Highly Frequent Mutations in Negative Regulators of Multiple Virulence Genes in Group A Streptococcal Toxic Shock Syndrome Isolates

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Abstract

Streptococcal toxic shock syndrome (STSS) is a severe invasive infection characterized by the sudden onset of shock and multiorgan failure; it has a high mortality rate. Although a number of studies have attempted to determine the crucial factors behind the onset of STSS, the responsible genes in group A Streptococcus have not been clarified. We previously reported that mutations in csrS/csrR genes, a two-component negative regulator system for multiple virulence genes of Streptococcus pyogenes, are found among the isolates from STSS patients. In the present study, mutations of another negative regulator, rgg, were also found in clinical isolates of STSS patients. The rgg mutants from STSS clinical isolates enhanced lethality and impaired various organs in the mouse models, similar to the csrS mutants, and precluded their being killed by human neutrophils, mainly due to an overproduction of SLO. When we assessed the mutation frequency of csrS, csrR, and rgg genes among S. pyogenes isolates from STSS (164 isolates) and non-invasive infections (59 isolates), 57.3% of the STSS isolates had mutations of one or more genes among these genes, while isolates from patients with non-invasive disease had significantly fewer mutations in these genes (1.7%). The results of the present study suggest that mutations in the negative regulators csrS/csrR and rgg of S. pyogenes are crucial factors in the pathogenesis of STSS, as they lead to the overproduction of multiple virulence factors.

Introduction

Streptococcus pyogenes (group A Streptococcus; GAS) is one of the most common human pathogens. It causes a wide variety of infections, ranging from uncomplicated pharyngitis and skin infections to severe and even life-threatening manifestations, such as necrotizing fasciitis (NF) and bacteremia. Several streptococcal virulence factors, including pyrogenic exotoxins, streptokinase, and streptolysins, are reportedly involved in these diseases. Streptococcal toxic shock syndrome (STSS) is a severe invasive infection that has been recently characterized by the sudden onset of shock and multiorgan failure; it has a high mortality rate, ranging from 30% to 70% [1]. There is controversy as to whether the cause of STSS largely depends on host factors or bacterial factors. Although many studies have sought to determine the crucial factors behind the onset of STSS, the responsible GAS genes have not been clarified.

Recently, we and others have reported that mutations in the csrS (covS) gene—a sensor gene of a two-component regulatory system—were detected in a panel of clinical isolates from severe invasive streptococcal infections, but not in non-STSS isolates [2–4]. Mutations in the gene caused an increased expression of various virulence genes; the upregulation of streptolysin O (SLO) induced necrosis of neutrophils and prompted the escape of covS mutated strains from being killed by neutrophils, resulting in increased virulence in lethality in the mouse model [2]. Complementation of the wild csrS gene into csrS-mutated STSS isolates dramatically decreased their virulence in lethality [2]. Similarly, csrR (covR) mutations were found in the clinical isolates of STSS patients [5]. Such results suggest that csrS/csrR mutations are closely associated with the onset of STSS.

However, several study groups that investigated the csrS/csrR gene sequence in each STSS isolate [3,4,6,7] also report that there is no mutation in the csrS/csrR gene of STSS isolates [4]. These results raise questions as to how frequently STSS isolates have mutations in the csrS/csrR genes in a mass of isolates, and what mutations other than csrS/csrR genes may be responsible for the onset of STSS.

In this study, we sequenced the csrS/covR genes of 164 GAS strains that have been isolated from STSS patients in Japan since 1992. Almost one-half of the STSS isolates had a mutation in the csrS/covR genes. In addition, we found a mutation in the rgg (topB) gene, instead of the csrS/covR genes, in the clinical isolates of patients with STSS. Since the rgg gene negatively regulates various virulence genes in a manner similar to that of the csrS gene, a mutation of the rgg gene in STSS clinical isolates increased the...
Highly Frequent Mutations in STSS Isolates

Author Summary

Group A streptococcus (GAS) causes life-threatening severe invasive diseases, including necrotizing fasciitis and streptococcal toxic shock-like syndrome. Although many studies have attempted to determine factors that are crucial for the onset of streptococcal toxic shock syndrome (STSS), bacterial factors responsible for it have not been clarified. By comparing genome sequences of clinical GAS isolates from STSS with those of non-invasive infections, we showed that mutations of negative regulator genes (csrS, csrR, rgg) were detected at a high frequency of more than 50% in STSS isolates, but at a low frequency of less than 2% in non-invasive isolates. These mutations of negative regulators were found in various emm-genotyped STSS isolates but not in a particular emm genotype. These mutants enhanced virulence in mouse models. Such results indicated that mutations of bacterial negative regulators are crucial for the pathogenesis of STSS due to the overproduction of multiple virulence factors under the de-repressed conditions.

expression of several virulence genes and virulence in lethality in the mouse model. Such mutations were detected at a high frequency in more than 50% of STSS isolates. These findings suggest that mutations in the negative regulators such as csrS or rgg of S. pyogenes bring about overproduction of a number of virulence factors, such as SLO, and play a crucial role in the onset of STSS.

Results

Mutation frequency of the csrS/csrR gene in STSS isolates

In our previous study, we reported that there were various types of mutations in the csrS gene of emm49 clinical isolates from STSS patients [2] and in the csrR gene in emm3 clinical isolates from STSS patients [5]. These findings strongly suggest that csrS/csrR mutations play important roles in the pathogenesis of STSS. To evaluate the frequency of these csrS/csrR mutations in isolates from clinical cases of STSS [8], we sequenced the csrS and csrR genes in STSS clinical isolates from sterile sites (164 isolates) and non-STSS clinical isolates from non-sterile sites (59 isolates). The diagnoses, sites of bacteria isolation, and characteristics of S. pyogenes isolates are described in Table 1. Of the 164 STSS clinical S. pyogenes isolates, 55 isolates (csrS, 46 isolates; csrS + rgg, 9 isolates) (33.5%) had mutations in the csrS gene, 19 isolates (csrR, 13 isolates; csrR + rgg, 6 isolates) (11.6%) had mutations in the csrR gene, and 2 isolates (1.2%) had mutations in both genes (Tables 1 and 2). The csrS and csrR genes of these isolates had deletions, point mutations, or insertions that created non-functional CsrS and CsrR products, as shown previously [2,4,5]. Therefore, 76 isolates (46.3%) had mutations in the csrS and/or csrR genes, while the remaining 88 STSS isolates (53.7%) had mutations in neither csrS nor csrR (Tables 1 and 2). On the other hand, non-STSS GAS isolates had a significantly lower number of mutations in the genes [csrS mutation, 1.69% (1/59); csrR mutation, 0% (0/59); total, 1.69% (1/59); p = 0.00000000062 by χ² analysis]. Although csrS/csrR mutations were more common among STSS isolates examined than among non-STSS isolates, they were not present in all STSS isolates. This may suggest that mutations in other regulatory genes may also be found among STSS isolates.

rgg or csrS mutations in STSS clinical isolates

To identify novel bacterial factors that may contribute to the pathogenesis of STSS, we next investigated the expression pattern of virulence factors in S. pyogenes isolates. We determined the sequence of the csrS/csrR genes from a panel of emm-matched GAS isolated from STSS patients; NIH1 (also called SSI-1), NIH3, NIH8, NIH34, NIH152-3, NIH249, and NIH352 were clinical isolates of the emm3 genotype from STSS and C500, OT22, and K33 were emm3 non-STSS isolates (Tables 1 and S1). A mutation in the csrS gene was found in NIH152-3, NIH249, NIH152, and NIH332 of the STSS isolates; however, the other STSS and non-STSS GAS isolates had mutations in neither the csrS nor the csrR gene (data not shown). To determine whether other emm3 STSS strains have possible mutations in their genomes, we used comparative genome sequencing (CGS) [9], a microarray hybridization-based method developed to search for single-nucleotide polymorphisms (SNPs) and insertion-deletion sites within a genome between emm3 STSS and non-STSS isolates. We found several genes with SNPs in the NIH1 genome in comparison with that of non-invasive isolates K33. Three (csdR, csrR and rgg) of them had non-synonymous amino acid change in NIH1 but not in K33 and C500 (Table S2). We further sequenced these 3 genes in other non-invasive isolate, OT22 and STSS isolates, NIH3, NIH8 and NIH34. A couple of genetic differences which affect amino acid sequence were detected between the STSS and non-STSS GAS isolates (Table 3).

Increased SLO expression in STSS isolates with csrS or rgg mutations

We [2] and others [4] have previously reported that STSS emm49 and emm1 clinical isolates exhibit a higher expression of the SLO gene (slo) than non-STSS isolates, due to a mutation in the csrS gene. Therefore, we examined whether a panel of emm3-genotyped STSS isolates possessing mutations in the csrS or rgg gene and emm3 non-STSS isolates lacking mutations could produce SLO (i.e., secretion of SLO in the culture supernatant). The comparison of the supernatants at the same growth condition (data not shown) showed that larger amounts of SLO were secreted by STSS isolates possessing mutations in the csrS gene (NIH152, NIH249, NIH152, and NIH352) or rgg gene (NIH1, NIH3, NIH8, and NIH34) had some difference in SP1742 (Rgg but not in non-STSS isolates C500, OT22, and K33) (Table 3). SP1742 is identified as the rgg gene, a transcriptional regulator of multiple genes [10–13], although the role of the rgg gene is controversial [14].

Enhanced expression of various virulence genes in STSS isolates is attributed to mutation of the rgg gene

It has been reported that Rgg influences the transcription of many virulence-associated genes in S. pyogenes [10–13]. To test the possibility that the transcriptional expression levels of virulence genes are regulated by the function of the rgg gene, we performed...
### Table 1. Clinical isolates used in this study.

| Diagnosis | NIH No. Strain name | Site of bacterial isolation | emm type | csrR | csrS | rgg | Increased SLO, production | Csrs/CsrR and Rgg amino acid sequence alterations | Accession No. | Reference |
|-----------|----------------------|-----------------------------|----------|------|------|----|--------------------------|-----------------------------------------------|--------------|-----------|
| STSS | NIH136 | blood | 1 | mut | + | + | + | CsrR, Arg→Ser at aa 119 | CsrS, delete Glu at aa 252, and Leu→Val at aa 253 | CsrS, NIH136-1 | This study |
| | NIH447 | blood | 1 | mut | + | + | + | CsrR, Asp→Glu at aa 53 | CsrS, NIH447 | This study |
| | NIH73 | blood | 1 | + | mut | + | + | CsrS, 5 bp delete = stop at aa 407 | CsrS, NIH73 | This study |
| | NIH4 | blood | 1 | + | mut | + | + | CsrS, 5 bp delete = stop at aa 407 | CsrS, NIH4 | This study |
| | NIH102 | ascites | 1 | + | mut | + | + | CsrS, 1 bp delete = stop at aa 76 | CsrS, NIH102 | This study |
| | NIH205 | soft tissue | 1 | + | mut | + | + | CsrS, Gln→Arg at aa 388 | CsrS, NIH205 | This study |
| | NIH200-2 | blood | 1 | + | mut | + | + | CsrS, 1 bp delete = stop at aa 35 | CsrS, NIH200-2 | This study |
| | NIH213-3 | blood | 1 | + | mut | + | + | CsrS, NIH213-3 | This study |
| | NIH220-1 | blood | 1 | + | mut | + | + | CsrS, Gly→Val at aa 457 | CsrS, NIH220-1 | This study |
| | NIH222 | soft tissue | 1 | + | mut | + | + | CsrS, Ala→Ser at aa 206 | CsrS, NIH222 | This study |
| | NIH235 | blood | 1 | + | mut | + | + | CsrS, NIH235 | This study |
| | NIH243-1 | blood | 1 | + | mut | + | + | CsrS, NIH243-1 | This study |
| | NIH253-1 | blood | 1 | + | mut | + | + | CsrS, NIH253-1 | This study |
| | NIH286 | blood | 1 | + | mut | + | + | CsrS, NIH286 | This study |
| | NIH314 | rubor site | 1 | + | mut | + | + | CsrS, NIH314 | This study |
| | NIH390 | soft tissue | 1 | + | mut | + | + | CsrS, NIH390 | This study |
| | NIH409 | blood | 1 | + | mut | + | + | CsrS, NIH409 | This study |
| | NIH445 | blood | 1 | + | mut | + | + | CsrS, NIH445 | This study |
| | NIH75 | blood | 1 | mut | mut | + | + | CsrR, NIH75 | This study |
| | NIH381-1 | wound | 1 | + | mut | + | + | CsrS, NIH381-1 | This study |
| | NIH366 | blood | 1 | + | mut | mut | + | CsrS, NIH366 | This study |
| | NIH117 | blood | 1 | + | + | + | - | WT sequence | CsrS, NIH117 | This study |
| | NIH68 | blood | 1 | + | + | + | - | WT sequence | CsrS, NIH68 | This study |
| | NIH9 | blood | 1 | + | + | + | - | WT sequence | CsrS, NIH9 | This study |
| | NIH111 | blood | 1 | + | + | + | - | WT sequence | CsrS, NIH111 | This study |
| | NIH35 | soft tissue | 1 | + | + | + | - | WT sequence | CsrS, NIH35 | This study |
| | NIH50 | Joint fluid | 1 | + | + | + | - | WT sequence | CsrS, NIH50 | This study |
| | NIH53 | wound | 1 | + | + | + | - | WT sequence | CsrS, NIH53 | This study |
| | NIH165-1 | blood | 1 | + | + | + | - | WT sequence | CsrS, NIH165-1 | This study |
| | NIH185 | blood | 1 | + | + | + | - | WT sequence | CsrS, NIH185 | This study |
| | NIH187 | blood | 1 | + | + | + | - | WT sequence | CsrS, NIH187 | This study |
| | NIH195 | amniotic fluid | 1 | + | + | + | - | WT sequence | CsrS, NIH195 | This study |
| | NIH201 | Joint fluid | 1 | + | + | + | - | WT sequence | CsrS, NIH201 | This study |
| | NIH204-1 | muscle | 1 | + | + | + | - | WT sequence | CsrS, NIH204-1 | This study |
| | NIH214 | blood | 1 | + | + | + | - | WT sequence | CsrS, NIH214 | This study |
### Table 1. Cont.

| Diagnosis | NIH No. Strain name | Site of bacterial isolation | emm type | csrR | csrS | rgg | Increased SLO, production | CsrR/CsrS and Rgg amino acid sequence alterations | Accession No. | Reference |
|-----------|---------------------|-----------------------------|----------|------|------|-----|--------------------------|-----------------------------------------------|--------------|-----------|
| NIH223    | soft tissue         | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH224    | effusion            | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH225    | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               | [33]        |           |
| NIH242    | soft tissue         | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | [36]      |
| NIH270    | pleural effusion    | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH261    | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH291-1  | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH298    | soft tissue         | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH304    | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH315    | fluid               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH320    | soft tissue         | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH324-2  | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH342    | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH338    | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH344-1  | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH354    | fascia              | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH361    | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH363    | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH380-2  | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH392    | serum               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH388-2  | ascites             | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH395-1  | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH399-1  | pleural effusion    | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH413    | soft tissue         | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH415    | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH417-3  | blood               | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH418    | soft tissue         | 1                           | +        | +    | +    | +   | WT sequence              |                                               |              | This study |
| NIH436    | soft tissue         | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH444    | soft tissue         | 1                           | +        | +    | +    | -   | WT sequence              |                                               |              | This study |
| NIH9      | blood               | 3                           | mut      | +    | +    | +   | CsrR, Gln→Pro at aa 216  | CsrR, AB219966                                 | [33]        |           |
| NIH212    | soft tissue         | 3                           | mut      | +    | +    | +   | CsrR, Asp→Tyr at aa 60   | CsrR, AB517826                                 |              | This study |
| NIH216    | fascia              | 3                           | mut      | +    | +    | +   | CsrR, Trp→Cys at aa 184  | CsrR, AB517827                                 |              | This study |
| NIH259    | blood               | 3                           | mut      | +    | +    | +   | CsrR point mutation = stop at aa 45 | CsrR, AB517839                                 |              | This study |
| NIH300    | blood               | 3                           | mut      | +    | +    | +   | CsrR, Arg→Leu at aa 119  | CsrR, AB517850                                 |              | This study |
| NIH404    | soft tissue         | 3                           | mut      | +    | +    | +   | CsrR, 1 bp insert = stop at aa 146 | CsrR, AB517867                                 |              | This study |
| TK800     | blood               | 3                           | mut      | +    | +    | +   | CsrS point mutation = stop at aa 131 | CsrS, AB517803                                 |              | This study |
| NIH152-3  | blood               | 3                           | mut      | +    | +    | +   | CsrS point mutation = stop at aa 160 | CsrS, AB517820                                 | [37]        |           |
| NIH249    | blood               | 3                           | mut      | +    | +    | +   | CsrS, 1 bp delete = stop at aa 35 | CsrS, same as NIH156-1                          |              | This study |
| NIH424-1  | blood               | 3                           | mut      | +    | +    | +   | CsrS, 11 bp insert = stop at aa 39 | CsrS, AB517873                                 |              | This study |
| NIH453    | effusion            | 3                           | mut      | +    | +    | +   | CsrS, 1 bp delete = stop at aa 180 | CsrS, AB517875                                 |              | This study |
| NIH3      | blood               | 3                           | +        | +    | mut  | +   | Rgg, Tyr→Cys at aa 31    | Rgg, AB17795                                   | [35]        |           |
| NIH8      | blood               | 3                           | +        | +    | mut  | +   | Rgg, Ile→Phe at aa 162   | Rgg, AB17798                                   | [35]        |           |
| TK3       | soft tissue         | 3                           | +        | +    | mut  | +   | Rgg, Tyr→Cys at aa 31    | Rgg, same as NIH3                               |              | This study |
| TK64      | fascia              | 3                           | +        | +    | mut  | +   | Rgg, Ile→Phe at aa 162   | Rgg, same as NIH8                               |              | This study |
| NIH34     | blood               | 3                           | +        | +    | mut  | +   | Rgg, Ile→Phe at aa 162   | Rgg, same as NIH8                               | [35]        |           |
| TK1153    | blood               | 3                           | +        | +    | mut  | +   | Rgg, Ile→Phe at aa 162   | Rgg, same as NIH8                               | [35]        |           |
| NIH357    | soft tissue         | 3                           | +        | +    | mut  | +   | Rgg, Phe→Tyr at aa 161   | Rgg, AB17856                                   |              | This study |
| NIH1      | fascia              | 3                           | mut      | +    | mut  | +   | CsrR, Arg→Cys at aa 118; Rgg, Tyr→Cys at aa 31 | BA000034                                     | [35]        |           |
| Diagnosis | NIH No. | Strain name | Site of bacterial isolation | emm type | csrR | csrS | rgg |Increased SLO, production| CsrS/CsrR and Rgg amino acid sequence alterations | Accession No. | Reference |
|-----------|---------|-------------|-----------------------------|----------|------|------|-----|----------------------|-----------------------------------------------|---------------|----------|
| NIH307 | wound | 12 | + + + + - | WT sequence | This study |
| NIH432 | Joint fluid | 4 | + + + + - | WT sequence | This study |
| NIH296 | blood | 6 | + + + + | WT sequence | This study |
| NIH323-1 | lung | 11 | + + + + | WT sequence | This study |
| NIH49 | soft tissue | 11 | + + + + | WT sequence | This study |
| NIH325-1 | blood | 11 | + + + + | WT sequence | This study |
| NIH50 | blood | 12 | + + + + - | Rgg, Glu →Asp at aa 89; Rgg, Glu →Asp at aa 89 | Rgg, AB517811 | This study |
| NIH61 | soft tissue | 12 | + + + + | WT sequence | This study |
| NIH109 | Joint fluid | 12 | + + + + - | Rgg, Glu →Asp at aa 89; Rgg, Glu →Asp at aa 89 | Rgg, AB517811 | This study |
| NIH120 | soft tissue | 12 | + + + + - | Rgg, Glu →Asp at aa 89; Rgg, Glu →Asp at aa 89 | Rgg, AB517811 | This study |
| NIH277 | blood | 12 | + + + + - | Rgg, Glu →Asp at aa 89; Rgg, Glu →Asp at aa 89 | Rgg, AB517811 | This study |
| NIH383 | blood | 12 | + + + + | WT sequence | This study |
| NIH391 | blood | 12 | + + + + - | Rgg, Glu →Asp at aa 89; Rgg, Glu →Asp at aa 89 | Rgg, AB517811 | This study |
| NIH398-2 | blood | 12 | + + + + | WT sequence | This study |
| NIH419 | soft tissue | 12 | + + + + - | Rgg, Glu →Asp at aa 89; Rgg, Glu →Asp at aa 89 | Rgg, AB517811 | This study |
| NIH263-2 | blood | 12 | + + + + | WT sequence | This study |
| NIH43 | effusion | 18 | + + + + | CsrR, Ser→Pro at aa 154; CsrR, Cys→Arg at aa 227 | Rgg, AB517807; Rgg, AB517808 | This study |
| TK76 | soft tissue | 22 | + + + + | CsrR, 5 bp delete = stop at aa 407 | Rgg, AB517800 | This study |
| NIH160 | blood | 22 | + + + + | CsrR, 1 bp delete = stop at aa 35 | CsrR, AB517810; CsrR, AB517810 | This study |
| NIH172 | blood | 22 | + + + + | CsrR, 1 bp delete = stop at aa 35 | CsrR, AB517810; CsrR, AB517810 | This study |
| NIH403 | blood | 22 | + + + + | CsrR, point mutation = stop at aa 369 CsrR, AB517802 | CsrR, AB517802 | This study |
| NIH236 | blood | 22 | + + + + | CsrR, Change TTTTT to GAGG = stop at aa158; Rgg, Phe→Leu at aa 150 | CsrR, AB517831; Rgg, AB517832 | This study |
| NIH98 | blood | 22 | + + + + - | WT sequence | This study |
| NIH229 | blood | 22 | + + + + - | WT sequence | This study |
| NIH35 | blood | 28 | + + + + - | WT sequence | This study |
| NIH40 | blood | 28 | + + + + | WT sequence | This study |
| NIH400 | Joint fluid | 28 | + + + + | WT sequence | This study |
| NIH440 | soft tissue | 28 | + + + + | WT sequence | This study |
| NIH423-1 | blood | 28 | + + + + | WT sequence | This study |
| NIH142-5 | blood | 28 | + + + + | WT sequence | This study |
| NIH316 | soft tissue | 28 | + + + + - | WT sequence | This study |
| NIH204-4 | blood | 49 | + + + + | WT sequence | This study |
| NIH230 | blood | 49 | + + + + | WT sequence | This study |
| NIH269 | soft tissue | 49 | + + + + | WT sequence | This study |

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| NIH No. | Strain name | Site of bacterial isolation | emm type | csrR | csrS | rgg | Increased SLO, production | CsrR/CsrS and Rgg amino acid sequence alterations | Accession No. | Reference |
|---------|-------------|-----------------------------|----------|------|------|-----|--------------------------|-----------------------------------------------|---------------|-----------|
| NIH346 | blood       | 49  +  +  +  -             | WT sequence | This study |
| NIH410 | soft tissue | 49  +  +  +  -             | WT sequence | This study |
| NIH389 | soft tissue | 53  +  mut  +  +       | CsrS, 11 bp insert = stop at aa 39 | CsrS, same as NIH250-2 | This study |
| TX65   | fascia      | 58  +  +  mut  +   | Rgg, Cys→Phe at aa 85; | Rgg, AB517799 | This study |
| NIH273 | blood       | 58  mut  +  +  +     | CsrR, Gly→Ser at aa 95; | CsrR, AB517842; Rgg, AB517843 | This study |
| NIH701 | blood       | 60  +  mut  +  +     | CsrS, point mutation = stop at aa 282 | CsrS, AB517852 | This study |
| NIH929 | soft tissue | 77  +  mut  +  +   | CsrS, Thr→Ile at aa 266 | CsrS, AB517849 | This study |
| NIH258 | soft tissue | 78  +  +  +  -     | WT sequence | This study |
| TK929  | blood       | 81  mut  +  +  +    | CsrR, Arg→Ser at aa 118 | CsrR, AB517804 | This study |
| NIH156-1 | blood    | 81  +  mut  +  +  | CsrS, 1 bp delete = stop at aa 35 | CsrS, AB517821 | This study |
| NIH268 | soft tissue | 81  +  mut  +  +  | CsrS, Arg→Cys at aa 241 | CsrS, AB517841 | This study |
| NIH1101 | soft tissue | 81  +  +  +  -   | WT sequence | This study |
| NIH283-1 | blood     | 87  +  mut  +  +  | CsrS, Pro→Leu at aa 16 | CsrS, AB517844 | This study |
| NIH437 | blood       | 87  +  mut  +  +     | WT sequence | CsrS, AB517862 | This study |
| NIH371 | blood       | 87  +  mut  mut  + | CsrS, 5 bp delete = stop at aa 407; | CsrS, AB517858 | This study |
| NIH372 | blood       | 87  +  mut  mut  + | CsrS, point mutation = stop at aa 193; | Rgg, Ala→Thr at aa 245 | CsrS, AB517859; Rgg, AB517860 | This study |
| NIH157 | blood       | 89  mut  +  +  +     | CsrR, Asp→Tyr at aa 10 | CsrR, AB517822 | This study |
| NIH5   | blood       | 89  +  mut  +  +     | CsrS, 5 bp insert = stop at aa 459 | CsrS, AB517796 | [35] |
| NIH58  | Joint fluid | 89  +  mut  +  +   | CsrS, Val→Ala at aa 423 | CsrS, AB517812 | This study |
| NIH238 | soft tissue | 89  +  mut  +  +     | CsrS, Ser→Arg at aa 204 | CsrS, AB517833 | This study |
| NIH421 | blood       | 89  +  mut  +  +     | CsrS, Arg→Cys at aa 229 | CsrS, AB517871 | This study |
| NIH118 | blood       | 89  +  +  mut  +   | Rgg, Asp→Tyr at aa 174 | Rgg, AB51818 | This study |
| NIH345 | wound       | 89  mut  +  mut  + | CsrR, Arg→Cys at aa 94; | CsrR, 1 bp delete = stop at aa 35 | CsrS, same as NIH252-2; CsrS, AB517855 | This study |
| NIH250-2 | blood     | 89  +  mut  mut  + | CsrS, 11 bp insert = stop at aa 39; | Rgg, Tyr→His at aa 135 | CsrS, AB517836; Rgg, AB517837 | This study |
| NIH208 | blood       | 89  +  +  +  -     | WT sequence | This study |
| NIH256 | blood       | 89  +  +  +  -     | WT sequence | This study |
| NIH252-2 | muscle    | 91  mut  +  +  +   | CsrR, Arg→Cys at aa 94 | CsrR, AB517838 | This study |
| NIH287-1 | soft tissue | 112  +  mut  +  + | CsrS, 11 bp insert = stop at aa 39 | CsrS, AB517846 | This study |
| NIH433 | blood       | 113  +  mut  +  +   | CsrS, 3 bp delete = delete Asp at aa 470 | CsrS, AB517874 | This study |
| K01    | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| K02    | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| K03    | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| K04    | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| K11    | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| K12    | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| K13    | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| K14    | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| S1393  | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| S2582  | bronchitis  | 1  +  +  +  -     | WT sequence | This study |
| S2638  | bronchitis  | 1  +  +  +  -     | WT sequence | This study |
| OS02   | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| OS06   | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| OS15   | pharyngitis | 1  +  +  +  -     | WT sequence | This study |
| Diagnosis   | NIH No. Strain name | Site of bacterial isolation | emm type | csrR | csrS | rgg | Increased SLO, production | CsrS/CsrR and Rgg amino acid sequence alterations | Accession No. | Reference |
|------------|---------------------|----------------------------|----------|------|------|-----|--------------------------|-----------------------------------------------|--------------|-----------|
| OS17       | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OT3        | vaginitis           | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OT7        | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OT8        | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OT5        | tonsillitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OT110      | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OT24       | scarlet fever       | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| S1         | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| S4         | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| S13        | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| S14        | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| S15        | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| S16        | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| S25        | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| Se235      | pharyngitis         | 1                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| Se202      | tonsillitis         | 3                          | +        | mut | +    | +   | CsrS, Val→Leu at aa 25, Leu→His at aa 26 and Phe→Leu at aa 28 | CsrS, AB517643 | This study |
| K22        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| K23        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| K24        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| K25        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| K31        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| K32        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| K33        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| K34        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| K35        | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              | [35]                                          |              |           |
| OT22       | tonsillitis         | 3                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OS29       | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OT24       | tonsillitis         | 3                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| OT28       | scarlet fever       | 3                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| F495       | pharyngitis         | 3                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| Se230      | pharyngitis         | 4                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| F2362      | pharyngitis         | 4                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| Se242      | pharyngitis         | 6                          | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| F2446      | pharyngitis         | 11                         | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| Se157      | pharyngitis         | 11                         | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| Se233      | pharyngitis         | 12                         | +        | +    | mut | -   | Rgg, Glu→Asp at aa 89    | Rgg, same as NIH50                            |              | This study |
| F2369      | pharyngitis         | 12                         | +        | +    | mut | -   | Rgg, Glu→Asp at aa 89    | Rgg, same as NIH50                            |              | This study |
| StNo.205   | pharyngitis         | 22                         | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| Se172      | pharyngitis         | 28                         | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| F2324      | pharyngitis         | 28                         | +        | +    | +    | -   | WT sequence              |                                              |              | This study |
| 1566       | pus                 | 49                         | +        | +*   | +    | -   | WT sequence              | [38]                                          |              |           |
| Kurume51   | pus                 | 49                         | +        | +*   | +    | -   | WT sequence              | [38]                                          |              |           |
| KH1651     | pus                 | 49                         | +        | +*   | +    | -   | WT sequence              | [38]                                          |              |           |
| S26        | pharyngitis         | 58                         | +        | +    | +    | -   | WT sequence              |                                              |              | This study |

STSS, streptococcal toxic shock-like syndrome; mut, mutation; SLO, streptolysin O; aa, amino acid; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine;
real-time polymerase chain reaction (RT-PCR) with specific primers for each virulence-associated gene. The amounts of mRNA of protein G-related alpha2-macroglobulin-binding protein (gyrA), nicotine adenine dinucleotide glycohydrolase (gyrB), streptodornase (peptidase) (scpA), degrading protease of GAS, Mac-1-like protein (mac), C5a peptidase (scpC), IL-8 protease (scpF), superantigen (scpE), and DNA gyrase (gyrB) genes in the STSS isolate of NIH34 were less than a half of those in the non-STSS isolate of K33 (Figure 2). On the other hand, the amounts of mRNA of the cystein protease (speB) and streptolysin S (sagA) genes in the STSS isolate of NIH34 were less than those of the pharyngitis isolate of K33 with the intact rgg gene (Figure 2). The amounts of mRNA of the IgG-isolate of NIH34 were less than a half of those in the non-STSS isolate of NIH34. The amounts of mRNA of the IgG-isolate of NIH34 were less than a half of those in the non-STSS isolate of NIH34 (Figure 2). The amounts of mRNA of the IgG-degrading protease of GAS, Mac-1-like protein (mac), C5a peptidase (scpC), IL-8 protease (scpF), superantigen (scpE), and DNA gyrase (gyrB) genes in the STSS isolate of NIH34 were almost the same as those in K33 (Figure 2 and data not shown). NIH34+rgg-suppressed the expression of virulence-associated genes to the levels found in non-STSS isolates; further, the expression of speB and sagA genes was increased to levels observed in non-STSS isolates (Figure 2). Additionally, the expression pattern of the virulence genes in K33+rgg was similar to that in the STSS isolate NIH34 (Figure 2). These findings suggest that the transcriptional expression of multiple virulence genes, including the slo gene in GAS, was strongly influenced by the mutation in the rgg gene.

**rgg mutation is important in the pathogenesis of invasive infections in mouse models**

To elucidate the role of rgg in infections in vivo, we used GAS intraperitoneal injections to compare the lethality and histopathology of NIH34 with that of the K33 strain in a mouse model. The NIH34 strain showed significantly higher lethality than the K33 strain ($p = 0.00027$) (Figure 3A). Introduction of the rgg mutation in the K33 strain (K33+rgg) resulted in higher lethality among infected mice than the K33 strain ($p = 0.00067$) and exerted a level of lethality similar to NIH34. The NIH34 strain into which an intact rgg gene (NIH34+rgg) had been introduced exhibited less lethality than the NIH34 strain ($p = 0.000097$) and possessed the same level of lethality as the K33 strain. We confirmed that bacteria isolated from kidney or liver of infected mice at day 6 retained the mutation (data not shown). Therefore, the mutation of the rgg gene in the STSS isolates contributed to enhanced lethality in the mouse model. Histopathological examination of mice infected with NIH34 or K33+rgg strains was carried out. Scattered multiple inflammatory foci containing bacterial colonies were observed in the kidney. The foci were accompanied with neutrophil infiltration, cell debris and hyalinization (Figure 3B). In contrast, no significant pathological change was observed in mice inoculated with the K33 or NIH34+rgg strains (Figure 3B). In another mouse model of soft-tissue infections, subcutaneous infection with NIH34 or K33+rgg resulted in significantly larger lesions as compared to the infection with NIH34+rgg or K33 ($p<0.01$) (Figure 3C). Bacteria were isolated from spleen and kidney after the subcutaneous infection with NIH34 or K33+rgg, but not from NIH34 or K33 strains. We confirmed that bacteria isolated from lesions retained the mutation (data not shown). This showed that subcutaneous inoculation of mice led to the systemic spreading in the rgg mutant. These results suggest that the rgg-mutated strains isolated from STSS patients are more virulent in vivo than strains from patients with non-invasive infections, and that the increase in virulence in vivo is canceled by introducing an intact rgg gene.

**Mechanism of the resistance of rgg mutants to killing by human neutrophils**

In our previous study, using the Transwell system, we showed that SLO, which causes necrosis in neutrophils, and an IL-8 protease of ScpC are important for bacterial resistance to killing by

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**Table 1.** Cont.

| Isolates from patients with: | No. of strains with mutation(s) in gene(s)(%) | Cont. |
|----------------------------|---------------------------------------------|-------|
|                            | crsS | crsR | rgg | crsS+crsR | crsS+rgg | crsR+rgg | crsS+crsR+rgg | none | Total |
|-----------------------------|------|------|-----|----------|---------|---------|--------------|------|-------|
| STSS                        | 46 (28.0) | 13 (7.9) | 27 (16.5) | 2 (1.2) | 9 (5.5) | 6 (3.7) | 0 (0) | 61 (37.2) | 164 (100) |
| SLO (+)                     | 46   | 28   | 18  | 2         | 9       | 6       | 0            | 1    | 91    |
| Non-functional mutation     | 46 (28.0) | 13 (7.9) | 18 (11.0) | 2 (1.2) | 9 (5.5) | 6 (3.7) | 0 (0) | 0 (0) | 94 (57.3) |
| Non-invasive diseases       | 1 (1.7) | 0 (0) | 2 (3.4) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 56 (94.9) | 59 (100) |
| SLO (+)                     | 1    | 1    | 0   | 0         | 0       | 0       | 0            | 0    | 1     |
| Non-functional mutation     | 1 (1.7) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (1.7) |

*crsS mutation affects significantly the expression of slo gene [18], and the mutant shows mucoid colony because the expression of the capsule synthesis operon is increased [4,39]. The function of CrsS was determined by colony morphology and by increase SLO production.

*crsR mutation does not affect significantly the expression of slo gene [18]. The cause of SLO increase in the 9 crsR mutants was described in the section of Discussion.

The crsR mutant shows mucoid colony because the expression of the capsule synthesis operon is increased [4,39]. The function of crsR was determined by colony morphology.

*rgg mutation affects significantly the production of slo gene [Figure 1, [16]] and SpeB [14,16–17]. The function of Rgg was determined by the increase of SLO production and the decrease of SpeB production.

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**Table 2.** Mutation frequency in the crsS/crsR and rgg genes.

| Isolates from patients with: | No. of strains with mutation(s) in gene(s)(%) | Cont. |
|-----------------------------|---------------------------------------------|-------|
|                            | crsS | crsR | rgg | crsS+crsR | crsS+rgg | crsR+rgg | crsS+crsR+rgg | none | Total |
|-----------------------------|------|------|-----|----------|---------|---------|--------------|------|-------|
| STSS                        | 46 (28.0) | 13 (7.9) | 27 (16.5) | 2 (1.2) | 9 (5.5) | 6 (3.7) | 0 (0) | 61 (37.2) | 164 (100) |
| SLO (+)                     | 46   | 28   | 18  | 2         | 9       | 6       | 0            | 1    | 91    |
| Non-functional mutation     | 46 (28.0) | 13 (7.9) | 18 (11.0) | 2 (1.2) | 9 (5.5) | 6 (3.7) | 0 (0) | 0 (0) | 94 (57.3) |
| Non-invasive diseases       | 1 (1.7) | 0 (0) | 2 (3.4) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 56 (94.9) | 59 (100) |
| SLO (+)                     | 1    | 1    | 0   | 0         | 0       | 0       | 0            | 0    | 1     |
| Non-functional mutation     | 1 (1.7) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 1 (1.7) |

*crsS mutation affects significantly the expression of slo gene [18], and the mutant shows mucoid colony because the expression of the capsule synthesis operon is increased [4,39]. The function of CrsS was determined by colony morphology and by increase SLO production.

*crsR mutation does not affect significantly the expression of slo gene [18]. The cause of SLO increase in the 9 crsR mutants was described in the section of Discussion.

The crsR mutant shows mucoid colony because the expression of the capsule synthesis operon is increased [4,39]. The function of crsR was determined by colony morphology.

*rgg mutation affects significantly the production of slo gene [Figure 1, [16]] and SpeB [14,16–17]. The function of Rgg was determined by the increase of SLO production and the decrease of SpeB production.

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neutrophils [2]. Here, we examined the effect of rgg mutation on resistance to killing by neutrophils. As shown in Figure 4A, the migration ability of human neutrophils in response to chemokine IL-8 did not significantly differ between K33 and K33rgg or between NIH34 and NIH34rgg. Furthermore, the scpC mutation in the NIH34 strain did not have a significant influence on the migration of human neutrophils, compared to the csrS mutation, as previously reported (Figure 4A). This finding is in accordance with the less influence of ScpC expression in the rgg mutation (Figure 2). Collectively, the mutation of the rgg gene had little influence on the migration of human neutrophils in response to IL-8. As previously reported [2], migrated neutrophils may be killed by the STSS GAS isolates via enhanced SLO production, and therefore we reported [2], migrated neutrophils may be killed by the STSS GAS isolates via enhanced SLO production, and therefore we reported [2]. The neutrophil-killing activity was significant (Figure 2), we examined the lethal activity of the rgg mutant in vitro. In our previous study, a csrS mutation in the emm49-genotyped strains was a key to the onset of severe invasive streptococcal infections [2]. The csrS mutant showed higher lethality in a mouse model and more efficiently killed human neutrophils than the non-mutated strain [2]. Therefore, we next compared the effect of the mutation in the csrS gene with that in the rgg gene, in terms of in vivo virulence in lethality and impairment of neutrophil function in vitro. Intraperitoneal infection of mice with the csrS mutant (K33csrS) caused earlier death and higher lethality than did infection with the rgg mutant (K33rgg) (p = 0.017) (Figure 3A). Furthermore, K33csrS strains decreased the migration ability of neutrophils in response to IL-8, and they induced necrosis of migrated neutrophils to a greater degree than did the rgg mutants (Figures 4A, B). These and the aforementioned results suggest that the rgg mutant can escape being killed by neutrophils only because of the SLO function, and not because of ScpC, whereas both SLO and ScpC in the csrS mutant contribute to the escape. This suggests that the csrS mutant may be more virulent in systemic infections than the rgg mutant, owing to its ability to up-regulate more virulence factors such as ScpC (Figures 2 and 3A, B).

### Comparison of virulence between the csrS and rgg mutations

In our previous study, a csrS mutation in the emm49-genotyped strains was a key to the onset of severe invasive streptococcal infections [2]. The csrS mutant showed higher lethality in a mouse model and more efficiently killed human neutrophils than the non-mutated strain [2]. Therefore, we next compared the effect of the mutation in the csrS gene with that in the rgg gene, in terms of in vivo virulence in lethality and impairment of neutrophil function in vitro. Intraperitoneal infection of mice with the csrS mutant (K33csrS) caused earlier death and higher lethality than did infection with the rgg mutant (K33rgg) (p = 0.017) (Figure 3A). Furthermore, K33csrS strains decreased the migration ability of neutrophils in response to IL-8, and they induced necrosis of migrated neutrophils to a greater degree than did the rgg mutants (Figures 4A, B). These and the aforementioned results suggest that the rgg mutant can escape being killed by neutrophils only because of the SLO function, and not because of ScpC, whereas both SLO and ScpC in the csrS mutant contribute to the escape. This suggests that the csrS mutant may be more virulent in systemic infections than the rgg mutant, owing to its ability to up-regulate more virulence factors such as ScpC (Figures 2 and 3A, B).

### Table 3. Amino acid difference in comparison with intact ORF of SF370.

| Isolates     | Strain name | SPs0322 (CodY) | SPs1615 (CsrR) | SPs1742 (Rgg) | CsrS |
|--------------|-------------|----------------|----------------|--------------|------|
| Non-STSS     | C500        | +              | +              | +            | +    |
|              | OT22        | +              | +              | +            | +    |
|              | K33         | +              | +              | +            | +    |
| STSS         | NIH1        | -              | -              | -            | +    |
|              | NIH3        | -              | +              | -            | +    |
|              | NIH8        | +              | +              | -            | +    |
|              | NIH34       | +              | +              | -            | +    |

(+) the same as the intact ORF of SF370 (accession No. AE004092).
(-): difference from the intact ORF.

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**Figure 1. More SLO is secreted in STSS isolates than in isolates from non-invasive infections.** The supernatants from an overnight culture (OD600 = 1.0) of emm3 S. pyogenes clinical isolates (non-STSS: C500, OT22, and K33; STSS: NIH1, NIH3, NIH8, NIH34, NIH152, NIH249, NIH327, and NIH352; non-STSS isolates with the mutated rgg gene: OT22rgg and K33rgg; STSS isolates complemented with the intact rgg gene: NIH8rgg+ and NIH34rgg+) were concentrated with trichloroacetic acid, and 5 μl of each sample was analyzed by western blotting with rabbit anti-SLO polyclonal antibody. Representative data of two independent experiments are shown.

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Figure 2. Mutation of the rgg gene influences expression of virulence-associated genes. The expression of virulence-associated genes in non-STSS, STSS GAS isolates, and strains into which an intact gene or mutant rgg or mutant csrS gene had been introduced was analyzed by RT-PCR; columns represent the relative mRNA expression levels of virulence-associated genes of each strain: nicotine adenine dinucleotide glycohydrolase (nag), streptolysin O (slo), streptokinase (skr), protein G-related alpha2-macroglobulin-binding protein (grab), streptodornase (phage-associated) (sdn), streptolysin S (sagA), streptococcal pyrogenic endotoxin (speB), and IL-8 protease (scpC). The expression level of K33 strain is shown as 1. Values are means ± SD (n = 4).

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Mutation frequency of the rgg and the csrS/csrR genes in STSS clinical isolates

In this study, we found that there are mutations in the rgg gene or the csrS/csrR genes in STSS clinical isolates. We sequenced the rgg gene in strains isolated from sterile sites of STSS patients and found that 42 of 164 (25.6%) isolates carried some mutations (deletion, point mutation, or insertion) in the rgg gene. To determine whether these mutations contributed to a loss of Rgg function, we examined the level of SLO and SpeB [14] secretion and compared it with that in non-STSS isolates because overproduction of SLO [This study, 16] and less secretion of SpeB are also reported in the rgg mutation [14,16–17]. We defined these phenotyped isolates as Rgg non-functional mutants. In 33 of 42 isolates, SLO production had increased and SpeB production had decreased (Tables 1 and 2 and data not shown). All of remaining nine rgg mutants (strains with mutation only in rgg) showing no increase of SLO expression were emm12-genotyped strains and had a mutation at the same position in comparison with other non-invasive strains. This mutation was synonymous in the level of amino acid, so we defined the mutants are functional as shown in Table 2. Collectively, 11.0%, 28.0%, 7.9%, 1.2%, 5.5%, and 3.7% of the 164 STSS clinical isolates carried non-functional mutations in the rgg, csrS, csrR, both csrS and csrR, both csrS and rgg, and both csrR and rgg genes, respectively, so that a total of 57.3% of the STSS isolates carried mutations in one or more of these negative regulator genes (Tables 1 and 2). On the other hand, the frequency of mutations in these genes was very low (1.7%) in non-invasive isolates (Tables 1 and 2). Therefore, the incidence of mutations in these genes is higher in STSS isolates than in non-invasive isolates (p<0.01 by χ² analysis). This finding suggests that mutations in the csrS/csrR genes or the rgg gene are crucial factors causing severe invasive infections, such as STSS.

Discussion

Since the late 1980s, STSS caused by S. pyogenes has become a serious health problem in both developed and developing countries. In this study, we found a high frequency of mutations of negative regulators in STSS clinical isolates. The rgg mutant killed human neutrophils, impaired multiple organs, and enhanced lethality in the mouse model, similar to the csrS mutant. These findings suggest that the impairment of negative regulators of S. pyogenes virulence genes induces neutrophil incompetence and subsequent STSS infection. This study is the first to show that clinical S. pyogenes isolates from STSS patients have mutations in one or more of genes—rgg, csrS, and csrR—which are involved in the negative regulation of multiple virulence genes.

In our previous study, we found mutations in the csrS/csrR genes of 5 emm49 strains isolated from patients with severe invasive infections [2]. In the present study, we further examined whether STSS isolates other than the emm49 genotype possess mutations in the csrS and csrR genes: 46.3% of the STSS isolates including various emm genotypes had non-functional mutations in one or more of the csrS/csrR genes. This finding suggests that mutations in the csrS/csrR genes are commonly recognized in STSS clinical isolates with various emm genotypes.

We have shown that the amount of SLO protein produced in STSS isolates is greater than that in non-STSS isolates, and that this effect is due to mutations in both the rgg and csrS genes of the isolates. The loss of function incurred by the mutation in the rgg gene in emm3-genotyped S. pyogenes affected the regulatory network of the virulence-associated genes; hence, the mutated strains could resist killing by neutrophils and caused damage to various organs in the mouse models. Therefore, the mutated emm3-genotyped S. pyogenes strains may potentially cause severe infections such as STSS in humans. Hollands et al. [14] reported that a mutation of the rgg (npB) gene reduces M1T1 group A streptococcal virulence.

We examined the contribution of Rgg to the pathogenesis of systemic infections by using a clinical emm1-genotyped STSS isolate, NIH1186, and an emm1-genotyped pharyngitis isolate, Se235. NIH1186 and Se235; both of which had a mutation in the rgg gene, showed higher lethality than NIH1186rgg and Se235, in both of which the rgg gene is intact (data not shown). These mutants impaired neutrophils to a greater extent than the rgg-intact strains did (Figure S1); this finding suggests that rgg mutants are more virulent than rgg-intact strains, in the emm1 genotype. Therefore, the discrepancy between the finding in this study and that of Hollands et al. [14] may be attributed to modified regulation of SLO expression in rgg-mutated isolate in the latter, but not downregulation of speB and sagA operons.

Rgg is reported to regulate the transcription of many virulence-associated genes in S. pyogenes [10–13], and its regulatory profile varies among strains used [16–17]. Nevertheless, up-regulation of the slo, nga and ska genes and down-regulation of the speB gene are commonly found in the rgg mutation of emm3-genotyped isolates (Figure 2) and of M49 serotyped-strains, NZ131 and CS101 [16–17], suggesting they are the Rgg core regulon of GAS strains.

In recent studies, it has been reported that expression of the rgg gene is positively regulated by CsrS [4], while it is negatively regulated by CsrR [16]. Expression of the slo gene is enhanced in the csrS mutant (Figure 2) [2,4], but not in the csrR mutant [18]. In this study, the expression of the slo gene was enhanced in the rgg mutant (Figure 2), suggesting that the enhancement of the slo gene may serve as the same regulatory pathway as the effect of the csrS mutation. These findings suggest that CsrS affects the Rgg regulon as well as the CsrR regulon (Figure 3); in the csrS mutant, CsrR is not phosphorylated by CsrS, and Rgg expression is suppressed.

It has been reported that the csrR null-mutation does not affect the expression of SLO [18]; however, Treviño et al. [19] reported that SLO production increases as a result of a csrR mutation in which histidine replaces arginine at position 119 of the CsrR protein; however the protein retained DNA-binding activity. The strains carrying such a kind of mutation are phenotypically identical to the csrS mutants [19]. Nine csrR mutants in this study showed increased SLO production (Tables 1 and 2), 2 (NIH1136 and NIH300) of which had an amino acid replacement at position 119 of CsrR protein. Other 7 isolates showed mutation in the N-terminal amino acid of CsrR, but the exact mechanism of the CsrR mutant remains to be solved.

The csrS/csrR and rgg genes negatively regulate various virulence genes; however, they regulate different virulence genes. The slo,
Figure 3. Mutation of \textit{rgg} gene enhances the lethality and histopathology of GAS in mouse \textit{in vivo} infection models. (A) Survival curves of mice infected with each strain. Mice were intraperitoneally inoculated with $1 \times 10^7$ CFU of each GAS, and mouse survival was observed for 7 days post-infection. Mortality differences were statistically significant ($P < 0.01$), as determined by a log-rank test. Survival curves were generated from 3 independent experiments using a total of 10–16 ddY mice for each strain. (B) Histopathological changes in the kidneys of mice infected with GAS. Tissue was extracted at 24 h after the intraperitoneal injection of GAS ($1 \times 10^7$ CFU). The black arrows indicate clusters of bacteria with filtrated inflammatory cells. The triangle heads indicate fibrous debris. (C) Lesion areas of subcutaneous infection in hairless mice injected with GAS. $1 \times 10^7$ CFU in 100 \(\mu\text{L}\) suspension of GAS in PBS was injected subcutaneously, and the lesion area and body weight were measured each day after infection. The peak values are shown as means ± SD ($n = 5$). *The skin-lesion area in \textit{rgg} mutant strains-infected mice was significantly larger than that in \textit{rgg} intact strains ($p < 0.05$), as estimated by ANOVA. doi:10.1371/journal.ppat.1000832.g003
nga, and ska genes are negatively regulated by both CsrS/R and Rgg. The grab gene is negatively regulated by Rgg, while the mac, scpA, and scpC genes are negatively regulated by CsrS [2] (Figure 2). Thus, in terms of impairing neutrophil function, the csrS mutant inhibits the migration of neutrophils due to the destruction of IL-8 by the increased expression of scpC (Figure 5) [2]; meanwhile, the rgg mutant does not significantly affect the expression of scpC. On the other hand, since both rgg and csrS genes negatively regulate the expression of slo, infections with these mutants result in damage of neutrophils due to the increased production of SLO in the foci. This may explain why neutrophils are observed histopathologically in some cases of severe invasive infection, but are not in others. Indeed, our mouse model shows that neutrophils clustered around the foci of bacteria in the kidney infected by the rgg mutant (Figure 3B) but not by the csrS mutant [2].

The slo, nga, and ska genes are negatively regulated by both CsrS and Rgg [2] (Figure 2). We previously reported that SLO is an important virulence factor for the necrosis of neutrophils, which leads to higher lethality of infected mice [2]. Nucleosidase (NADase), which is encoded by the nga gene, contributes to severe invasive infections by GAS in the murine model of infection [20]. Streptokinase, which is encoded by the ska gene, has an important role in GAS invasion and proliferation [21]. STSS isolates carrying mutations in the csrS gene and/or the rgg gene commonly increased the expression of these genes [2; this study]. Thus, overproduction of these factors in the mutants could cooperatively contribute to increased virulence, thus causing the onset of STSS. Notably, the mutation frequency of these genes in STSS isolates (57.3%) was much higher than that in non-invasive isolates (1.7%). These results suggest that mutations in the negative regulators of various virulence genes are important to the STSS onset. However, 42.7% of the STSS isolates did not have mutations in the csrS/ csrR or rgg genes. Such strains may have mutations in other various other two-component regulatory systems or regulators in the S. pyogenes genome [22], which would be the focus of our research. We could not exclude the possibility that clinical severity of infection by strains lacking any mutations in the three genes depends on host factors, and not on bacterial factors. Specific human leukocyte antigen class II haplotypes are associated with a risk of disease severity [23], and the importance of both host and environmental factors has been reported [24].

In the mouse model, the csrS mutant (K33csrS) showed higher lethality than the rgg mutant. However, in the present study, the mortality rate of STSS patients infected with the rgg mutant was 60.9%, while that of patients infected with the csrS mutant was 47.2% (data not shown). These findings suggest that the rgg mutant also causes high lethality in humans, which may indicate differences in disease severity between humans and mice. Streptokinase is highly specific for human plasminogen, exhibiting little or no activity to those of other animal species [25].
expression. In the lin-resistant Recently, it has been reported that community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) causes severe invasive infections such as STSS. The change of expression of the virulence of CA-MRSA has been linked to an overproduction of PSMs is regulated under the strict control of the coordinated overexpression of multiple virulence factors that are affected by the global regulatory network.

Methods

Ethic statement

This study complies with the guidelines of the declaration of Helsinki. This study protocol was approved by the institutional individual ethics committees for the use of human subjects (the National Institute of Infectious Diseases Ethic Review Board for Human Subjects) and the animal experiments (the National Institute of Infectious Diseases Animal Experiments Committee). Written informed consent was obtained from all study participants or their legal guardians for the patients who died. All clinical samples and healthy human neutrophils were stripped of personal identifiers not necessary for this study. All animal experiments were performed according to the Guide for animal experiments performed at National Institute of Infectious Diseases, Japan.

Bacterial strains and culture conditions

The S. pyogenes strains and plasmids used in this study are described in Tables 1 and S1. The STSS criteria in this study are based on those proposed by the Working Group on Severe Streptococcal Infections (8). The clinical isolates used were isolated from sterile sites of patients with STSS (164 isolates; age 0–99 years) and from non-sterile sites of patients with non-invasive infections (59 isolates; ages 1–67 years). The isolates from STSS and non-invasive infections were collected by the Working Group for Beta-hemolytic Streptococci in Japan, as previously reported (30). Escherichia coli DH5α was used as a host for plasmid construction and was grown in a Luria-Bertani liquid medium with shaking or on agar plates at 37°C. S. pyogenes was cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY medium) without agitation or on tryptic soy agar supplemented with 5% sheep blood. Cultures were grown at 37°C in a 5% CO2 atmosphere. When required, antibiotics were added to the medium at the following final concentrations: erythromycin, 300 µg/mL for E. coli and 1 µg/mL for S. pyogenes; and spectinomycin (Sp), 25 µg/mL for each of E. coli and S. pyogenes. The growth of S. pyogenes was turbidimetrically monitored at 600 nm, using a MiniPhoto 518R (Taitec, Tokyo, Japan).

DNA sequencing and data deposit

The nucleotide sequences of the csrS, csrR, and rgg genes were determined by automated sequencers, i.e., an Applied Biosystems 3130xl Genetic Analyzer and an Applied Biosystems 3130 Genetic Analyzer (both Applied Biosystems, Tokyo, Japan). Sequencing data were deposited in the DNA Data Bank of Japan (DDBJ).

Animals

Male five to six-week-old outbred ddY and hairless mice were purchased from SLC (Shizuoka, Japan) and maintained in specific pathogen-free conditions at the National Institute of Infectious Diseases, Tokyo, Japan.

Human-specific pathogenic factor(s) may influence virulence in cases of infection with the rgg mutant.

Collectively, we showed that mutations of negative regulators that result in the overproduction of multiple virulence factors are important to the onset of severe invasive infections such as STSS. Recently, it has been reported that community-associated methicillin-resistant Staphylococcus aureus (CA-MRSA) causes severe invasive infections, resulting in NF or even death [26,27]. The enhanced virulence of CA-MRSA has been linked to an overproduction of leukolytic peptides, phenol-soluble modulins (PSMs) [28,29]. The production of PSMs is regulated under the strict control of agr [29]. The change of expression of the agr regulator results in increased virulence factors and increased virulence. Severe invasive infections are caused not only by S. pyogenes but also by other bacteria such as other Streptococcus, Staphylococcus aureus, Vibrio vulnificus, and Aeromonas spp. Such severe invasive infections may be caused by the coordinated overexpression of multiple virulence factors that are affected by the global regulatory network.

Figure 5. Schema of regulatory network and its dysfunction in STSS isolates leading to host evasion. CsrS phosphorylates CsrR, and the CsrR represses expression of a number of virulence genes including rgg and scpC[18]. CsrS also positively regulates the expression of rgg [4], which suppresses slo gene expression (Figure 2). The rgg mutation causes an overexpression of SLO, which kills neutrophils, but has no influence on ScpC expression. In the csrS mutant, overproduced ScpC inhibits the migration of neutrophils, and upregulated Rgg reduces the slo gene expression. In the csrS mutant, inactive form of CsrR leads to the overproduction of ScpC, which inhibits the migration of neutrophils, and decrease of Rgg leads to the overproduction of SLO, which kills neutrophils.

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Construction of deletion or deficient mutants

(i) Construction of the rgg mutant. A 692-bp DNA fragment containing the internal region of rgg was amplified from the NIH34 (for emm3) and NIH186 (for emm1) chromosomal DNA, using the primers of rgg-del1 and rgg-del2 (Table S3). The PCR products were digested by BamHI and EcoRI. This fragment was then cloned into the integration shuttle vector pSF152 [31] to create the plasmid pSF152_{rgg}9 and pSF152_{rgg}1, respectively, which was then used for the chromosomal inactivation of the rgg gene, as described previously [31]. The inactivated mutant strains K33_{rgg}, OT22_{rgg}, S1_{rgg}, Sc253_{rgg} and F402_{rgg} (rgg::aad9 Sp') were then selected by using spectinomycin-containing agar plates. Deficiency of the native rgg gene was verified by PCR.

(ii) Construction of the csrS mutant. A 930-bp DNA fragment containing the internal region of csrS was amplified from the K33 chromosomal DNA, using the primers of csrS-def1 and csrS-del2 (Table S3). The PCR products were digested by BamHI and EcoRI. This fragment was then cloned into the integration shuttle vector pSF152 to create the plasmid pSF152_{csrS}, which was then used to create K33_{csrS}, as described above.

(iii) Construction of the slo mutant. A 1,061-bp DNA fragment containing the internal region of slo was amplified from the NIH34 chromosomal DNA, using the primers of slo-del3 and slo-del4 (Table S3). The PCR products were digested by BamHI and EcoRI. This fragment was then cloned into the integration shuttle vector pSF152 to create the plasmid pSF152_{slo}, which was then used to create NIH34_{slo}, as described above.

(iv) Construction of the scpC mutant. A 1,240-bp DNA fragment containing the internal region of scpC was amplified from the NIH34 chromosomal DNA, using the primers of scpC-del5 and scpC-del6 (Table S3). The PCR products were digested by BamHI and EcoRI. This fragment was then cloned into the integration shuttle vector pSF152 to create the plasmid pSF152_{scpC}, which was then used to create NIH34_{scpC}, as described above.

(v) Construction of the sdn mutant. A 693-bp DNA fragment containing the internal region of sdn was amplified from the NIH34 chromosomal DNA, using the primers of sdn-del3 and sdn-del2 (Table S3). The PCR products were digested by BamHI and EcoRI. This fragment was then cloned into the integration shuttle vector pSF152 to create the plasmid pSF152_{sdn}, which was then used to create NIH34_{sdn}, as described above.

(vi) Construction of the nga mutants. A 1,071-bp DNA fragment containing the 5' terminal of nga and the adjacent upstream chromosomal DNA was amplified from the NIH34 chromosomal DNA, using the primers of ngad11 and ngad2 (Table S3); additionally, a 775-bp fragment containing the 3' terminal of nga and the adjacent downstream chromosomal DNA was amplified from the NIH34 chromosomal DNA, using the primers of ngad3 and ngad4 (Table S3). These two PCR products were digested by BamHI and EcoRI and by EcoRI and PstI, respectively. The digested fragments were cloned into the erythromycin-resistant and temperature-sensitive shuttle vector pJR5235 [32], to create the plasmid pJR5SAnga. This plasmid was then introduced into the strain NIH34 by electroporation, and transformants were selected on erythromycin agar plates at 30°C. To create an integration of pJR5SAnga with the chromosome, transformants were grown at 39°C and selected with erythromycin. Replacement of the native nga gene by the nga-deleted mutant allele was verified by PCR, and the resultant strain was named NIH34nga.

Construction of strains integrating the intact rgg gene

The replacement of a mutated rgg gene by an intact rgg gene was performed by allelic recombination. Specifically, the chromosomal DNA derived from the GAS strains K33 (for emm3) and F402 (for emm1) was purified and used as a template for the PCR amplification of the intact rgg gene. The primers used were 5'-GGGGATCCCTATGGCTATATCATAGCTG-3' (sense) and 5'-GGGAATTCCTGAGATATAACTACACC-3' (antisense). The PCR fragment was ligated into the plasmid pSF152, and the resultant plasmids pSF_{rgg}9 (for emm3) and pSF_{rgg}1 (for emm1) were used for chromosomal integration into the mutated rgg gene of isolates from STSS patients, as described previously [31]. The integrated strains (Sp') were then selected by using spectinomycin (Sp)-containing agar plates. Integration of the intact rgg gene was confirmed by PCR.

Western blotting

A total of 1 mL of the supernatant of an overnight bacterial culture (OD_{600}=1.0) was passed through a 0.45-mm pore size membrane filter (Nippon Millipore, Tokyo, Japan), to remove the remaining cells. Proteins in the resulting cell-free supernatant were precipitated with 10% trichloroacetic acid and resuspended in a sample loading buffer containing a saturated Tris base. Samples were heated at 100°C for 3 min and separated on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels. To detect SLO, the proteins on the gels were electrophoretically transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat milk and then incubated with primary anti-SLO polyclonal antibody (American Research Products, Belmont, MA, USA), secondary antibody peroxidase-conjugated anti-rabbit Ig (GE Healthcare, Tokyo, Japan), and an ECL Plus Western blotting Detection System (GE Healthcare).

Complete-genome comparisons

Complete-genome comparisons were performed with an array-based service (CGS) provided by NimbleGen Systems Inc. (Madison, WI, USA) [9]. The reference genome sequence used in the microarray was that of S. pyogenes SSI-1 (GenBank accession No. BA000034).

Quantitative RT-PCR analysis

Total RNA was extracted from bacterial cells using the RNeasy Protect Bacteria Mini Kit (QIAGEN, Tokyo, Japan), according to the manufacturer’s instructions. Complementary DNA synthesis was performed with the PrimeScript RT reagent kit (Perfect Real Time) (Takara Bio, Otsu, Japan), also following the manufacturer’s instructions. Transcript levels were determined using the ABI PRISM Sequence Detection System 7000 (Applied Biosystems) and Premix Ex Taq (Perfect Real Time) (Takara). For real-time amplification, the template was equivalent to 5 ng of total RNA. Measurements were performed in triplicate; a reverse-transcription-negative blank of each sample and a no-template blank served as negative controls. The primers and probes used are listed in Table S4.

GAS infection in a mouse model

GAS was grown to late-log phase (OD_{600}=0.6–0.8) at 37°C in a 5% CO_{2} atmosphere, pelleted by centrifugation, washed twice with sterile phosphate-buffered saline (PBS), suspended in sterile PBS. A total of 1×10^{6} CFU of GAS suspended in 0.5 mL of PBS was injected intraperitoneally into five to six-week-old ddY outbred male mice (10–16 mice/GAS isolate). The number of surviving mice was compared statistically, using the Kaplan-Meier

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log-rank test. For the subcutaneous infection model, male hairless mice Hos:Hr-1 were injected with 1 x 10^7 CFU of GAS in a 100-µl suspension of GAS in PBS. The lesion area was measured daily and analyzed. Dissemination in kidney and spleen of GAS was evaluated by colony counting at day 7 post-infection.

**Histopathological examination**

For histopathological analysis, the tissues from GAS-infected mice were directly fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E).

**Isolation of human neutrophils**

Human neutrophils were isolated from the venous blood of five healthy volunteers, in accordance with a protocol approved by the Institutional Review Board for Human Subjects, National Institute of Infectious Diseases [2]. This study complies with the guidelines of the declaration of Helsinki.

**Migration assay**

Chemotaxis assays were performed as previously described [2]. Briefly, 5 x 10^4 neutrophils in Roswell Park Memorial Institute (RPMI) medium containing 25 mM HEPES and 1% FCS in Transwell inserts (3-µm pore size; Coraster, Corning, NY, USA) were placed in 24-well plates containing 600 µl medium or 100 nM interleukin (IL)-8 solution (Pertec, London, UK); the plates were then incubated with or without 5 x 10^6 bacteria for 1 h at 37°C, in advance of the assay. After 1 h of incubation, cells in the lower wells were collected and 10^4 10-µm microsphere beads (Polysciences Inc., Warrington, MA, USA) were added. Cells were stained with propidium iodine (Sigma, St Louis, MO, USA) for flow cytometry to quantify the viable neutrophils; analysis was performed, using the FACS Calibur (BD Biosciences, San Jose, CA, USA).

**Accession numbers**

The DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/index-e.html) accession numbers for the genes and gene products discussed in this paper are: TK283 csrR locus - AB517797; TK292 csrR locus - AB517804; NIH43 csrR locus - AB517807; NIH75 csrR locus - AB517814; NIH136 csrR locus - AB517819; NIH157 csrR locus - AB517822; NIH212 csrR locus - AB517826; NIH216 csrR locus - AB517827; NIH252-2 csrR locus - AB517838; NIH259 csrR locus - AB517839; NIH275 csrR locus - AB517842; NIH300 csrR locus - AB517850; NIH301 csrR locus - AB517851; NIH323-1 csrR locus - AB517853; NIH381-1 csrR locus - AB517863; NIH404 csrR locus - AB517867; NIH406 csrR locus - AB517868; NIH447 csrR locus - AB517877; NIH5 csrS locus - AB517796; TK76 csrS locus - AB517800; NIH18 csrS locus - AB517801; TK280 csrS locus - AB517803; NIH35 csrS locus - AB517805; NIH44 csrS locus - AB517809; NIH49 csrS locus - AB517810; NIH55 csrS locus - AB517812; NIH75 csrS locus - AB517815; NIH102 csrS locus - AB517817; NIH152-3 csrS locus - AB517820; NIH156-1 csrS locus - AB517821; NIH205 csrS locus - AB517823; NIH220-4 csrS locus - AB517825; NIH220-1 csrS locus - AB517828; NIH222 csrS locus - AB517829; NIH230 csrS locus - AB517830; NIH236 csrS locus - AB517831; NIH236 csrS locus - AB517833; NIH243 csrS locus - AB517834; NIH253-1 csrS locus - AB517835; NIH250-2 csrS locus - AB517836; NIH263-2 csrS locus - AB517840; NIH266 csrS locus - AB517841; NIH283-1 csrS locus - AB517844; NIH286 csrS locus - AB517845; NIH287-1 csrS locus - AB517846; NIH296 csrS locus - AB517847; NIH297 csrS locus - AB517849; NIH317 csrS locus - AB517852; NIH325-1 csrS locus - AB517854; NIH345 csrS locus - AB517855; NIH372 csrS locus - AB517859; NIH437 csrS locus - AB517873; NIH433 csrS locus - AB517874; NIH453 csrS locus - AB517875; NIH620 ggg locus - AB517799; NIH118 ggg locus - AB517802; NIH157 ggg locus - AB517811; NIH160 ggg locus - AB517813; NIH91 ggg locus - AB517816; NIH118 ggg locus - AB517818; NIH186 ggg locus - AB517824; NIH256 ggg locus - AB517832; NIH258-2 ggg locus - AB517837; NIH273 ggg locus - AB517843; NIH289 ggg locus - AB517848; NIH357 ggg locus - AB517856; NIH366 ggg locus - AB517857; NIH371 ggg locus - AB517858; NIH372 ggg locus - AB517860; NIH374-2 ggg locus - AB517861; NIH381-1 ggg locus - AB517864; NIH390 ggg locus - AB517865; NIH406 ggg locus - AB517869; NIH409 ggg locus - AB517870; NIH422 ggg locus - AB517872; NIH445 ggg locus - AB517876.

**Supporting Information**

| Table S1 | Strains of emm3 and emm1 genotype S. pyogenes and plasmids used in this study |
|----------|----------------------------------------------------------------------------------|
| Found at: | doi:10.1371/journal.ppat.1000832.s001 (0.06 MB DOC) |

| Table S2 | Amino acid difference in comparison with K33 |
|----------|----------------------------------------------------------------------------------|
| Found at: | doi:10.1371/journal.ppat.1000832.s002 (0.04 MB DOC) |

| Table S3 | Primers used for the construction of deletion mutants |
|----------|----------------------------------------------------------------------------------|
| Found at: | doi:10.1371/journal.ppat.1000832.s003 (0.03 MB DOC) |

| Table S4 | Primers used for RT-PCR |
|----------|----------------------------------------------------------------------------------|
| Found at: | doi:10.1371/journal.ppat.1000832.s004 (0.06 MB DOC) |

**Figure S1** Effect of ggg mutation of emm1-genotyped S. pyogenes on survival of human neutrophils. Human neutrophils migrated in the lower wells of a Transwell system in response to IL-8. The migrated human neutrophils were brought into contact with various emm1 GAS strains (S1, Se235, and F482; non-STSS clinical isolates, NIH60 and NIH186; and STSS isolates and their ggg mutants) (Table S1), and then the remaining viable neutrophils were counted. Values shown are means ± SD. *p<0.05, as estimated by Student’s t test. The results shown are representative of one of four individual experiments, all of which had similar results. Found at: doi:10.1371/journal.ppat.1000832.s005 (0.05 MB TIF)

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**Author Contributions**

Conceived and designed the experiments: TI MA TM HK HW. Performed the experiments: TI MA TM HH. Analyzed the data: TI MA TM HH. Contributed reagents/materials/analysis tools: TI MA TM HH. Wrote the paper: TI MA TM HH KK HW.

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