Analysis of two-component sensor proteins involved in the response to acid stimuli in *Streptococcus pyogenes*

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The virulence of *Streptococcus pyogenes* depends on proteins that are produced by this bacterium. The production of virulence proteins depends on environmental factors, and two-component regulatory systems are considered to be involved in sensing these factors. One of the environmental factors is acid stimuli. We established knockout strains in all speculated two-component regulatory sensor proteins of the M1 clinical strain of *S. pyogenes* and examined their relevance to acid stimuli. The parental strain and its derived knockout strains were cultured in a medium adjusted to pH 7.6 or 6.0, and their growth in broth was compared. The *spy1622* sensor knockout strain showed significant growth reduction compared with the parental strain in broth at pH 6.0, suggesting that the Spy1622 two-component sensor protein is involved in sensing acid stimuli. To further examine the role of the Spy1622 two-component sensor protein in virulence, blood bactericidal assays and mouse infection model experiments were performed. We found that the *spy1622* knockout strain was less virulent than the parental strain, which suggests that the Spy1622 two-component sensor protein could play an important role in virulence.

INTRODUCTION

Streptococcus pyogenes is a Gram-positive bacterium that infects the upper respiratory tract, for example the tonsils and pharynx, and causes serious post-infectious diseases such as rheumatic fever and glomerulonephritis. Furthermore, *S. pyogenes* causes even more serious human diseases such as streptococcal toxic shock syndrome (STSS), whose mortality rate is considerable (Cone *et al.*, 1987; Cunningham, 2000). Because of the worldwide prevalence of STSS, a number of studies have been undertaken to identify relevant virulence factors (Hauser *et al.*, 1991; Reichardt *et al.*, 1992). These factors include M protein, streptococcal inhibitor of complement, streptococcal pyrogenic toxins, haemolysins, and several DNases (Cunningham, 2000).

The production of virulence proteins depends on environmental factors such as pH. Bacterial growth and the host environment, such as that in phagocytic cells, can cause local and temporal pH changes, and bacteria can subsequently sense the pH change as a signal to modify their growth mode. In addition, bacteria can modulate their

gene expression in response to changes in pH (Loughman & Caparon, 2006). Hence, pH is considered to play an important role in host colonization and infection by *S. pyogenes*.

The regulation of virulence protein expression in *S. pyogenes* is mainly controlled by stand-alone response regulators and two-component regulatory systems (TCSs). Stand-alone transcriptional regulators have no identified sensory domain (Kreikemeyer *et al.*, 2003). The three characterized stand-alone regulators include Mga, Rgg and RofA-like family members (McIver *et al.*, 1995; Beckert *et al.*, 2001; Chaussee *et al.*, 1999; Lyon *et al.*, 1998; Molinari *et al.*, 2001; Podbielski *et al.*, 1999). A TCS consists of a sensor protein and a regulatory protein. Thirteen TCSs have been identified in the available *S. pyogenes* genome sequences from strains of serotypes M1, M2, M3, M4, M5, M6, M12, M18 and M28 (Sumby *et al.*, 2005; Beres *et al.*, 2006, 2002; Nakagawa *et al.*, 2003; Holden *et al.*, 2007; Banks *et al.*, 2004; Smoot *et al.*, 2002; Green *et al.*, 2005; McShan *et al.*, 2008). Some studies have analysed the involvement of TCSs in virulence; however, most experiments were carried out from the viewpoint of the regulators. In this study, we focused on sensor proteins of TCSs, established 13 types of sensor knockout mutants, and analysed their involvement in the acid response and their relevance to virulence using *in vitro* blood bactericidal assays and mouse infection model experiments.

Abbreviations: HK, histidine kinase; STSS, streptococcal toxic shock syndrome; TCS, two-component regulatory system.

Supplementary methods and a supplementary table are available with the online version of this paper.

METHODS

***S. pyogenes* strains.** The *S. pyogenes* strains used in this study were SF370 (Ferretti *et al.*, 2001) and the 1529 clinical isolate from a Japanese patient with STSS. As far as *CovS* is concerned, comparison of the *covS* sequence with that of SF370 indicated that the mutation in 1529 is the same as the one previously reported [994(A→G), relative to the *covS* start site, amino acid change I→V] (Sumby *et al.*, 2005; Hasegawa *et al.*, 2010b).

Culture conditions. An aliquot of bacterial stock solution stored frozen at -80°C was inoculated into 4 ml Todd–Hewitt broth (Becton Dickinson) supplemented with 0.3% yeast extract (Becton Dickinson) (TH-YE) and cultured overnight at 37°C without agitation. A 60–80 μl volume of overnight culture was added to 4 ml fresh TH-YE. The growth of bacteria was then monitored by a colorimeter (Asahi Science) via OD_{660} . After confirmation that the growth of the bacteria was approximately the same, the bacteria were again freshly cultured in TH-YE broth, the pH of which was adjusted to 7.6 or 6.0 for 18 h, and the growth was monitored. We checked the relationship between c.f.u. and optical density in all strains used, and confirmed that the cell numbers estimated by optical density were applicable to a comparison of the maximal growth of each strain. The mean estimated cell number observed from the optical density at pH 7.6 of each strain was defined as 100% growth rate, and the growth rate at pH 6.0 was calculated from the estimated maximal cell numbers at pH 6.0 for each strain. The experiments were carried out at least in duplicate, and repeated at least three times independently.

Creation of the knockout strain. A nonpolar inactivated mutant was constructed through double-crossover allelic replacement in the chromosomes of *S. pyogenes* 1529 and SF370. The method was virtually the same as that by which the *covS* knockout strain was constructed previously (Sawai *et al.*, 2007). The plasmids used for each sensor protein were constructed as described in Supplementary Methods and Supplementary Table S1. All PCRs were carried out with Pyrobest DNA polymerase or PrimeSTAR HS DNA polymerase (Takara), yielding blunt-ended DNA. The *spc1*, *spc2* and *spc3* DNA fragments containing *aad9* (promoterless spectinomycin-resistance gene) were obtained from *SmaI*-digested fragments of pSL60-1, -2 and -3, respectively (Lukomski *et al.*, 2000). All plasmids for knockout were suicide vectors for *S. pyogenes*. For preparation of competent cells, strain 1529 or SF370 was harvested at early to mid-exponential phase (OD_{660} 0.4–0.5) and washed twice with 0.5 M sucrose buffer. The constructed suicide vector was transformed into the strain by electroporation. Conditions of electroporation were 1.25 kV mm^{-1} , 25 μF capacitance and 200 Ω resistance, using a Gene Pulser II (Bio-Rad). After incubation at 37°C for 3 h, competent cells were spread onto brain heart infusion-yeast extract (BHI-YE; Eiken Chemical) agar plates containing spectinomycin (final concentration 100 $\mu\text{g ml}^{-1}$). Selected colonies on the plates were cultured. Cultured bacteria were washed once with saline, resuspended in 10 mM Tris/1 mM EDTA, and boiled for 10 min. Genomic DNA was obtained from the supernatant of boiled bacteria. Double-crossover replacement with genomic DNA was analysed by PCR. Successful double-crossover replacement was further confirmed by DNA sequencing.

To construct a plasmid for *spy1622* complementation, the DNA fragment containing *spy1622* was amplified with oligonucleotide primers 1622-n4Nhe and 1622-c1 (see Supplementary Table S1) with PrimeSTAR HS DNA polymerase. The fragment digested with *RsaI*, which contained all of the coding region of *Spy1622*, was ligated into the *SmaI* site of the pLZ12-Km2 plasmid (Okada *et al.*, 1998). The protocol for transformation was the same as that described above, except that the competent cells were spread onto BHI-YE agar plates

containing spectinomycin and kanamycin (final concentrations 100 and 200 $\mu\text{g ml}^{-1}$, respectively).

Lancefield bactericidal assay. Lancefield bactericidal assays (Loughman & Caparon, 2006; Lancefield, 1957) were performed as described previously (Hasegawa *et al.*, 2010a). In brief, approximately 1000 c.f.u. of cells from the late-exponential phase of growth were added to 1 ml heparinized human whole blood, which had been pre-treated for 15 min with or without 10 μM cytochalasin D (Wako Pure Chemical Industries) (von Delwig *et al.*, 2002) to clarify the mechanism of neutrophil killing, and were rotated at 37°C . In addition, we carried out another experiment in which the strains were incubated in human serum inactivated at 65°C for 30 min before the bactericidal assay. Diluted samples of blood were plated on BHI-YE agar plates at the indicated time points to determine the number of c.f.u. The growth rate was calculated from the number of c.f.u. at a given time point divided by the initial c.f.u. at time zero. The growth rate data at each time point were tested for significance using analysis of variance (ANOVA; Tukey's method). Data are the mean growth ratios from three independent experiments. Error bars depict SEM.

Mouse model of invasive skin and soft tissue infection. The procedure used was the same as that described previously (Hasegawa *et al.*, 2010a; Tatsuno *et al.*, 2010). In brief, parental, mutant and complemented cells of *S. pyogenes* were harvested after 16 h of growth on BHI-YE agar plates. The cells were harvested with appropriate antibiotics into 1 ml PBS ($\times 1$, pH 7.4) and then centrifuged at 2000 g for 2 min. The pellets were diluted to 1×10^8 c.f.u. in 100 μl PBS and then injected (with a 25-gauge needle) under the skin surface of 3 week-old female Slc:ICR mice after they had been anaesthetized by ketamine–xylazine injection. Ten mice of each strain were used for this experiment. The number of c.f.u. injected was verified for each experiment by plating bacteria on BHI-YE agar plates and then counting c.f.u. The mice were observed daily for lesion and necrosis formation. Survival data were assessed by Kaplan–Meier survival analysis and tested for significance by the logrank test. Lesion sizes (length $\times$ width) were measured, with the length determined as the longest dimension of the lesion at day 3 or at the death time point. The data for lesion size were tested for significance using ANOVA (Tukey's method). All animal procedures were approved by the Institutional Animal Care and Use Committee at Nagoya City University.

RESULTS

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

To test the effect of sensor proteins of *S. pyogenes* on growth in acidic pH conditions, we first attempted to establish knockout mutants in all 13 speculated sensor proteins. We successfully established all sensor protein knockout strains derived from strain 1529. We cultured the parental strain and derived knockout strains in medium whose pH was adjusted to 7.6 or 6.0, and the decreases in growth rates were compared. As shown in Fig. 1, the growth of all strains was inhibited at pH 6.0 compared with that at pH 7.6. However, the decrease in the growth rate of *spy1622* (60.8%) was significantly greater than that of the parental strain 1529 (50.6%) (two-way repeated measures ANOVA, $P=0.0024$). There was no statistically significant difference in the extent of decrease in the other two-component sensor knockout strains. However, in the

Fig. 1. Analysis of the growth of sensor knockout strains cultured under acidic conditions. Wild-type strain 1529 and its derived sensor knockout strains were cultured in broth at pH 7.6 or 6.0, and growth was assessed after 18 h. The percentage of growth of each strain cultured at pH 7.6 was defined as 100% and the percentage of growth at pH 6.0 was calculated from this.

case of the *spy0529* knockout, the decrease in growth rate (46.1 %) was less than that of the wild-type strain 1529 (50.6 %) ($P=0.0084$).

In this study, we focused on Spy1622, because this protein seems to play a role in acid resistance though not in acid tolerance. To rule out effects related to the specificity of the strain, we established a *spy1622* knockout strain from strain SF370. Complemented strains derived from knockout strains SF370 and 1529 were also established, and the same experiments were performed. As shown in Fig. 2(a), a significant decrease in the growth of the SF370 *spy1622* knockout after 18 h incubation could be confirmed (56.8 % compared with 41.0 % for the SF370 wild-type) ($P<0.0001$). A smaller decrease in growth in the complemented strain than in the knockout strain was confirmed (SF370 complemented strain 48.3 %) ($P=0.0011$) (Fig. 2a); however, in the case of strain 1529, we detected only a partial effect of complementation (knockout 56.7 %, complemented 54.4 %) (Fig. 2b). In addition, the speed of growth of the wild and knockout strains was similar, and lower growth of the knockout was also obtained after 6 and 24 h incubation (data not shown). Thus these results suggest that the lower growth levels of the *spy1622* mutants were not due to slower overall growth, and that the effect is not strain-dependent but Spy1622-dependent.

In vitro blood bactericidal assay

Acid conditions play an important role in the destruction of bacteria by phagocytosis. To determine whether Spy1622 affected the bactericidal function of human

leukocytes, we performed bactericidal assays with human blood. For this purpose, we used the parental strain 1529, its derived *spy1622* knockout mutant strain, and a *spy1622*-complemented strain. Fig. 3(a) shows that the growth rate (y axis) of the *spy1622* knockout was less than that of the parental strain after 30, 60, 90 and 120 min ($P<0.05$ at 120 min). The growth rate of the complemented strain was similar to that of the parental strain. We also performed a bactericidal assay in which neutrophil phagocytosis was inhibited by cytochalasin D to see whether the killing was extracellular or intracellular. As shown in Fig. 3(b), all the growth rates were almost the same, suggesting that the neutrophil killing was intracellular. Another experiment in which the strains were incubated in plasma was carried out, but there was no significant survival difference among the strains (data not shown). These results imply that the *spy1622* knockout strain is destroyed intracellularly more efficiently by human blood cells than the parental strain.

Analysis of virulence in a mouse infection model

To examine virulence, we performed mouse infection model experiments using the parental M1 serotype 1529 and knockout strains. As shown in Fig. 4(a), mice were injected subcutaneously with the parental, *spy1622* knockout and complemented strains, and it was found that the mice injected with the knockout strain survived longer than those injected with the parental strain ($P=0.0104$). The complemented strain slightly rescued survival, although there was no significant difference. The local skin lesions were smaller in mice injected with the

Fig. 2. Analysis of the growth of wild-type, *spy1622* knockout and complemented strains cultured under acidic conditions. Wild-type strains SF370 (a) and strain 1529 (b), and their derived *spy1622* knockout and complemented (comp) strains, were cultured in broth at pH 7.6 or 6.0, and growth was assessed after 18 h. The percentage of growth of each strain cultured at pH 7.6 was defined as 100% and the percentage of growth at pH 6.0 was calculated from this.

knockout strain ($P < 0.01$). Furthermore, the local skin lesions caused by the complemented strain were significantly larger than those of the knockout strain ($P < 0.01$) (Fig. 4b). These results suggest that the Spy1622 sensor protein plays an important role in virulence in mouse infection model experiments.

DISCUSSION

S. pyogenes produces many types of protein, including virulence proteins, in response to environmental stresses. By using 2D gel electrophoresis (2-DE) and mass analysis, we have so far identified several DNases (Hasegawa *et al.*, 2002a, b, 2010a). We have also studied the influence of various culture conditions on the production of these exoproteins (Nakamura *et al.*, 2004; Tanaka *et al.*, 2005; Sawai *et al.*, 2007; Minami *et al.*, 2010). One of the mechanisms by which protein expression is controlled depends on TCSs; in particular, sensor proteins sense alterations on the outer surface of cells. In this study, we examined whether any sensor proteins (histidine kinases; HKs) of TCSs could play a role in response to acid stimuli, because acid stimuli are considered to play an important role in virulence. One of the defence mechanisms of the

host against bacteria, including *S. pyogenes*, is phagocytosis. Phagosome maturation can be viewed as the end of a phagocytic process during which internalized bacteria are efficiently degraded. Acidification is essential during phagosome maturation. If the phagosome does not reach a sufficiently low pH, the lytic activity of a variety of degradative enzymes is impaired and the generation of hydrogen peroxide is not promoted, and thus the process of degradation of phagocytosed bacteria cannot begin (Kinchin & Ravichandran, 2008; Huynh & Grinstein, 2007). *Mycobacterium tuberculosis* has been well studied in this regard. Acid resistance strategies in the phagosome that favour survival have been demonstrated (Ehrt & Schnappinger, 2009; Vandal *et al.*, 2009). Other than the involvement of phagocytosis, a study using *S. pyogenes* has shown that acidification resulting from fermentative sugar metabolism drives pilus expression, and the ability to form biofilms on abiotic surfaces and microcolonies on epithelial cells (Manetti *et al.*, 2010). Thus, the acid response is considered to be closely related to the virulence of *S. pyogenes*.

Although Sitkiewicz *et al.* (2010) have carried out transcriptome analysis to study the response of *S. pyogenes* to amniotic fluid and have shown that *spy1622* expression

Fig. 3. *In vitro* blood bactericidal assay. The wild-type strain 1529, a *spy1622* knockout strain or a *spy1622*-complemented (comp) strain (100 μ l) was mixed with human blood (200 μ l) without (a) or with (b) cytochalasin D, and was incubated at 37 °C for 30 min to yield a final concentration of 10^5 c.f.u. ml⁻¹. Aliquots were removed and plated on BHI-YE agar plates for quantification. The growth rate equals the number of c.f.u. at a given time point divided by the initial c.f.u. at time zero. Data represent mean values from three independent assays.

is increased, the functions of Spy1622 and what Spy1622 senses are not completely known. According to the DNA database, *spy1622* of *S. pyogenes* is designated *yvqE*. The *yvqE* gene is found in other bacteria such as *Bacillus subtilis* (Kunst *et al.*, 1997; Fabret *et al.*, 1999), *Enterococcus faecalis* (Hancock & Perego, 2002), *Streptococcus dysgalactiae* subsp. *equisimilis* and *Streptococcus equi* subsp. *zooepidemicus*. Mascher and co-workers renamed YvqEC as LiaRS (Mascher *et al.*, 2004), and showed that LiaRS is one of the three TCSs that respond to cell envelope stresses induced by cell wall-specific antibiotics such as bacitracin (Mascher *et al.*, 2003). Thus, Spy1622 is considered to be a homologue of the YvqE or LiaS sensor protein. LiaS-like intramembrane-sensing HKs are found in many bacteria (Mascher, 2006). A LiaRS homologue, the VraSR system of *Staphylococcus aureus*, has also been reported to contribute to antibiotic resistance in clinical isolates of *Staph. aureus* (Boyle-Vavra *et al.*, 2006; Gardete *et al.*, 2006; Kuroda *et al.*, 2003, 2000). Other than the cell wall stress responses, LiaS (formerly designated HK11) has been found to play a role in acid resistance; it may act as a pH sensor (Li *et al.*, 2002) and regulate virulence factor expression in *Streptococcus mutans* (Chong *et al.*, 2008). In addition, LiaS of *S. mutans* has also been shown to be involved in tolerance to various inhibitors that target protein synthesis, DNA synthesis and cell wall biosynthesis (Zhang & Biswas, 2009). The results of studies on *S. mutans* are in accordance with our data and may support our results, thus indicating that Spy1622 is involved in the response to acid stimuli.

Dalton & Scott (2004) have reported that the growth of a CovS knockout mutant strain derived from M6 serotype strain JRS4 is impaired at low pH. In addition, Santi *et al.* (2009) have reported that a CovRS homologue in *Streptococcus agalactiae* is involved in the pH-dependent regulation of gene expression. In this study, we did not identify a significant role for CovS but showed that Spy1622 could be involved in the response to acid stimuli. The reason why we could not identify clear growth differences between wild-type and *covS* knockout strains in our study remains unknown. Treviño *et al.* (2009) have stated that they also observed no difference in growth for several wild-type and *covS* mutant pairs grown under general stress conditions such as low pH. One possibility is the existence of differences between strains, a second possibility is that strain 1529 already has one amino acid mutation in CovS, and a third possibility is the existence of differences in the methods used to assess bacterial growth. We cannot completely rule out the possibility that CovS is involved in acid sensing in our experimental design. We believe further experiments are necessary to confirm the involvement of CovS.

In addition, our comprehensive sensor knockout analysis showed that Spy0529 (VicK) may also be involved in the response to acid stimuli. The involvement of the VicK sensor kinase of *S. mutans* in both acidogenicity and aciduricity has been observed, while an acid tolerance response assay has revealed that loss of the VicK sensor kinase significantly enhances the survival of *S. mutans*

Fig. 4. Mouse infection model experiments. (a) Survival after subcutaneous injection with wild-type strain 1529, the *spy1622* knockout mutant and the complemented strain (comp). Slc:ICR mice were injected subcutaneously with parental strain 1529, knockout and complemented strains. Mice were observed daily. Survival data were assessed by Kaplan–Meier survival analysis and tested for significance by the logrank test. (b) Analysis of lesions after subcutaneous challenge. Mice were observed daily for lesion and necrosis formation. Lesion size (length×width) was measured, with the length determined as the longest dimension of the lesion at day 3 or at the death time point. The data for lesion size were tested for significance using ANOVA (Tukey's method).

(Senadheera *et al.*, 2009). Walkmycin C (WKM C), an HK inhibitor isolated during the screening of inhibitors of WalK HK in *B. subtilis*, inhibits the *in vitro* autophosphorylation activity of the three *S. mutans* HKs, i.e. VicK, CiaH and LiaS. When cells are pretreated with WKM C, an increase in acid sensitivity is observed (Eguchi *et al.*, 2011). Thus, VicK of *S. pyogenes* could also be involved in the response to acid stimuli.

In conclusion, our results suggest that Spy1622 is involved in the response to acid stimuli, and thus plays an important role in the virulence of *S. pyogenes*. Further studies to examine the interaction of Spy1622 with other two-component sensor proteins, including VicK and CovS, will be necessary to reveal the details of the response to acid and its relationship with the virulence of *S. pyogenes*, and to

assess whether these proteins could be targets in the control of infectious streptococcal diseases.

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