Single colonies containing a recombinant plasmid identified as positive were amplified and induced for expression with 1 mM IPTG. After low-temperature induction, we used a nickel column (GE Healthcare) to purify the soluble His-tagged recombinant proteins.

To prepare the antisera against rPsePs, four rabbits were immunized subcutaneously with a mixture containing 1 ml purified rPsePs, protein (1mg) and 1 of Montanide™ ISA 206 VG (Seppic SA, France). The immunization procedure was repeated two more times (2 weeks apart). Two weeks after the last immunization, rabbit sera were collected.

EMSA

The BProm program (SoftBerry) was used to predict the promoter regions, and a

fragment from 16S rRNA was used as the negative control. The promoter variants were obtained by overlap extension PCR using the upstream and downstream sequences of the repeats. DNA probes were obtained by PCR amplification using specific primers (Table S2) and purified using a gel extraction kit (Vazyme, China). The EMSA reaction system was 20 μ L: 100 ng DNA fragment was mixed with more and more purified recombinant protein rXtrSs^{dimer} (0 to 250 ng) in the binding buffer (10 mM Tris, 20 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.05% NP-40, and 5% glycerol [vol/vol]) (Guyet et al. 2013; Liu et al. 2020). The EMSA mixtures were incubated at 37°C for 30 min, then loaded onto a 6% polyacrylamide gel in 0.5× TBE buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH 7.4) and electrophoresed at 200 V for 45 min. Finally, the gel was stained in 0.5× TBE buffer containing ethidium bromide for 15 min and the image was recorded.

Reporter plasmid construction and β-galactosidase assays

S. suis CZ130302 genomic DNA was as a template to amplify the fragments of different promoters with the appropriate primers (Table S2). After digestion with *SmaI*, the sequences were inserted into the pTCV-lac vector to generate different recombinant plasmids. Then the recombinant plasmids were transformed into CZ130302, Δ*xtrSs* and CΔ*xtrSs* by natural DNA transformation.

The β-galactosidase assays were performed with CZ130302, Δ*xtrSs* and CΔ*xtrSs* strains containing a certain recombinant plasmid. β-Galactosidase activities were determined using *o*-nitrophenyl-β-d-galactopyranoside as the substrate, normalized to the optical density at 600 nm, and expressed in Miller units. The β-galactosidase activity of each strain was calculated according to the following formula: (A_{420} ×1000 ×VE)/ (RT × VS × OD_{600}) (RT: reaction time; VE: End volume, Vs: The volume of each sample (Aviv and Gal-Mor 2018). At least three independent cultures of each strain were assayed in each experiment.

H_2O_2 susceptibility test

To investigate the role of XtrSs in oxidative stress, CZ130302 and its mutant strains were challenged with H_2O_2 according to a previously described protocol

(Minchington et al. 2020). Bacteria in log phase were washed and resuspended in THB and adjusted to a concentration of approximately 10^7 CFU/mL. H_2O_2 was added to the cell suspension to a final concentration of 20 Mm. The cells were incubated for 10 min at 37 ℃ at shaking.

Bioinformatics analysis

Sequences of the identified proteins were searched using the Quik Blastp server in NCBI to identify homologous sequences. According to 30% of the amino acid sequence homology as a threshold, the sequences from 87 *S. suis* strains were included and used to construct the phylogenetic trees. Amino acid sequences of *xtrSs* and *psePs* from each strain were edited, concatenated, aligned, and subjected to phylogenetic analysis using MEGA X by the neighbor-joining (NJ) method.

The DNA-palindrome finder (http://palindromes.ibp.cz/#/en/palindrome) was used to predict the palindromes of the promoter region. MEME database(https://meme-suite.org/meme/) was used to create an XtrSs-PsePs operator consensus sequence. DNA sequence logos were generated using the MEME.

We used local TBLASTN 2.10.1+ to compare the amino acid sequences of XtrSs and PsePs respectively with the whole genome database of *Streptococcus* species, published in NCBI by December 2021. Based on the data, we statistics the distribution of XtrSs and PsePs.

Statistical analysis

All experiments were repeated at least three times. Statistical significance was set to $P < 0.05$, and an unpaired two-tailed Student's t-test or the analysis of variance (ANOVA) test was applied to analyze the data.

Results

***xtrSs* mutant construction**

XtrSs is a putative transcriptional regulator of 333 bp, encoding 111 amino acids, with the typical HTH structure of the XRE family. To investigate the role of *xtrSs* in *S. suis*, we generated an *xtrSs* mutant strain (Δ*xtrSs*) by natural transformation as well as a genomic complemented strain (CΔ*xtrSs*) and confirmed them by PCR amplification using the respective primers (Fig. 1A), while the deletion of *xtrSs* did not disrupt the

promoters of upstream and downstream genes. As shown in figure 1B, there was no significant difference in the growth curves of strains CZ130302, Δ*xtrSs* and CΔ*xtrSs* during the 24-hour observation. The data showed that the bacterial growth was not affected by genetic manipulation on *xtrSs*.

XtrSs down-regulates the transcription and expression of upstream gene *psePs*

Firstly, to determine if the deletion of *xtrSs* had a polar effect, the transcription level of *xtrSs* and its flanking genes *psePs* and CVO91_RS12780 in the wild-type and mutant strains were analyzed by qRT-PCR. The results showed that the *xtrSs* gene was not transcribed in Δ*xtrSs*, and the transcription of the downstream gene CVO91_RS12780 was not affected by the *xtrSs* deletion. However, the transcription of upstream gene *psePs* increased significantly in Δ*xtrSs*. In strain CΔ*xtrSs*, the transcription levels of these genes were not increased compared to the wild-type CZ130302 (Fig. 2A). Meanwhile, we extracted RNA from strain CZ130302 during various growth phases and observed the transcription of *xtrSs* and *psePs* by qRT-PCR. The results showed that the transcription of *xtrSs* increased with bacterial growth, at the same time the transcription level of *psePs* correspondingly decreased，which also implied that *xtrSs* suppressed the transcription of *psePs* (Fig. 2B).

To confirm that *xtrSs* down-regulated the expression of *psePs* at the protein level, we expressed rPsePs (shown as Fig. 2C, about 120KDa) and prepared the mice polyclonal antibody against *psePs* protein. The website Psortb (https://www.psort.org/psortb/) predicted that the subcellular localization of protein *psePs* was extracellular. We extracted secreted proteins of the wild-type CZ130302, Δ*xtrSs* and CΔ*xtrSs*， and used *psePs* polyclonal antibody as the primary antibody for western blot analysis. The expression level of secreted protein *psePs* was higher in Δ*xtrSs* than that in CZ130302 and CΔ*xtrSs* (Fig. 2D). The result showed that the expression of the secreted protein *psePs* was suppressed by XtrSs.

XtrSs promotes self-transcription by directly binding to the overlapping promoter region

The *xtrSs* and *psePs* genes are divergently transcribed from overlapping promoters in CZ130302. Given that many transcriptional regulators always form homologous dimers to bind promoters, we expressed rXtrSs^{dimer} (shown as Fig. 3A, about 35KDa) to explore its affinity to the predicted promoter DNA sequence (250 bp) in *vitro*. Electrophoretic mobility shift assays (EMSA) showed that XtrSs specifically bound to the promoter and displayed a dose-dependent mobility shift (Fig. 3B). Conversely, there was no bind shift observed after XtrSs and 16S sequence were incubated.

Meanwhile, we constructed transcription fusion vectors by inserting the promoter sequences of the *xtrSs* and *psePs* into the pTCV-lac plasmid respectively. Subsequently, the corresponding recombinant plasmids were introduced into wild-type CZ130302, Δ*xtrSs*, and CΔ*xtrSs* by natural transformation. Interestingly, we found that the β-galactosidase activity of the *psePs* promoter in Δ*xtrSs* was notably increased compared to that in CZ130302 and CΔ*xtrSs* strains (Fig. 3C), implicating the promoter activity of *psePs* was inhibited by XtrSs when XtrSs bound the *psePs* promoter. However, the β-galactosidase activity of the *xtrSs* promoter in Δ*xtrSs* was significantly lower than that in CZ130302 and CΔ*xtrSs* (Fig. 3D), which suggested that XtrSs could autoregulate and promote *xtrSs*-self promoter transcriptional activity when *xtrSs* bound to itself promoter.

Identification of XtrSs binding sites

Many known transcriptional regulators have been reported to bind palindrome sequences on promoters of target genes. We found a series of palindromic sequences in the promoter sequence of *xtrSs* or *psePs* by bioinformatic analysis. Among them, the longest 16 bp palindromic sequence (5'-CAACTTTACAAAGTTG-3') contained the -35 boxes of the two genes, making it possible to be XtrSs binding sites, thus we mutated the palindromic sequence (Fig. 4A). Principally, the mutated promoter remained the CG content and unchanged the -35box sequence.

EMSA assay showed the promoter sequence was blocked by rXtrSs^{dimer}, but the mutant promoter sequence could not form the bind shift even if the concentration of rXtrSs^{dimer} reached 200 ng (Fig. 4B). The mutated promoter sequence was then cloned into the pTCV-lac plasmid, creating plasmid M*psePs*-pTCV-lac and M*xtrSs*-pTCV-lac,

and introducing them into *S. suis* strains CZ130302, Δ*xtrSs* and CΔ*xtrSs,* respectively. Galactose activity assay showed that there was no difference in the levels of β-galactosidase activity in strains CZ130302, Δ*xtrSs* and CΔ*xtrSs* with the M*psePs*-pTCV-lac plasmid (Fig. 4C). Consistently, no significant differences in the β-galactosidase activity of three strains carried the M*xtrSs*-pTCV-lac plasmid were observed (Fig. 4D). Based on these results, we believed that that palindrome (5'-CAACTTTACAAAGTTG-3') was the binding site for XtrSs. It is well known that -35 box and -10 box are binding sites for the RNA polymerase (RNAP). Our analysis found that the XtrSs target sequence located at the upstream and overlapped partly the -35 box of the XtrSs promoter, and concurrently covered fully the -35 box of the PsePs promoter (Fig. 4A). It implied that XtrSs promoted its own transcription by its interaction with the RNAP and prevented RNAP from initiating transcription of *psePs*.

XtrSs is involved in H2O2 stress by repressing *psePs*

To test whether XtrSs could affect oxidative tolerance of *S. suis*, we compared the survival rates of CZ130302, Δ*xtrSs* and CΔ*xtrSs* strains under H_2O_2 stress condition *in vitro*. When these three *S. suis* strains were challenged with H_2O_2 (10 mM H_2O_2 in THB), the survival of Δ*xtrSs* was significantly higher than CZ130302 and CΔ*xtrSs* strains (Fig. 5A). These results suggested that the transcription of *xtrSs* inhibited the resistance of *S. suis* to H_2O_2 stress. Given that XtrSs repress *psePs* significantly, we assumed that *psePs* might play an essential role when the strain was exposed in H_2O_2 stress. Therefore， we knocked out *psePs* in CZ130302 to generate strain Δ*psePs*. The H_2O_2 challenge assay showed that the survival rate of Δ*psePs* was significantly lower than that of CZ130302 as well as Δ*xtrSs* (Fig. 5A). This data confirmed our hypothesis that XtrSs was involved in H_2O2 stress by repressing *psePs*.

Analysis of the prevalence of XtrSs and PsePs in *S. suis*

The amino acid sequences of XtrSs were compared with the published protein database of *S. suis* by NCBI Quick Blastp. To analysis evolutionary relationships of XtrSs-PsePs in *S. suis*, we combined the amino acid sequences of XtrSs and PsePs from *S. suis* 87 strains sharing more than 30% per-identity and constructed a

phylogenetic tree by the NJ method. Based on the homology， XtrSs-PsePs was roughly divided into two branches. XtrSs-PsePs in CZ130302 is in class I, which is different from class II because the position of motif 9 and 10 was changed (Fig. 6A). Meanwhile, we compared all the palindromic sequences from the DNA sequence between *xtrSs* and *psePs* with the XtrSs binding sites "5'-CAACTTTACAAAGTTG-3'" identified in CZ130302, thus we could identify the palindromic sequence of the *xtrSs* suspected binding sites in *S. suis*. All binding sites were used to create logos by the MEME website, which showed a certain regularity in the binding sites, summarized as the sequence motif 5'-YAACTTTAYAARGYTG-3' in class I (Fig. 6B) and 5'-CAACTTTACAAGGTTC-3' in class II (Fig. 6C). Although CZ130302 was in class I, the logo in class II was highly conserved.

By bioinformatic analysis, XtrSs is widely distributed in *Streptococcus* species (Table 1), such as *Streptococcus pyogenes* (100%), *Streptococcus pneumoniae* (98.89%), *Streptococcus agalactiae* (52.34%), *Streptococcus salivary* (88.33%) and *Streptococcus oralis* (75%), implicating that XtrSs is relatively conserved among *Streptococcus* species. Moreover, we found that XtrSs and PsePs co-existed in diverse *Streptococcus* genomes, such as *S. pyogenes* (100%), *S. anginosus* (63.64%), *S. suis* (36.36%) and *S. agalactiae* (21.88%) (Table 1), which implied that the regulation mechanism (XtrSs down-regulated *psePs* and up-regulated itself) probably in common in *streptococcus* species.

Discussion

Bacteria can respond to environmental changes by using complex regulatory systems to influence the transcription of virulence or metabolic genes (Guyet et al. 2013; Trouillon et al. 2021). The XRE family transcriptional regulators contain the HTH domain that can bind to the promoter of the target genes and modulate their transcription. We previously characterized an XRE family transcriptional regulator XtgS that negatively regulates virulence in *Streptococcus agalactiae* (Liu et al. 2020). In this study, we demonstrated XtrSs, sharing less than 50% amino acid sequence homology with XtgS, negatively regulates bacterial resistance to H_2O_2 by binding to the promoter of the antioxidant gene *psePs* and repressing its

expression.

Notably, *Streptococcus* species are deficient in the catalase. It is necessary to identify *xtrSs* potential target genes associated with bacteria resistance to H_2O_2 in *S. suis*. RT-PCR and EMSA tests showed that *psePs* was directly regulated by XtrSs. According to NCBI-CDD results, *psePs* has been annotated as a SEC10/PgrA surface exclusion domain-containing protein. Although the SEC10/PgrA domain has been found in other species such as *Streptococcus pyogenes*, *Streptococcus gordoni* and *Enterococcus faecalis*, functions of the SEC10/PgrA domain have been reported in a few bacteria. Deletion of the SpyAD gene resulted in very long bacterial chains and attenuated *Streptococcus pyogenes* adhesion to host cells (Gallotta et al. 2014). PrgA can provide a distinct competitive advantage for *Enterococcus faecalis* and contribute to bacterial virulence and surface exclusion(Schmitt et al. 2020). However, the SpyAD and PrgA share only approximately 13.64% and 19.5% amino acid similarity with *psePs,* respectively. In our study, we for the first time revealed that *psePs* played a role in bacterial peroxide resistance. Based on the abovementioned results, we can explain that the increased resistance of Δ*xtrSs* strain to oxidative stress due to the transcriptional surge of *psePs* in Δ*xtrSs*.

Amino acid sequence comparison showed that XtrSs has low homology to that of known XRE regulators that have been reported to be associated with oxidative stress, such as *S. suis* SrtR (3.07%) (Hu et al. 2018) and *Corynebacterium glutamicum* MsrR (5.42%) (Fig. S1) (Si et al. 2020). MsrR in *C. glutamicum* was proved that as an oxidation-sensing regulator to upregulate the expression of adjacent genes, *3-mst* and *mfs* which were related to bacterial stress resistance (Si et al. 2020). Because cysteine sites coexisted in SrtR and MsrR, the authors suggested a thiol-based regulatory switch served to modulate oxidative stress resistance in the XRE family regulators. Similarly, many redox-sensitive regulators, such as OxyR, PerR, and OhrR, have been reported to specifically sense reactive oxygen species via a thiol-based mechanism (Lee et al. 2007; Zhang et al. 2012; Jo et al. 2015; Garnica et al. 2017)。 The thiol-based mechanism showed that when bacteria exposed to oxidative stress, regulators change morphology to form a homomultimer via intersubunit disulfide

bonds caused by cysteines oxidation, resulting in dissociating from or binding promoters of target genes (Liu et al. 2013; Ulrich et al. 2021). Coincidentally, considering that one cysteine residue was at position 62 (Cys62) in XtrSs, we postulated XtrSs maybe a novel oxidative sensor by a similar thiol-based redox modulation mechanism. Therefore, we will investigate the difference in resistance to oxidative stress between CZ130302 and Cys62 mutant and confirm this thiol-based mechanism of *S. suis* in the future.

Transcriptional regulators often can autoregulate, leading to signal amplification, producing a switch-like response, and promoting bistability (Vujanac et al. 2015; Gao and Stock 2018; Lu et al. 2019). For instance, the *spxA2* gene in *Bacillus anthracis* was highly induced in infected macrophages due to an autoregulatory positive-feedback loop driving *spxA2* transcription (Barendt et al. 2016). QseB in *E. coli* bound directly to two low-and high-affinity sites in its promoter simultaneity and activate its transcription (Wu et al. 2021). In contrast to the negative autoregulation of redox-sensitive regulators previously reported, such as PerR and OxyR (Zhang et al. 2012; Jo et al. 2015). However, XtrSs binding site was adjacent to or partly overlapped the RNAP binding sites, so that XtrSs played the positively autoregulation role on its own transcription.

Conclusion

In summary, our results provided insight into XtrSs, the XRE transcriptional regulator identified in *S. suis,* involved in the adaptation and oxidative stress of *S. suis* by repressing upstream gene *psePs,* which was shown to contribute to bacteria resist hydrogen peroxide stress. Our study on the binding sites of XtrSs may lead to a better understanding of the characteristics of *xtrSs* binding DNA sequences.

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Declarations

Conflict of interest None declared.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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Figure legends

Figure 1 Identification and characteristics of the *xtrSs* gene mutant and complemented strains. (A) A schematic chart and confirmation of the *xtrSs* gene mutant and complemented strains by PCR. (B） The growth curves of *S. suis* strain CZ130302, Δ*xtrSs*, CΔ*xtrSs* . Growth curves were started with ~5 × 10^5 CFU in THB at 37 °C, and the OD_{600} nm was measured each hour. Data are means and standard deviations (SD) from three independent experiments. The data were compared with that of strain CZ130302 and analyzed using the one-way ANOVA test.

Figure 2 XtrSs inhibits transcription and expression of upstream genes *psePs*. (A) Transcriptional level analysis of *xtrSs* and its flanking genes by qRT-PCR. (B) Transcriptional level analysis of *xtrSs* and *psePs* in different growth states of the wild-type strain CZ130302 by qRT-PCR. (C) SDS-PAGE analysis of rPsePs; (D) Western blot analyses identified the subcellular localizations of PsePs in the indicated strains. Each value represents the average of three independent experiments. The data are shown as the means and standard deviations of the results from three independent experiments performed in triplicate. Unpaired two-tailed Student's t-test: ***P < 0.001).

Figure 3 XtrSs promotes self-transcription by directly binding to the overlapping promoter region. (A) SDS-PSGE analysis of $rXtrSs^{dimer}$; (B) Electrophoretic mobility shift assay was performed in the presence of various amounts of His-tagged $rXtrSs^{dimer}$ using the promoters described in the text. DNA probes containing the 16S rRNA promoter region were used as a negative control. Only the *xtrSs/psePs* promoter region could be shifted by the $rXtrSs^{dimer}$ in EMSA. β-Galactosidase activities in CZ130302, Δ*xtrSs* and CΔ*xtrSs* strains containing *psePs*-promoter-pTCV-lacZ plasmid (C) and *xtrSs*-promoter-pTCV-lacZ plasmid (D). Each value represents the average of three independent experiments. Asterisks indicate significant differences (***, P < 0.001).

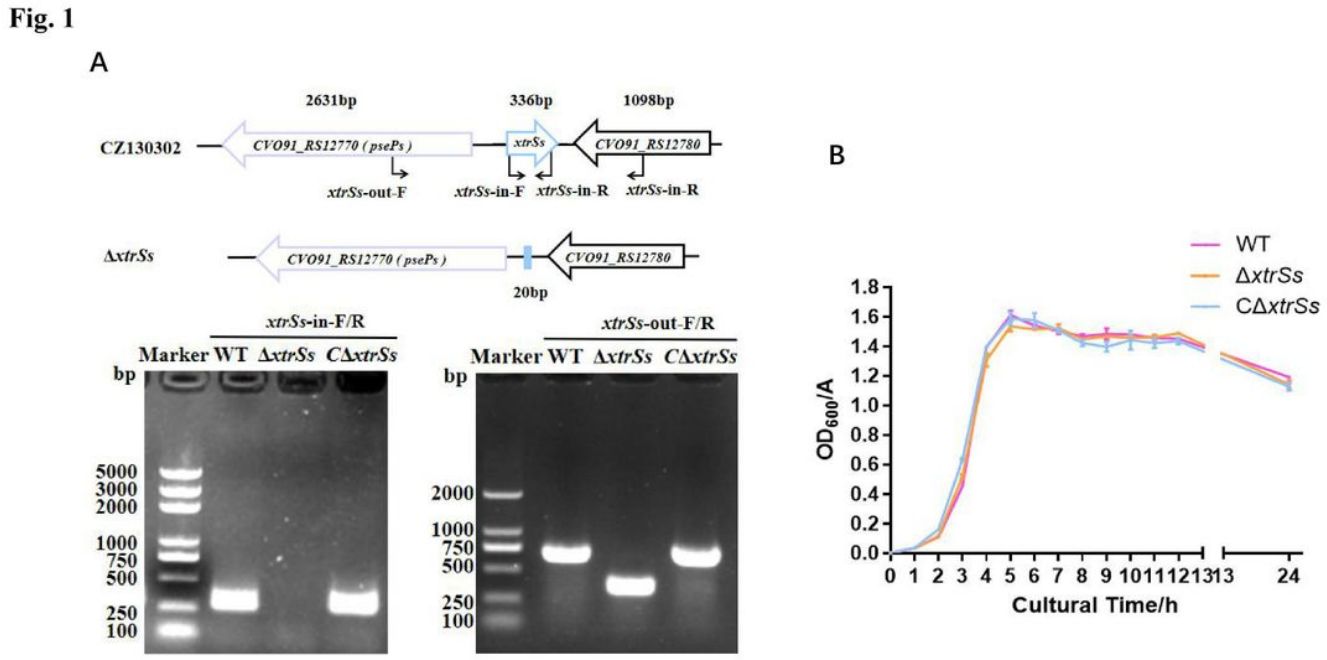
Figure 4 Identification of XtrSs binding sites. (A) Graphic representation of XtrSs binding to the *psePs* promoter and mutant palindrome sequence; (B) EMSA was performed in the presence of various amounts of His-tagged rXtrSs^{dimer} using the promoters described in the text. DNA probes containing the 16S rRNA promoter region were used as a negative control. DNA probes containing the *psePs* promoter region were used as a positive control. β-Galactosidase activities in CZ130302, Δ*xtrSs* and CΔ*xtrSs* strains containing M*psePs*-promoter-pCTV-lacZ plasmid (C) and M*xtrSs*-promoter-pTCV-lacZ plasmid (D). The experiments were repeated three times. No significant differences were detected using unpaired two-tailed Student's t-test (ns, $P > 0.05$).

Figure 5 H_2O_2 challenge assay. CZ130302, Δ*xtrSs* and CΔ*xtrSs* and Δ*psePs* in the mid-log phage were harvested and used to test the survival ability in a peroxidation environment. H_2O_2 was added to the cell suspension to create a final concentration of 20 mM and incubation for 10 min. Surviving cells were diluted appropriately, plated on THA plates. The percentage of the CFU was normalized to the wild-type group designed as 100%. The experiments were repeated three times. Asterisks indicate significant differences by Unpaired two-tailed Student's t-test (*, $P < 0.05$; **, $P < 0.01$).

Figure 6 Phylogenetic tree analysis of the prevalence of XtrSs and PsePs in *S. suis*. (A) A neighbor-joining tree (bootstrap n=1000; Poisson correction) was constructed with MEGA X based on a ClustalW alignment of the amino acid sequences of XtrSs and PsePs from 89 *S. suis* strains. All the strains are labeled. We added motif features of XtrSs-PsePs on the right of each phylogenetic tree. We divided the amino acids of XtrSs-PsePs into ten motifs according to their homology. The colors were used to distinguish different motifs. Two *S. suis* strains containing two different XtrSs-PsePs assemblies were tagged with "(1)" and "(2)" after their names. Bind sites consensus sequence was created by aligning palindrome sequences similar to the bind sites we identified in CZ130302 from 29 *S. suis* strains in class I (B) and 60 *S. suis* strains in class II (C） using the MEME website.

Table 1 Distribution of XtrSs and PsePs in the *Streptococcus* genome

Figures



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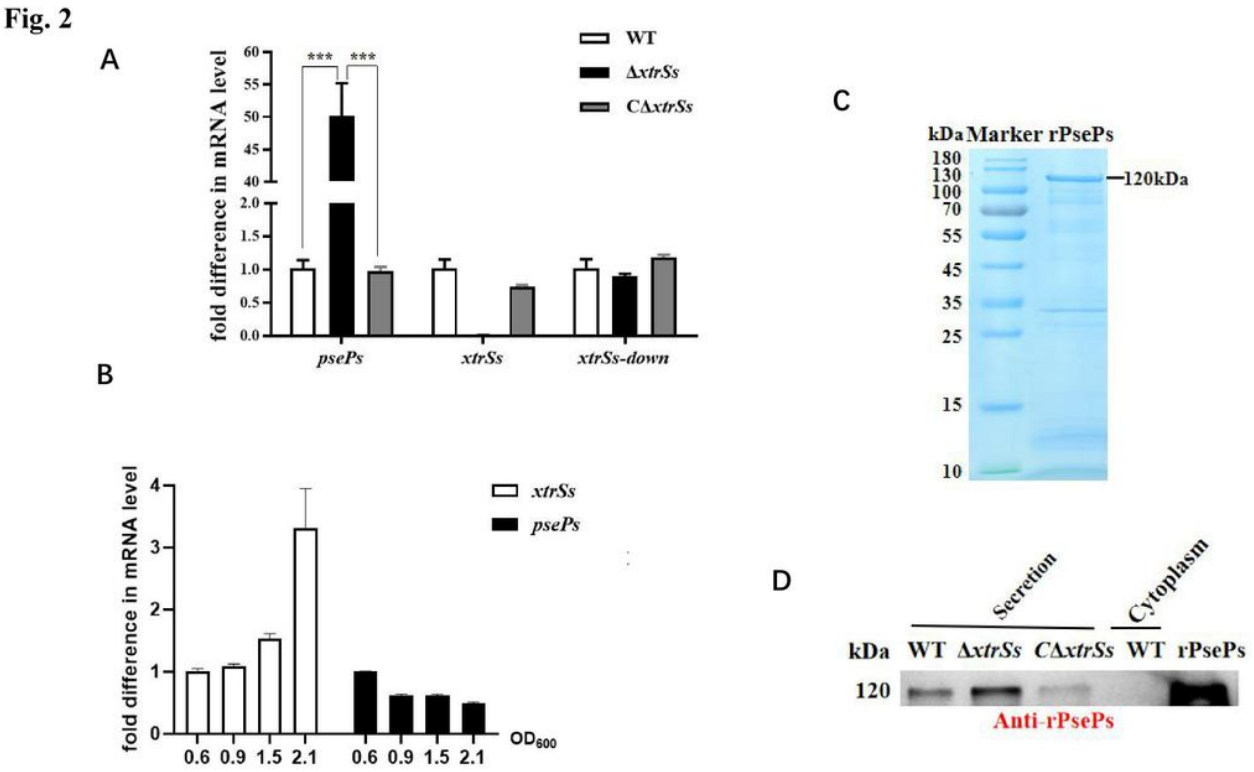

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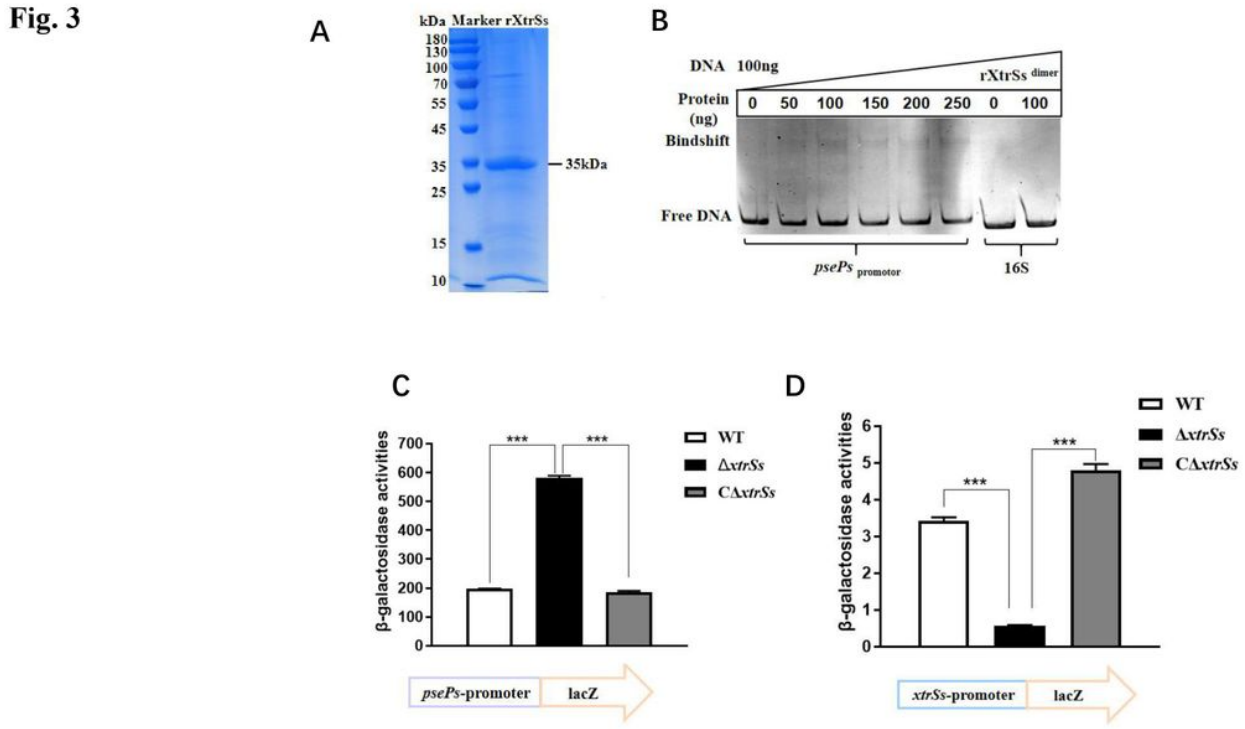

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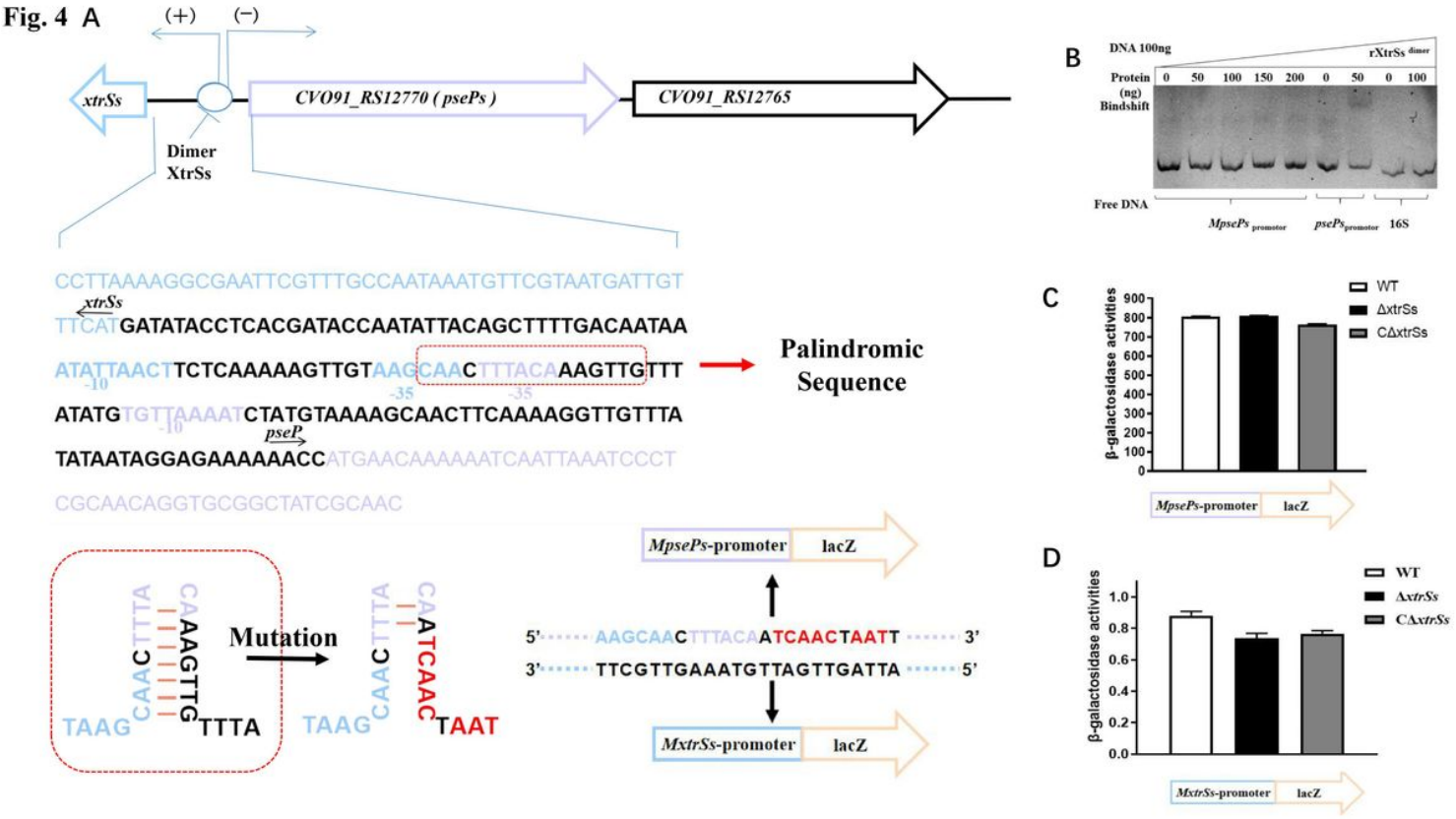

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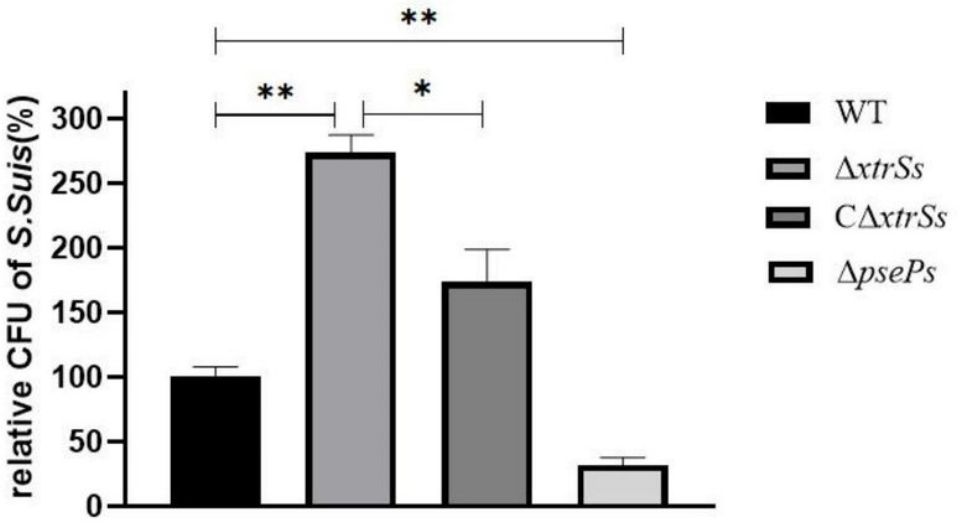
Fig. 5



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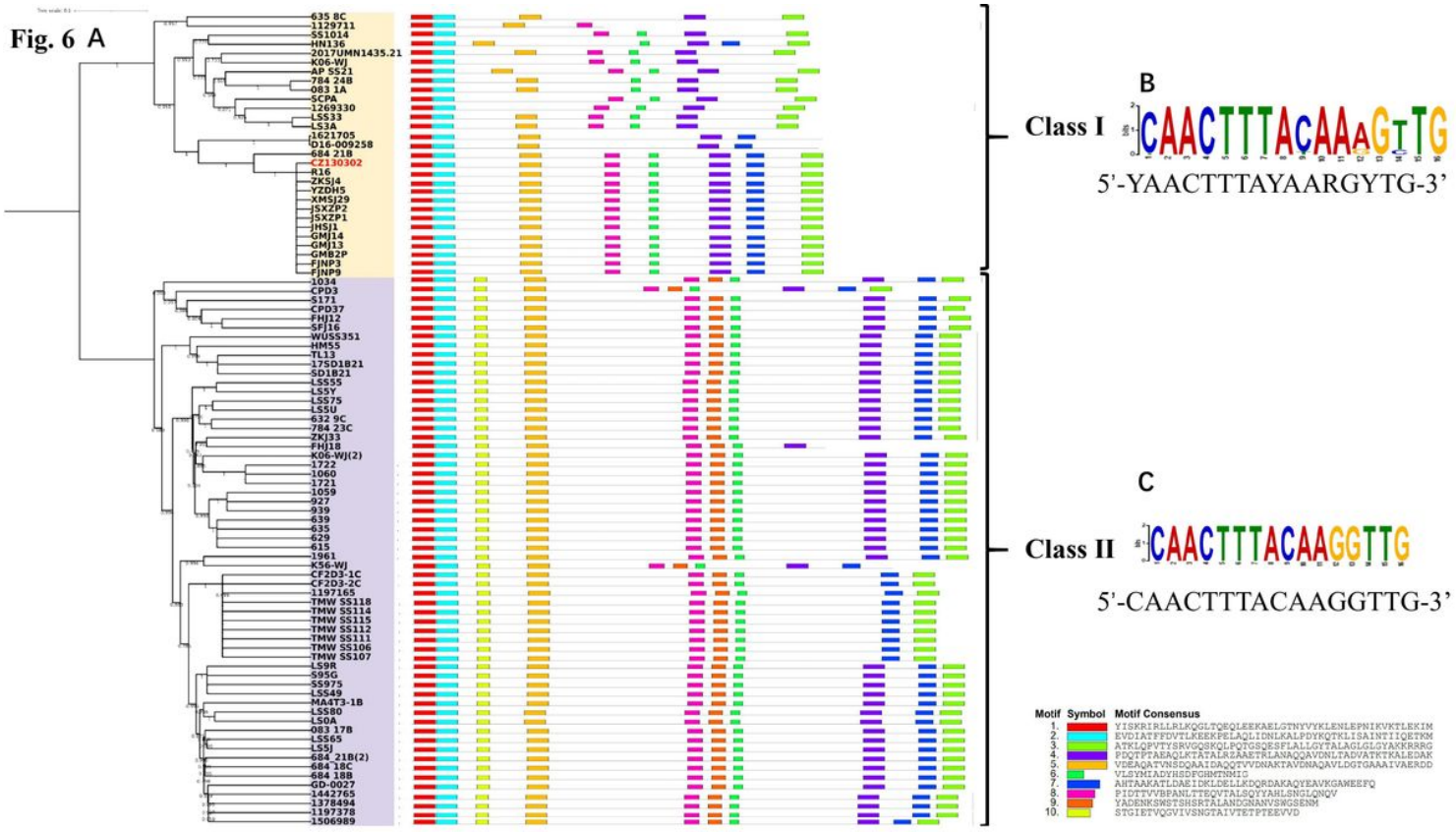

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Supplementary Files

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- Table.pdf
- 22.1.1SupplementaryMaterials.pdf