emm1/Sequence Type 28 Strains of Group A Streptococci That Express *covR* at Early Stationary Phase Are Associated with Increased Growth and Earlier SpeB Secretion[▽]

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Streptococcus pyogenes (group A streptococcus [GAS]) is a versatile human pathogen, and *emm1*/sequence type 28 (ST28) is the most frequently isolated type from GAS infections. The *emm1*/ST28 strain is associated with necrotizing fasciitis and streptococcal toxic shock syndrome. Growth-phase regulation is one of the important regulatory mechanisms in GAS, which controls gene expression at restricted phases of growth. CovRS, a two-component regulatory system, is considered the regulator of streptococcal pyrogenic exotoxin B (SpeB) and is thought to be activated in the exponential phase of growth. In the present study, Northern hybridization analysis showed that 52% of the analyzed GAS strains expressed *covR* at the exponential phase, but 48% of the strains expressed *covR* at the early stationary phase of growth. Strains transcribing *covR* at the early stationary phase showed better growth and earlier SpeB expression than the other group of strains. Multilocus sequence typing and pulsed-field gel electrophoresis analysis showed only *emm1*/ST28 strains (which comprise a clonal cluster) were expressing *covR* at the early stationary phase of growth, indicating that *emm1*/ST28 strains have special characteristics which may be related to their worldwide distribution.

Streptococcus pyogenes (group A streptococcus [GAS]) is an important human pathogen which causes a wide spectrum of diseases such as pharyngitis, cellulitis, necrotizing fasciitis, and streptococcal toxic shock syndrome (STSS). Although distinct serotypes of GAS have been isolated from invasive infections, MIT1 is the most persistent and frequently isolated serotype from invasive GAS infections worldwide (4, 16).

Currently, sequence-based *emm* typing is used to predict the Lancefield M serotypes and is one of most used typing methods with GAS. More than 177 *emm* types and 750 *emm* subtypes have been identified so far (36). The other two major typing methods for GAS are multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) typing. MLST is a method based on the nucleotide sequence of housekeeping genes and is primarily used for the identification of clusters among isolates (14). MLST sequence type 28 (ST28) is the most dominant type in the MLST GAS database (<http://www.mlst.net/>). Since horizontal gene transfer has frequently occurred within the GAS population, PFGE typing, which can probe the genomic DNA organization, is also an important tool for GAS typing (18).

Growth-phase regulation is one of the most important regulatory mechanisms in GAS (9, 44). Under the control of growth-phase regulation, any specific gene in GAS is transcribed during a restricted phase of growth. Since GAS has no

functional alternative sigma factors, it is believed that the control of gene expression is dependent on the complex interactions among transcriptional regulators and two-component regulatory systems (9, 34). Transcriptional regulators Mga, Rgg, the RofA/Nra regulator family, and the two-component regulatory system CovRS are associated with growth-phase-dependent virulence gene regulation in GAS (26). CovRS-regulated genes are expressed in both the exponential phase and stationary phase (17). In addition, the CovRS system has important roles for bacterial growth during general stress and amino acid starvation conditions (13, 37). Graham et al. further suggested that CovRS has important roles in linking key biosynthesis, catabolic, and virulence functions during transcriptome restructuring (21).

Streptococcal pyrogenic exotoxin B (SpeB) is a cysteine protease of GAS which degrades human immunoglobulins, complement components, and extracellular matrix proteins (15, 23, 27, 42). In addition, SpeB also degrades several bacterial proteins, including surface M proteins, protein F1 and C5a peptidase, and secreted proteins such as DNase and EndoS (2, 6, 33, 45). It is thought that SpeB enhances host tissue destruction and bacterial dissemination during GAS infection (6, 7, 23).

In the present study, we found that different GAS strains have different *covR* expression patterns during bacterial growth. The difference in the *covR* expression pattern among GAS strains is associated with different bacterial growth and SpeB expression patterns. In addition, *covR* expression at the early stationary phase is highly associated with *emm1*/ST28 strains. Our results show that epidemiologically important *emm1*/ST28 strains present unique *covR* and *speB* expression patterns and exhibit faster growth.

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TABLE 1. *emm* types, PFGE types, *covR* expression patterns, and MLST characteristics of 21 GAS strains

Strain	<i>emm</i> type	PFGE type	<i>covR</i> expression pattern	Collection yr	Allele profile (<i>gki</i> , <i>gtr</i> , <i>murI</i> , <i>mutS</i> , <i>recP</i> , <i>xpt</i> , <i>yiqL</i>)	MLST ST
113	4	H	Exponential phase	1995	5, 7, 8, 5, 15, 2, 65	391
133	4	I	Exponential phase	1995	5, 11, 8, 5, 15, 2, 1	39
261	12	Q	Exponential phase	1998	5, 2, 2, 6, 6, 2, 2	36
516	22	O	Exponential phase	1999	9, 8, 1, 1, 1, 3, 4	46
574	12	F	Exponential phase	2000	5, 2, 2, 57, 6, 2, 2	465
625	89	P	Exponential phase	2001	16, 2, 8, 3, 1, 75, 3	466
747	12	F	Exponential phase	2003	5, 2, 2, 57, 6, 2, 2	465
754	12	G	Exponential phase	2003	5, 2, 2, 6, 6, 3, 2	467
L-257	95	N	Exponential phase		2, 6, 8, 3, 9, 3, 66	400
L-259	73	M	Exponential phase		43, 2, 2, 47, 1, 3, 4	331
NZ131	49	L	Exponential phase		4, 6, 2, 7, 85, 7, 1	424
165	1	B	Early stationary phase	1997	4, 3, 4, 4, 2, 4	28
276	89	K	Early stationary phase	1998	16, 2, 8, 3, 2, 13, 1	384
602	1	A	Early stationary phase	2000	4, 3, 4, 4, 4, 2, 4	28
646	1	C	Early stationary phase	2002	4, 3, 4, 4, 4, 2, 4	28
654	1	A	Early stationary phase	2002	4, 3, 4, 4, 4, 2, 4	28
663	75	J	Early stationary phase	2002	102, 2, 44, 6, 51, 3, 74	468
A-20	1	A	Early stationary phase	1994	4, 3, 4, 4, 4, 2, 4	28
L-395	1	D	Early stationary phase	1995	4, 3, 4, 4, 4, 2, 4	28
L-435	1	A	Early stationary phase	2002	4, 3, 4, 4, 4, 2, 4	28
SF370	1	E	Early stationary phase		4, 3, 4, 4, 4, 2, 4	28

MATERIALS AND METHODS

Bacterial strains. The GAS clinical isolates analyzed in this study were randomly selected from the collection consecutively archived between 1994 and 2003 at National Cheng Kung University Hospital, Tainan, Taiwan (Table 1). GAS strain NZ131 (M49) was described previously (43). SF370 (M1T1) is strain ATCC 700294. All GAS strains were grown in tryptic soy broth (Becton, Dickinson and Company, Sparks, MD) supplemented with 0.5% yeast extract (TSBY).

RNA extraction and Northern hybridization. RNA extraction from GAS was performed by the method described previously (12). Briefly, the bacterial pellet was washed with ice-cold 0.2 M sodium acetate twice and resuspended in buffer (100 mM of Tris-HCl [pH 7.0], 1 mM EDTA, 25% [wt/vol] glucose) containing 40 μ l of lysozyme (20 mg/ml) and 10 μ l of mutanolysin (1 U/ μ l; Sigma Chemicals, St. Louis, MO) and then incubated at 37°C for 1 h. After incubation, bacterial pellets were collected and resuspended in 500 μ l of acetic buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA, 0.5% [wt/vol] sodium dodecyl sulfate) with 500 μ l of hot phenol. After vortexing, the mixtures were centrifuged at 14,000 $\times$ g for 20 min. The upper aqueous layer was collected, and RNAs were precipitated with isopropanol. RNAs were dissolved in diethyl pyrocarbonate-H₂O and treated with DNase (Promega, Madison, WI) at 37°C for 2 h. After DNase treatment, RNAs were obtained by using phenol-chloroform extraction and isopropanol precipitation. Northern hybridization analysis was performed as described previously (12). The probes used for detecting *covR*, *speB*, *sagA*, *emm*, and *scpA* expression are described in Table 2. After hybridization, the membranes were washed and the hybridized RNA transcripts were detected by a LAS3000 Intelligent Dark Box (Fujifilm, Japan). All Northern hybridization analyses were performed at least twice independently.

Western hybridization. Three hundred microliters of bacterial culture supernatant was mixed with 1.2 ml of acetone and incubated at -20°C for 1 h to precipitate the total protein. The precipitated protein was collected by centrifugation at 14,000 $\times$ g for 20 min at 4°C. The protein pellet was dissolved in 50 mM NaOH, and the protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Two micrograms of total protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by transfer to polyvinylidene difluoride membranes. The membrane was blocked with phosphate-buffered saline (PBS) containing 5% (wt/vol) skim milk at 37°C for 2 h and probed with anti-C192S mouse serum prepared as previously described (28). After three washes with PBS containing 0.05% Tween 20 (PBST), the membrane was incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody (Chemicon International, Inc., Temecula, CA). After incubation, the membrane was washed with PBST solution three times and then was treated with chemiluminescent substrate (Pierce ECL Western blotting substrate; Thermo Fisher Scientific, Inc., Rockford, IL) to visualize the signal. The signal was detected with the LAS 3000 Intelligent Box (Fujifilm, Japan). All Western hybridization analyses were performed at least twice independently.

Bacterial growth curve with kinetic parameters. To obtain bacterial growth curves, GAS strains were cultured in TSBY broth at 37°C for 16 h. One milliliter of overnight culture was transferred to 100 ml of fresh TSBY and incubated at 37°C without agitation. The optical density of the bacterial culture at 600 nm (OD₆₀₀) was measured by UV/visible spectrophotometer (GE Healthcare, United Kingdom) after 3 to 17 h of incubation.

The bacterial growth curve was fitted by using a modified logistic model to estimate the growth parameters asymptotes (*A*), maximum specific growth rate

TABLE 2. Primers used in this study

Primer	Sequence (5'→3')	Use	Source or reference
CovR-F	ATGACAAAGAAAATTTTAAT	<i>covR</i> probe	This study
CovR-R	TTATTTCTCACGAATAACGT	<i>covR</i> probe	This study
SpeB-6855	TACTAAGGTTTGATGCCTAC	<i>speB</i> probe	This study
SpeB-8195	CCGTGTTATTGTCTATTACC	<i>speB</i> probe	This study
SSA-1	CTCGCGTTCTTATCAGTTAC	<i>sagA</i> probe	This study
SSA-2	ACCTGGCGTATAAATTCC	<i>sagA</i> probe	This study
Emm1-1	CGATCGAAAAAGCAAACTTG	<i>emm1</i> probe	This study
Emm1-2	CTCCAGCTGTTGCCATAACA	<i>emm1</i> probe	This study
ScpA-1	CTAAAACTGCTGATACACCAGC	<i>scpA</i> probe	30
ScpA-2	ACCGTCTTTTCGACTGATAAAG	<i>scpA</i> probe	30

FIG. 1. *covR* expression patterns and growth rates in SF370 and NZ131. (A) *covR* expression pattern during bacterial growth in SF370 and NZ131. The RNAs were extracted after 3, 5, and 7 h of incubation, and *covR* expression in SF370 and NZ131 was analyzed by Northern hybridization with an alkaline phosphatase-labeled *covR* probe. *covR/S* represents the *covRS*-cotranscribed RNA; *covR* represents the *covR* monocistronic transcripts. The lower panels show the 23S rRNA and 16S rRNA as the internal controls. (B) Growth curves of SF370 and NZ131. The bacterial density was measured by spectrophotometer at OD₆₀₀ after 3 to 17 h of incubation. The data presented are from three independent experiments.

(μ_m), and lag time (λ), as described by Zwietering et al. (47). A is defined as the maximal value of bacterial growth, μ_m is defined as the tangent in the inflection point of the bacterial growth curve, and λ is defined as the x -axis intercept of this tangent. Experiments in duplicate were performed, and the averages were used for estimating growth parameters.

emm typing and PFGE typing. *emm* typing and PFGE typing were performed as previously described (46). The PFGE fragment patterns were interpreted in accordance with the criteria of Tenover et al. (41). PFGE fragment patterns were compared with the use of GelCompar II software (Unimed Healthcare, Inc., Houston, TX), by using the unweighted pair group method using average linkages (UPGMA) based on the Dice coefficient with a position tolerance of 1.5%. PFGE-based clusters were defined as isolates with a genetic relatedness of >80% on a dendrogram.

MLST. GAS genomic DNA was prepared as previously described (12). Seven housekeeping genes of GAS (*gki*, *gtr*, *murI*, *mutS*, *recP*, *xpt*, and *yiqL*) were amplified and sequenced using the primers and conditions described in the MLST database (www.mlst.net). New housekeeping allele sequences and STs generated from this study were submitted to the MLST database. To generate dendrograms, sequences of the seven housekeeping genes used in the MLST scheme were joined into a 3,134-bp concatenated sequence and a dendrogram was constructed from concatenated sequences by the UPGMA method and the Kimura two-parameter nucleotide substitution model with 1,000 bootstrap trials

(25). A clonal cluster was defined by bootstrap value of >95 and further verified by the eBURST program (eburst.mlst.net).

Statistics. Statistical analysis was performed by using Student's t test. A P value of <0.05 was taken as significant.

RESULTS

***covR* expression pattern in SF370 and NZ131.** To investigate the *covR* expression pattern in SF370 (*emm1*) and NZ131 (*emm49*) during bacterial growth, the RNAs were extracted from SF370 and NZ131 at different phases of growth and *covR* expression was analyzed by Northern hybridization with an alkaline phosphatase-labeled *covR* probe. *covR* and *covS* consist of an operon, the *covR*- and *covS*-cotranscribed RNA (*covRS*), and *covR* monocistronic transcripts (*covR*) were detected by a *covR* probe (Fig. 1A) (12). The results showed that SF370 had maximal *covR* RNA expression at the early stationary phase (after 5 h of incubation), whereas NZ131 had its maximal *covR* expression in the exponential phase of growth

(Fig. 1A and B). No difference was found in *covRS*-cotranscribed RNA between SF370 and NZ131 (Fig. 1A).

Two different *covR* expression patterns are distributed in GAS. Nineteen clinical strains collected from 1994 to 2003 were analyzed for their *covR* expression pattern by Northern hybridization with an alkaline phosphatase-labeled *covR* probe. Bacteria were cultured in TSBY broth, and *covR* expression was analyzed after 3, 5, and 7 h of incubation. We found nine strains expressed *covR* RNA maximally at the early stationary phase (*covR*-ST), and 10 strains expressed *covR* RNA at the exponential phase (*covR*-E). Among the *covR*-E strains, six different *emm* types were found (two of *emm4*, four of *emm12*, one of *emm22*, one of *emm73*, one of *emm89*, and one of *emm95*). All of the *emm1* strains were the *covR*-ST phenotype. In addition, one *emm75* strain and one *emm89* strain were the *covR*-ST type (Table 1).

The growth curves of the *covR*-E and *covR*-ST strains. SF370 and NZ131 expressed *covR* RNA at different phases of growth (Fig. 1A). In addition, the growth curves of SF370 and NZ131 had different characteristics (Fig. 1B). Apparently, SF370 had the better growth activity and spent less time reaching the stationary phase than did NZ131 (Fig. 1B). To further demonstrate that the *covR* expression pattern was associated with bacterial growth activity, we analyzed the growth curves of 21 strains (SF370, NZ131, and 19 clinical isolates) (Table 1). Bacteria were cultured in TSBY broth at 37°C, and the bacterial density was determined by spectrophotometer at 600 nm after 3, 5, 7, and 9 h of incubation. The average of the bacterial density of *covR*-E or *covR*-ST strains at each time point was calculated and plotted on a chart to represent the growth curve of *covR*-E and *covR*-ST strains (Fig. 2A). This chart showed that the *covR*-ST strains had faster growth and reached the stationary phase faster than did the *covR*-E strains (Fig. 2A).

To demonstrate the growth curve characteristics mathematically, we used the mathematic model to analyze the bacterial growth kinetics of our clinical isolates. The results showed the lag time (λ of 4.096 for *covR*-E strains and 4.234 for *covR*-ST strains) and the maximal value of bacterial growth (A of 1.977 for *covR*-E strains and 1.947 for *covR*-ST strains) were no different between *covR*-E and *covR*-ST strains ($P > 0.5$). However, the maximum specific growth rate (μ_m) of the *covR*-ST strains was significantly higher than that of the *covR*-E strains (Fig. 2B) ($P < 0.05$).

***speB* and *sagA* expression in *covR*-ST and *covR*-E strains.** Gene expression in GAS is controlled by growth-phase regulation (9, 43). The transcription of *speB* and *sagA* is restricted to the stationary phase, and expression of *emm* and *scpA* is restricted to the exponential phase. We hypothesized that the *covR*-ST strains, which had better growth activity, would express *speB* and *sagA* genes earlier than did the *covR*-E strains. To verify this hypothesis, SF370 and A-20 (*covR*-ST strains) and NZ131 and GAS516 (*covR*-E strains) were cultured for 6 to 10 h in TSBY broth and *speB* and *sagA* transcripts were analyzed by Northern hybridization with the alkaline phosphatase-labeled *speB* and *sagA* probes, respectively. The results showed that *speB* expression in SF370 was detected after 6 h of incubation, whereas *speB* expression in NZ131 was only detected after 10 h of incubation (Fig. 3A). Similarly, A-20 *speB* expression was detected after 6 h of incubation, and GAS516 *speB* expression was detected after 8 h of incubation (Fig. 3B).

FIG. 2. Growth curve characteristics of strains exhibiting two different *covR* expression patterns. Strains having *covR* expressed at the exponential phase are defined as the *covR*-E strains, and those with *covR* expressed at the early stationary phase are defined as the *covR*-ST strains. (A) Growth curves of the *covR*-E and *covR*-ST strains. The bacterial density (measured by spectrophotometer at OD₆₀₀) of the *covR*-E or *covR*-ST strains after 3, 5, 7, and 9 h of incubation was recorded, and the average value was calculated. The bacterial growth curve was plotted according to the calculated average values. Data for the growth kinetics for each strain were from two independent experiments. (B) Maximal specific growth rate (μ_m) of the *covR*-ST and *covR*-E strains. The mathematic model of bacterial growth curve was described in Materials and Methods. Statistical analysis was performed with Student's *t* test. *, $P < 0.05$.

However, the *sagA* expression pattern in these strains was similar. Expression was detected after 6 h of incubation and increased after 8 and 10 h of incubation in SF370, NZ131, and GAS516 (Fig. 3C).

To further clarify the *speB* expression pattern in SF370 and NZ131, SpeB protein secretion was detected by Western hybridization. SF370 and NZ131 were cultured in TSBY broth for 3 to 20 h, and SpeB protein in culture supernatant was analyzed by Western hybridization with anti-C192S mouse serum (C192S is a protease-negative recombinant SpeB protein). The results showed that the zymogen form of SpeB protein (42 kDa) was detected in SF370 culture supernatant after 8 h of incubation, whereas it was detected after 12 h of incubation in NZ131 (Fig. 3D). In addition, after 12 h of incubation, SF370 accumulated more SpeB protein in culture supernatant than did NZ131 (Fig. 3D).

***emm* and *scpA* expression in *covR*-E and *covR*-ST strains.** *emm* and *scpA* expression was restricted to the exponential phase of growth. We also hypothesized that *covR*-ST strains expressed these genes in a more restricted pattern during bac-

FIG. 3. *speB* and *sagA* expression patterns in *covR*-ST and *covR*-E strains. (A and B) *speB* expression pattern during bacterial growth. (C) *sagA* expression pattern during bacterial growth. RNAs were extracted after 6, 8, and 10 h of incubation, and *speB* and *sagA* expression was analyzed by Northern hybridization with the alkaline phosphatase-labeled *speB* and *sagA* probes, respectively. SF370 and A-20 are the *covR*-ST strains, and NZ131 and GAS516 are the *covR*-E strains. The lower panels show 23S rRNA and 16S rRNA as the internal controls. (D) SpeB protein in culture supernatant of SF370 (upper panel) and NZ131 (lower panel). The total proteins were collected from bacterial culture supernatant at different culture time points. The SpeB protein in culture supernatant was analyzed by Western hybridization with anti-C192S (SpeB protease mutant recombinant protein) mouse serum. The zymogen form of SpeB is 42 kDa, and the mature form of SpeB is 28 kDa.

terial growth than did *covR*-E strains. The SF370 and A-20 strains (*covR*-ST) and NZ131 (*covR*-E) were cultured for 3 to 9 h, and *emm* and *scpA* transcripts were analyzed by Northern hybridization with alkaline phosphatase-labeled *emm1* and *scpA* probes, respectively. The results showed that SF370, A-20, and NZ131 maximally expressed *emm* and *scpA* genes at 5 h of incubation and decreased expression after 7 h of incubation (data not shown).

MLST analysis. We found that all *emm1* strains expressed *covR* at the early stationary phase of growth. Therefore, we hypothesized that the strains expressing *covR* at the early stationary phase were clonally related. To verify this hypothesis, MLST analysis was used to analyze all 21 strains. The allelic profile, *emm* type, and epidemiologic information of strains in this study were posted on the MLST database (www.mlst.net). Four new MLST STs (465, 466, 467, and 468) were found in the present study. Thirteen STs were found in 21 GAS strains. Among them, all *emm1* strains were ST28 (Table 1). Strains 663 and 276, which expressed *covR* at the early stationary phase, were ST468 and ST384, respectively.

The MLST-based dendrogram analysis showed four major clonal clusters (Fig. 4). Eight *emm1* strains with identical STs (ST28) were defined as cluster 1 (Fig. 4). Cluster 2 and cluster 3 were composed of four *emm12* strains with three different STs (36, 465, and 467) and two *emm4* strains with two different STs (391 and 39), respectively (Fig. 4). L257 (*emm95*/ST400) was cluster 4. Furthermore, strains that shared more than five identical housekeeping loci were classified into one clonal cluster by eBURST analysis. eBURST analysis showed similar classification results except for cluster 4 (L257; data not

shown). Six strains (276, 625, 516, 663, L259, and NZ131) were singletons (Fig. 4). Other than strains 276 and 663, clusters 2, 3, and 4 and other singleton strains all expressed *covR* at the exponential phase of growth (Fig. 4).

PFGE type analysis. A PFGE-based dendrogram showed four major clusters (Fig. 5). The largest cluster (cluster P2) comprised 6 *emm1*/ST28 strains. Another *emm1*/ST28 strain, L395, has >75% genetic relatedness to cluster P2 strains (Fig. 5). Strains SF370 (*emm1*/ST28), 276 (*emm89*/ST384), and 663 (*emm75*/ST468), expressed *covR* at the early stationary phase and were not related to any specific clusters. Clusters P1 and P4 comprised two *emm4* and three *emm12* strains, respectively (Fig. 5). Cluster P3 comprised *emm73* and *emm95* strains with different STs. Except for cluster P2 strains, which expressed *covR* at the early stationary phase, cluster P1, P3, and P4 strains all expressed *covR* at the exponential phase of growth (Table 1).

DISCUSSION

Although *emm1*/ST28 is the predominant type isolated from GAS infections in many countries and associated with severe diseases such as necrotizing fasciitis and STSS, the underlying mechanism for these important epidemiological observations is still not clear. In the present study, we show two different *covR* expression patterns among GAS strains during their growth. Strains with *covR* expression at the early stationary phase are highly correlated with the *emm1*/ST28 type and associated with faster growth than the strains with *covR* expression at the exponential phase. In addition, we found that

FIG. 4. MLST-based dendrogram of 21 GAS strains and their STs and *emm* types. The dendrogram was generated by comparing the concatenated housekeeping genes sequence by UPGMA with the Kimura two-parameter method. A clonal cluster (no. 1 to 4) was verified by a bootstrap value of >95 and eBURST analysis. The value shown on nodes of the branches is the bootstrap value. The scale bar represents the sequence similarity. Asterisks indicate the strains with *covR* expression at the early stationary phase of growth.

the different growth activity affects the *speB* expression pattern, but not *sagA*, *emm*, and *scpA* expression. To our knowledge, this is the first demonstration that *covR* is expressed at different growth phases among the GAS population and that the different expression is significantly associated with different growth rates and *speB* expression patterns.

CovRS is a global regulatory system and regulates about 15% of gene expression in GAS. Previously, we found that the *covRS* operon transcribes three different RNA transcripts, which are *covRS* cotranscribed RNA and two different sizes of *covR* monocistronic transcripts (12). CovRS was thought to be expressed at the exponential phase (20). In this study, we found two *covR* expression patterns among GAS and showed that the different *covR* expression patterns are significantly associated with bacterial growth rates (Fig. 2). The different growth kinetics and *covR* expression patterns between *covR*-E and *covR*-ST strains suggest that the virulence gene expression patterns may be different between these two groups of strains. However, the results showed that *emm*, *scpA*, and *sagA* expression patterns in SF370, A-20, NZ131, and GAS516 were similar, whereas the *speB* expression pattern was different between *covR*-ST (SF370 and A-20) and *covR*-E (NZ131 and GAS516) strains (Fig. 3). One possibility to explain the different *SpeB* expression patterns in *covR*-ST and *covR*-E strains is that the bacterial-density-related factors are required for triggering *SpeB* expression. In culture broth, bacteria consume the carbohydrates and release metabolic products, causing a decrease of the broth pH. The acidic pH of the broth and carbohydrate

starvation are signals for triggering *SpeB* expression (10, 29). Therefore, these positive signals might induce *SpeB* expression at an early culture stage in the *covR*-ST strains. Although the interaction between CovR and *SpeB* is controversial, CovR is considered the regulator to control *SpeB* expression (17, 21, 38). We have constructed the *covR* isogenic mutation in SF370, A-20, NZ131, and GAS516 strains and analyzed *speB* and *sagA* expression pattern. In SF370, A-20, and GAS516 *covR* mutants, *speB* and *sagA* expression were increased (data not shown). However, we found in NZ131 (*covR*-E strain) *covR* isogenic mutant, *speB* expression was similar when compared with that of the wild-type strain, but *sagA* expression was increased in the mutant strain (data not shown). These results suggest that the different *speB* expression patterns in these strains are not directly responsible for different *covR* expression patterns.

SpeB is a cysteine protease which degrades several host and bacterial proteins (35). Recently, *SpeB* was shown to degrade GAS-secreted DNase to decrease the neutrophil resistance ability of GAS (45). These reports indicate that *SpeB* has broad biological effects during GAS infection. Therefore, the timing of *SpeB* expression during infection would be critical for GAS to establish a successful infection. In this study, we showed that the *SpeB* expression pattern is different in *covR*-ST and *covR*-E strains. SF370 expresses *SpeB* earlier than NZ131 and also accumulates more *SpeB* protein in culture supernatant during the same incubation period (Fig. 3A and D). Different *SpeB* expression patterns could cause differ-

FIG. 5. Dendrogram and PFGE band patterns of SmaI-digested chromosomal DNA of 21 GAS isolates. The dendrogram was constructed by using UPGMA based on the Dice coefficient with a position tolerance of 1.5%. Clusters P1 to P4 were defined by genetic relatedness of >80% on a dendrogram. Asterisks indicate the strains with *covR* expression at the early stationary phase.

ent levels of bacterial cell surface protein integrity and might also contribute to different degrees of tissue damage.

emm1 GAS is the predominant clone isolated from GAS infections in many countries and is associated with severe infections such as necrotizing fasciitis and STSS (4, 16). In general, *emm1* strains have several superantigen genes, such as *speA*, *speF*, *speG*, and *smeZ* (8, 24). Furthermore, the presence of the prophage-associated *speA* gene is statistically associated with strains recovered from patients with severe or recurrent GAS infections (5, 32, 40). In addition, the chromosomal *sic* gene (coding for streptococcal inhibitor of complement) is also considered to be present in all *emm1* but not other *emm* type strains (1, 22). In the present study, we found *covR* expression at the early stationary phase of growth is highly associated with *emm1*/ST28 strains (Table 1). These results suggest that, along with the presence of the *speA* and *sic* genes, *covR* expression at the early stationary phase of growth is one of the specific characteristics of *emm1*/ST28 strains.

MLST analysis showed all *emm1* strains comprised a clonal cluster with identical STs (Fig. 4). PFGE analysis further showed that, except for SF370, all *emm1* strains have similar PFGE band patterns (Fig. 5). SF370 has no *speA* gene, which is carried on a prophage (3). The lack of the prophage integration in the SF370 genome could be the reason for the completely different PFGE band pattern compared with other *emm1*/ST28 strains. *emm1* isolates were randomly chosen from our collections from 1994 to 2002, suggesting that these isolates were not collected from a single clonal outbreak (Table 1). These results indicate that the *emm1*/ST28 strains should be

highly homogenous, which supports the observations of previous studies (8, 31, 39). Importantly, *emm1*/ST28/PFGE type A is also the dominant type found in Canada, Portugal, and northern Taiwan, reinforcing its epidemiologic significance on a global scale (8, 11, 19). However, due to the limited geographical origin of these strains, the phenomenon of our finding requires further study on strains from different parts of the world.

In summary, we found that two different *covR* expression patterns were distributed among GAS strains. Strains with *covR* expression at the early stationary phase are highly correlated with the *emm1*/ST28 type and exhibited faster bacterial growth and earlier SpeB expression patterns than the other group of strains. Since *emm1*/ST28 is a predominant type of GAS isolates, we suggest that the specific *covR* expression pattern, faster growth, and earlier SpeB expression in *emm1*/ST28 strains could be a potential key phenotype found in global distribution of *emm1* *S. pyogenes*.

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