Relevance of the two-component sensor protein CiaH to acid and oxidative stress responses in *Streptococcus pyogenes*

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**Abstract**

**Background:** The production of virulence proteins depends on environmental factors, and two-component regulatory systems are involved in sensing these factors. We previously established knockout strains in all suspected two-component regulatory sensor proteins of the *emm1* clinical strain of *S. pyogenes* and examined their relevance to acid stimuli in a natural atmosphere. In the present study, their relevance to acid stimuli was re-examined in an atmosphere containing 5% CO2.

**Results:** The *spy1236* (which is identical to *ciaHpy*) sensor knockout strain showed significant growth reduction compared with the parental strain in broth at pH 6.0, suggesting that the Spy1236 (CiaHpy) two-component sensor protein is involved in acid response of *S. pyogenes*. CiaH is also conserved in *Streptococcus pneumoniae*, and it has been reported that deletion of the gene for its cognate response regulator (*ciaRpn*) made the pneumococcal strains more sensitive to oxidative stress. In this report, we show that the *spy1236* knockout mutant of *S. pyogenes* is more sensitive to oxidative stress than the parental strain.

**Conclusions:** These results suggest that the two-component sensor protein CiaH is involved in stress responses in *S. pyogenes*.

**Background**

*Streptococcus pyogenes*, a Gram-positive bacterium that infects the upper respiratory tract, including the tonsils and pharynx, and is responsible for post-infection diseases such as rheumatic fever and glomerulonephritis. *S. pyogenes* also causes severe invasive diseases including necrotizing fasciitis and streptococcal toxic shock syndrome (STSS) [1-5].

*S. pyogenes* is exclusively a human pathogen and it possesses many virulence factors that help it to resist host defense systems. The production of these factors is precisely regulated in response to host environmental conditions, such as different infection sites or host immune system induction levels [6-8]. In prokaryotes, the regulation of protein production in response to fluctuating environmental conditions depends primarily on two-component regulatory systems, which consist of a sensor histidine kinase and its cognate response regulator [9]. Thirteen two-component regulatory systems have been described in *S. pyogenes*, of which the CovRS system (also known as the CsrRS system) mediates the control of several virulence factors [10-15]. Analysis of the other two-component regulatory systems is still incomplete. In addition, most experiments have been performed from the viewpoint of the response regulators. Therefore, it is still unclear which signals the sensor proteins sense.

In a previous study, we focused on the sensor proteins of two-component regulatory systems, establishing 13 types of sensor knockout mutants, analyzing their involvement in the acid response in a “natural” atmosphere, and proposing that the Spy1622 two-component sensor protein is involved in sensing acid stimuli [16]. In contrast to the natural atmosphere used in our previous study, an atmosphere containing 5% CO2 is often used to culture *S. pyogenes* [10,17,18]. The CO2 concentration in deeper tissues is higher than its concentration at the epithelial surface of the host [19]. This can cause certain genes—for example, the gene encoding M protein—to...
be stimulated by carbon dioxide [20]. Therefore, it is possible that the genes involved in the acid response are also stimulated differently under natural atmospheric conditions than they are in an atmosphere containing 5% CO₂. In the present study, we reanalyzed the involvement of 13 sensor proteins in the acid response in an atmosphere containing 5% CO₂.

**Methods**

**S. pyogenes strains**

Streptococcal strains 1529, MDYK, and MDN were isolated from Japanese patients with STSS [21,22]. *S. pyogenes* (GAS) strain SF370, which is currently the most prevalent database reference isolate (accession number NC_002737), was provided by J. J. Ferretti [23,24]. As shown in Figure 1, 13 sensor knockout mutants derived from the strain 1529 have previously been constructed [16]. These strains were cultured in either brain–heart infusion (E-MC62, EIKEN Chemical Co., Tokyo, Japan) supplemented with 0.3% yeast extract (BD, Sparks, MD, USA) broth (BHI-Y), or Todd Hewitt broth (BD) supplemented with 0.3% yeast extract broth (TH-YE), unless otherwise stated.

**Culture conditions for growth assay**

Streptococcal strains were cultured using a previously described strategy [16], with certain modifications. In brief, an aliquot of frozen bacterial stock solution that had been stored at −80°C was inoculated into the TH-YE broth and cultured overnight (about for 18 h) at 37°C without agitation. A 70 μL sample of the overnight culture was added to fresh TH-YE broth (4 mL, pH 7.6 or 6.0), cultured in an atmosphere containing 5% CO₂ for 23 h, and then the viable cells were counted by plating onto blood agar and BHI-Y agar plates. The experiments were repeated at least three times, independently.

**Production of spy1236 knockout strains**

We constructed an *S. pyogenes* strain 1529Δspy1236 as described previously [16]. Strains MDYKΔspy1236 and MDNΔspy1236 were constructed using the same strategy. To construct a plasmid for spy1236 complementation (pLZ-spy1236), the DNA fragment was amplified using oligonucleotide primers 1236-n2 (5′-GTGGTTGACTTAGCTCGAAA-3′) and 1236-c2 (5′-AAAATTCATTGAACCTACAC-3′), strain 1529 genomic DNA as template, and PrimeSTAR HS DNA polymerase (Takara, Ohtsu, Japan). Digestion with *Pvu* II produced a fragment containing *spy1236*, which was treated with T4 polynucleotide kinase and ligated into the *Sma*I site of the plasmid pLZ12-Km2 [25].

**BLAST analysis**

The Basic Local Alignment Search Tool (BLAST) was used for homology search (http://blast.ncbi.nlm.nih.gov/).

**The sensitivity of Δspy1236 mutants and derivative strains to H₂O₂**

Assays were performed as described previously [26]. In brief, aliquots of bacterial cultures grown to an OD₆₆₀ of ~0.3 were exposed to 61 mM H₂O₂ for 15 min at room temperature. Viable cells were counted by plating onto blood agar and BHI-Y agar plates before and after exposure to H₂O₂, and the result was expressed as percent survival.

**Plasmids having htrA gene**

Plasmids pLZ-htrAforward and pLZ-htrAreverse were constructed as described in Additional file 1: Figure S1. In brief, a DNA fragment encoding the *htrA* gene was amplified using oligonucleotide primers htrA-F3 (5′-CATTACTTTTTACACAAATTTATCCACAAGT-3′) and htrA-R1 (5′-GTAGGTCTATCAATAATTCTTTGTCAAAAAT-3′), strain1529 genomic DNA as template, and
Changes in the levels of gene expression were calculated was determined automatically using a real-time 7900HT machine; Applied Biosystems) using the Sybr green detection system (Applied Biosystems). Primers for the genes of interest and the internal control gene to assess genomic DNA contamination. The cDNA was also used without reverse transcription, as a control to assess genomic DNA contamination. The cDNA and the control were then used as templates for quantitative RT-PCR (qRT-PCR) (real-time 7900HT PCR machine; Applied Biosystems) using the Sybr green detection system (Applied Biosystems). Primers for the genes of interest and the internal control gene gyrA are shown in Table 1. PCR conditions included incubation at 50°C for 2 min, followed by incubation at 95°C for 10 min, and finally 40-cycles of amplification (95°C for 15 s and 60°C for 1 min). The signal was standardized to that of the gyrA gene, where the cycle threshold (CT) was determined automatically using a real-time 7900HT PCR software (Applied Biosystems) after 40 cycles. Changes in the levels of gene expression were calculated using the $\Delta\Delta Ct$ method [27,28]. Each assay was repeated using at least three independent RNA samples. Product specificity was evaluated using both melting-curve analysis [29] and 2% agarose gels.

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted from bacterial cells grown as described above for the H2O2 sensitivity assay. The purity and concentration of the RNA were determined by gel electrophoresis and spectrophotometry, respectively. Extracted total RNA was employed as the template for random-primed first-strand cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s instructions. It was also used without reverse transcription, as a control to assess genomic DNA contamination. The.cDNA and the control were then used as templates for quantitative RT-PCR (qRT-PCR) (real-time 7900HT PCR machine; Applied Biosystems) using the Sybr green detection system (Applied Biosystems). Primers for the genes of interest and the internal control gene gyrA are shown in Table 1. PCR conditions included incubation at 50°C for 2 min, followed by incubation at 95°C for 10 min, and finally 40-cycles of amplification (95°C for 15 s and 60°C for 1 min). The signal was standardized to that of the gyrA gene, where the cycle threshold (CT) was determined automatically using a real-time 7900HT PCR software (Applied Biosystems) after 40 cycles. Changes in the levels of gene expression were calculated using the $\Delta\Delta Ct$ method [27,28]. Each assay was repeated using at least three independent RNA samples. Product specificity was evaluated using both melting-curve analysis [29] and 2% agarose gels.

### Table 1 Sequences of primers used in qRT-PCR

| Primer          | Sequence (5'-3')            |
|-----------------|----------------------------|
| GyrA-1584 F     | ACGTGGCGTGCAAGGAACCT        |
| GyrA-1709 R     | TGCTAACGTCTTCAACCGATAGACA   |
| HtrA-R4         | ATCGAAGGACGCTAAACCAGATTG    |
| HtrA-R4         | TCAGCTCTCAAATTTTACAAAC      |
| Nrd-R           | ACCAGTACGACAGCTGACGGGC      |
| Nrd-R           | AGGCAGTGATGACACTGGACATCT    |
| Emm1-31 F       | TGCTACTCCGCTGTGCCATA        |
| Emm1-98R        | ACAGTGGACGACGCTAACCATTCT   |
| MGAS5005 polA-F | GCGGCGAAACGCCCTTTA          |
| MGAS5005 polA-R | GCGGCGACCGCCCTTTA           |

Primer sequences were designed using Primer Express® software version 3.0 and 2.0, and with technical support of life technologies Japan.

**Statistical analysis**

Survival times were analyzed by using nonparametric Mann–Whitney U analysis and unpaired t test. P values of <0.05 for both analyses were considered statistically significant.

**Results and discussion**

Analysis of the effect of sensor proteins on the growth of bacteria cultured at pH 7.6 or 6.0

To test the effect of *S. pyogenes* sensor proteins on growth under acidic pH conditions, we first used the previously established knockout mutants lacking all 13 suspected sensor proteins [16]. We cultured the parental and derived knockout strains in a medium with the pH adjusted to 6.0, and in an atmosphere containing 5% CO2. As shown in Figure 1 and Table 2, the CFU (colony forming units)/ml for overnight cultures of strain 1529Δspy337 (covS) and 1529Δspy1236 were lower than that of the parental strain 1529. However, the CFU/ml for overnight cultures of strain 1529Δspy1622 was not lower than that of the parental strain 1529 under this experimental condition.

In this study, we focused on Spy1236 more than CovS, for the following three reasons. First, we have already analyzed 1529ΔcovS and have showed that 1529ΔcovS had lower growth ability than the parental wild type strain 1529 even at pH 7.6 [17]. Therefore, the lower growth ability of the covS mutated strain shown in Figure 1 may not have been caused by the fact that the medium was adjusted to pH 6.0. Second, CFU/ml (or OD$_{660}$) for overnight cultures of strain 1529Δspy1236 was similar to that of the parental strain 1529, when cultured in a medium with pH adjusted to 7.6 and in an atmosphere containing 5% CO2 (Table 2). Finally, BLAST analysis showed that Spy1236 (436 amino acids) shares 58% identity with CiaH of *Streptococcus mutans* (referred to as CiaH$_m$). It is known that the CiaH$_m$ sensor kinase is involved in a response to acid stress in *S. mutans* [27,30,31].

In order to further investigate the effect of Spy1236 sensor kinase on growth under acidic conditions, we

**Table 2 Growth of spy1236 knockout mutant in acidic (pH 6.0) media in an atmosphere containing 5% CO2**

| Strains | OD$_{660}$ | Av. CFU/ml |
|---------|-----------|-----------|
|         | (pH7.6)   |           |
| 1529wt  | 0.906 ± 0.009 | 3.4 ± 0.7 x 10$^3$ |
| 1529Δspy1236 | 0.927 ± 0.025 | 2.0 ± 0.6 x 10$^3$ |
|         | (pH6.0)   |           |
| 1529wt  | 0.662 ± 0.009 | 4.1 ± 1.6 x 10$^4$ |
| 1529Δspy1236 | 0.571 ± 0.039 | 2.3 ± 1.0 x 10$^4$ |

The experiment was performed as described in Figure 1. At least three independent experiments were performed and they always yielded essentially the same results. Values expressed are the means (±SEM).
next established a strain in which the spy1236 knockout was complemented using an appropriate plasmid, and performed the same experiments at pH 6.0 and 5% CO2. As shown in Figure 2, spy1236 cloned into a plasmid vector (pLZ-spy1236) complemented the lower growth ability of 1529Δspy1236. These results suggested that the lower growth level of the spy1236 mutant was Spy1236-dependent, at least in this strain.

To examine the effects related to strain specificity, we established additional spy1236 knockout strains derived from strains MDYK and MDN (MDYKΔspy1236 and MDNΔspy1236, respectively). When the same experiments were performed at pH 6.0 and 5% CO2, the CFU/ml for overnight cultures of MDYKΔspy1236 and MDNΔspy1236 were lower than those of the parental strains (Figures 3a and c). In addition, the CFU/ml for overnight cultures of MDYKspy1236 (pLZ-spy1236) and MDNΔspy1236 (pLZ-spy1236), in which the spy1236 deletions were complemented with pLZ-spy1236, were higher than for MDYKΔspy1236 (pLZ12-km2) and MDNΔspy1236 (pLZ12-km2), which harbor a control vector, respectively (Figure 3b and d). Thus, Spy1236 (also referred to as CiaHpy) may be involved in the response to acid stress in some S. pyogenes strains, as it is in S. mutans, in an atmosphere containing 5% CO2.

Meanwhile, the empty-vector complementation resulted in the increased acid-resistance compared to the mutant strain (Figure 3). Kanamycin added to the complementation assay might induce some stress responses including the acid-resistance.

Sensitivity of the spy1236 knockout strains to oxidative stress

The CiaH sensor kinase is also conserved in Streptococcus pneumoniae (51% identical with Spy1236 by BLAST analysis). Ibrahim et al. [26] showed that deletion of the gene encoding the cognate response regulator CiaRpn made a pneumococcal strain more sensitive to oxidative stress. Therefore, we were interested to learn whether the Spy1236 sensor kinase is involved in the response to oxidative stress, and performed essentially the same experiments using S. pyogenes Δspy1236 mutants as were previously done using S. pneumoniae [26]. As shown in Figure 4a, 1529Δspy1236 was significantly more sensitive to hydrogen peroxide than the parental strain 1529, and the complemented strain 1529Δspy1236 (pLZ-spy1236) was more resistant to hydrogen peroxide than 1529Δspy1236 (pLZ12-Km2, the control vector) (Figure 4b). To examine the effects related to strain specificity, we also performed this experiment in strain MDYK. The knockout strain MDYKΔspy1236 was more sensitive to hydrogen peroxide than its parental strain, MDYK (Figure 4c). Thus, the CiaHpy sensor kinase of S. pyogenes may contribute to oxidative stress tolerance.

The percent survival of 1529Δspy1236 (pLZ12-Km2) (Figure 4b) seems to be similar to that of wild-type strain 1529 (Figure 4a). One of the differences in their experimental settings is that 1529Δspy1236 (pLZ12-Km2) was grown in broth supplemented with kanamycin. This might induce some stress responses to increase the survival rate of the 1529Δspy1236 (pLZ12-Km2). At least, we did not find a potential region to confer the ability, when the pLZ12-Km2 sequence was analyzed using BLAST.

Contribution of the CiaH sensor kinase to oxidative stress tolerance may not be mediated via HtrA

Ibrahim et al. [26] also demonstrated that the contribution of the CiaHpn sensor kinase to oxidative stress tolerance was mediated by the HtrA protein in S. pneumoniae, based on the following evidence: (i) the sensitivity of the S. pneumoniae strain D39ΔciaR to oxidative stress can be restored by complementation with HtrA, and (ii) the expression of htrA in the CiaR-null mutant was down-regulated. HtrA, also known as DegP or DO protease [32], is a stress-induced serine protease that manifests both general molecular chaperone and proteolytic activities, and switches from chaperone to protease in a temperature-dependent manner [33]. HtrA is also conserved in S. pyogenes and is known to be essential for oxidative tolerance in S. pyogenes [34]. Therefore, we were interested to learn whether the contribution of the
Figure 3 Analysis of the growth of wild-type, spy1236 knockout and complemented strains cultured in acidic media (pH 6.0) in an atmosphere containing 5% CO2. The experiment was performed as described in Figures 1 and 2. At least three independent experiments were performed and they always yielded essentially the same results. The error bars indicate the standard error of the mean. (a) The CFU/ml broth culture of wild-type strain MDYK and its derived strain MDYKΔspy1236 are shown. (b) The CFU/ml culture of MDYKΔspy1236 (pLZ-spy1236) and MDYKΔspy1236 (pLZ12-Km2) grown in a broth supplemented with 62.5 mg/mL kanamycin are shown. Viable counts were performed on BHI-Y agar plates supplemented with 125 mg/mL kanamycin. (c) The CFU/ml broth culture of wild-type strain MDN and its derived strain MDNΔspy1236 are shown. (d) The CFU/ml culture of MDNΔspy1236 (pLZ-spy1236) and MDNΔspy1236 (pLZ12-Km2) grown in a broth supplemented with 62.5 mg/mL kanamycin are shown. Viable counts were performed on BHI-Y agar plates supplemented with 125 mg/mL kanamycin.

Figure 4 H2O2 sensitivity assays for 1529Δspy1236 and MDYKΔspy1236 strains. H2O2 sensitivity assays were performed for wild-type 1529 and 1529Δspy1236 strains (a), 1529Δspy1236 (pLZ-spy1236) and 1529Δspy1236 (pLZ-12-Km2) strains (b), or wild-type MDYK and MDYKΔspy1236 strains (c). 1529Δspy1236 (pLZ-spy1236) and 1529Δspy1236 (pLZ-12-Km2) strains were grown in broth supplemented with 62.5 mg/mL kanamycin. H2O2 (61 mM) was added to 1-mL aliquots of culture grown to an OD660 of ~0.3. After 15 min at room temperature (~22°C), viable counts were performed on BHI-Y and sheep blood agar plates before and after the addition of peroxide, and the percentages of survival were calculated. The BHI-Y agar plates were supplemented with 125 mg/mL kanamycin for the 1529Δspy1236 (pLZ-spy1236) and 1529Δspy1236 (pLZ-12-Km2) strains. Values expressed are the means (± SEM) of three independent experiments.
Spy1236 (CiaHpy) sensor kinase to oxidative stress tolerance was also mediated by the HtrA protein in S. pyogenes (HtrApy). We first attempted to determine whether the sensitivity of the 1529Δspy1236 strain to oxidative stress could be restored by complementation with HtrApy; i.e., to investigate whether the first evidence shown in S. pneumoniae is also true in S. pyogenes. For this purpose, htrApy of S. pyogenes was cloned into pLZ12-Km2 to yield pLZ-htrAforward and pLZ-htrAreverse (Additional file 1: Figure S1). The htrApy gene is cloned into pLZ-htrAreverse in the direction opposite to that in pLZ-htrAforward. When pLZ-htrAforward was introduced into 1529Δspy1236, the sensitivity of the resultant strain 1529Δspy1236 (pLZ-htrAforward) to hydrogen peroxide was not significantly different than that of 1529Δspy1236 (control vector) (Figure 5a). This may have resulted from the insufficient expression of HtrApy by the pLZ-htrAforward contained within 1529Δspy1236. In addition, we hypothesized that level of HtrA expression from pLZ-htrAreverse was greater than that from pLZ-htrAforward (See Additional file 1: Figure S1 for detailed explanation). As shown in Figure 5 (b), 1529Δspy1236 having pLZ-htrAreverse was more resistant to hydrogen peroxide than 1529Δspy1236 having pLZ12-Km2 (control vector). This result suggests that the overexpression of htrA may contribute the oxidative tolerance in that spy1236 mutant.

Next, we attempted to investigate the down-regulation of HtrA in the CiaH-null mutant; i.e., to determine whether the second evidence shown in S. pneumoniae is also true in S. pyogenes. For this purpose, expression of htrApy was measured using qRT-PCR. Surprisingly, expression of htrApy was not decreased in strains 1529Δspy1236 and MDYKΔspy1236, compared with the parental strains 1529 and MDYK (Figure 6a and b). Therefore, we could not conclude that the contribution of the Spy1236 sensor kinase to oxidative stress tolerance was mediated by the HtrA protein in S. pyogenes. This result evoked further questions about what mediates control of oxidative stress tolerance by Spy1236 in S. pyogenes. In addition to HtrA, probably, there are at least two systems (NrdR- and PolA1-dependent, respectively) for oxidative stress tolerance in S. pyogenes. NrdR is a transcription factor first described in Streptomyces coelicolor [35] that regulates the expression of ribonucleotide reductase genes [36]. The ribonucleotide reductase genes are involved in the proliferation of Salmonella Typhimurium inside macrophages [37]. PolA1, a putative DNA polymerase I, has been reported to contribute to peroxide stress defenses in S. pyogenes [38]. Therefore, we measured the expression of nrdR and polA1 (Figure 6a and b), and observed that their expression levels were slightly decreased in strains 1529Δspy1236 and MDYKΔspy1236, compared with the parental strains (Figure 6a and b).

Thus, the contribution of Spy1236 (CiaHpy) sensor kinase to oxidative stress tolerance may not be mediated via HtrA in S. pyogenes (Additional file 2: Figure S2). If this hypothesis is true, what factor mediates the contribution of Spy1236 to the tolerance? The slightly decreased expression of polA1 (and/or nrdR) may be insufficient to

![Figure 5](image_url)
explain why spy1236 mutant that the expression of htrA is increased is lower than the parental wild type in the oxidative tolerance ability. Our next experiments will attempt to identify a Spy1236 regulon.

Conclusions

In this study, we have demonstrated that the CiaH py sensor kinase of *S. pyogenes* is involved in the response to acid and/or oxidative stresses, as are the related sensor kinases in *S. mutans* and/or *S. pneumoniae*. However, an important subject remains to be solved; i.e., it is still unclear how the CiaR/H py two-component regulatory system is involved in the virulence of *S. pyogenes*, whereas the CiaR/H two-component regulatory systems in *S. mutans* and *S. pneumoniae* are already known to be involved in regulating virulence. At least using a mouse infection model, the virulence of Δspy1236 mutants is not significantly different from that of the parental *S. pyogenes* strains (Tatsuno et al., unpublished results). However, the infection model seems to investigate the middle to late, but not the early stages of infection, because more than 10⁷ CFU of bacteria are inoculated into each mouse (10–12 g) [17], and this number is equivalent to >10¹⁰ CFU in a human. Therefore, the CiaH py sensor kinase of *S. pyogenes* may not be important for virulence in the late stage of the infection, whereas it is still possible that CiaH py confers some benefits to *S. pyogenes* in earlier infection stages, as proposed for the CovS sensor kinase in a previous study [17].

The present and previous studies suggested that CO₂ condition is important for the triggering the function of Spy1236, but not of Spy1622 [16]. There are some helpful reports to discuss the potential mechanism about why CO₂ is required for triggering Spy1236 regulatory function [39,40]. M1 and PrtF1/SfbI are both fibronectin binding proteins, which are required for *S. pyogenes* invasion of mammalian cells. PrtF1/SfbI expression is enhanced in an O₂-rich environment, while M1 expression is greater at higher CO₂ partial pressure [20,41]. It has been explained that the differential regulation of these two Fn-binding proteins in high O₂ or high CO₂ may allow *S. pyogenes* to adapt to several different *in vitro* environments, such as those on the skin, on mucosal surfaces, and within the tonsils. When *S. pyogenes* encounters acid stress at the epithelial surface of the host, the bacterium might need the expression of different genes, compared with the genes required when exposed to acid and/or oxidative stresses in deeper tissues. If this hypothesis is true, the Spy1236 regulon should be different from a Spy1622 regulon. Although CovR was already found to influence transcription of 15% of all chromosomal genes using DNA microarrays [42], such analysis has not been adopted for the other two-component systems in *S. pyogenes*. Our next experiments will attempt to identify the Spy1236 and the Spy1622 regulons.

Not only the present study but also previous studies have not ever determine whether CiaH directly senses acidic signal [26,27,30,31]. Although the established method to address this question does not exist as far as we know, if the phosphorylation status or regulatory activity of cognate response regulator, or the expression of Spy1236-regulated genes is demonstrated, they may provide some answers to the question.

Availability of supporting data

There are three supporting data of Additional file 1: Figure S1, Additional file 2: Figure S2, and Additional file 3: Figure S3.

Additional files

**Additional file 1: Figure S1.** Schematic representations of the pLZ-htrAforward and pLZ-htrAreverse plasmids used for overexpression of the htrA gene in *S. pyogenes*.

**Additional file 2: Figure S2.** Hypothetical working model for the response to oxidative stress mediated by the two-component system.
Spy1236-1237. HtrA is regulated by systems other than Spy1236 in S. pyogenes. Dotted arrows indicate hypothetical pathways.

Additional file 3: Figure S3. Expression levels of htrA and polA1 in 1529Δspy1236 (pLZ-htrAforward) relative to those in 1529Δspy1236 (pLZ-htrAforward) evaluated using qRT-PCR. The expression of htrA in 1529Δspy1236 (pLZ-htrAforward) was 1.5 ± (±0.1) times that in 1529Δspy1236 (pLZ-htrAforward), while the expression of polA1 in 1529Δspy1236 (pLZ-htrAforward) was 0.94 ± (±0.08) times that in 1529Δspy1236 (pLZ-htrAforward). Error bars represent the SEM of four experiments.

Abbreviations

BHI-Y: Brain-heart infusion yeast; TH-YE: Todd Hewitt yeast.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

IT conceived the study. IT, RO, and TH designed and performed the experimental work with help by YZ and MI. All authors contributed to the results of a nationwide investigation in Japan. All authors read and approved the final manuscript.

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