

## Effects of Oligopeptide Permease in Group A Streptococcal Infection

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The oligopeptide permease (Opp) of group A streptococci (GAS) is a membrane-associated protein and belongs to the ATP-binding cassette transporter family. It is encoded by a polycistronic operon containing *oppA*, *oppB*, *oppC*, *oppD*, and *oppF*. The biological function of these genes in GAS is poorly understood. In order to understand more about the effects of Opp on GAS virulence factors, an *oppA* isogenic mutant was constructed by using an integrative plasmid to disrupt the *opp* operon and confirmed by Southern blot hybridization. No transcript was detected in the *oppA* isogenic mutant by Northern blot analysis and reverse transcriptase PCR. The growth curve for the *oppA* isogenic mutant was similar to that for wild-type strain A-20. The *oppA* isogenic mutant not only decreased the transcription of *speB*, *speX*, and *rofA* but also increased the transcription of *speF*, *sagA* (streptolysin S-associated gene A), *slo* (streptolysin O), *pel* (pleiotrophic effect locus), and *dppA* (dipeptide permease). No effects on the transcription of *emm*, *sda*, *speJ*, *speG*, *rgg*, and *csrR* were found. The phenotypes of the *oppA* mutant were restored by the *oppA* revertant and by the complementation strain. The *oppA* mutant caused less mortality and tissue damage than the wild-type strain when inoculated into BALB/c mice via an air pouch. Based on these data, we suggest that the *opp* operon plays an important role in the pathogenesis of GAS infection.

Group A streptococci (GAS) are important human pathogens that cause pharyngitis, impetigo, and many other human respiratory tract and soft tissue infections. A number of regulators, such as Mga, RofA, Rgg, Dpp, Nra, CsrS/CsrR, Pel, Fas, RelA, and oligopeptide permease (Opp), are likely to have regulatory roles in the expression of the virulence genes of GAS (3, 6, 13, 17, 18, 29, 35, 36, 38, 44). The expression of M protein, C5a peptidase, serum opacity factor, streptococcal A, and streptococcal pyrogenic exotoxin B (SpeB) is positively regulated by Mga, a transcriptional activator protein (37) which is predicted to be a response regulator of the streptococcal two-component system (38). In addition to being positively regulated by Mga, the expression of SpeB is also positively regulated by Rgg, Opp, and Dpp (6, 35, 36) and negatively regulated by the two-component system CsrS/CsrR (13).

Opp has been identified in several gram-negative and gram-positive bacteria (21). It belongs to the ATP-binding cassette (ABC) transporter superfamily (33). The *opp* operon encodes five proteins, including a periplasmic binding protein (OppA), two transmembrane proteins (OppB and OppC) believed to form a channel for passage of the substrate, and two membrane-associated cytoplasmic ATPases (OppD and OppF) (36). ABC transporters have been shown to be important in the *Agrobacterium tumefaciens* response to opines (9), the competence of *Streptococcus pneumoniae* (14), and the uptake of

oligopeptides of three to six amino acids in *Salmonella enterica* serovar Typhimurium, *Bacillus subtilis*, *Lactococcus lactis*, and *Streptococcus agalactiae* (7, 21, 42). The biological functions of Opp in GAS are not clear yet. Previously, Podbielski et al. (36) demonstrated that mutations in *oppD* and *oppF* decrease the expression of SpeB.

In this study, a conserved region of sensor regulators in bacterial two-component system genes was used to screen the GAS genomic library, and the *opp* gene was detected. Furthermore, mutation of the *oppA* gene not only affects *speB* expression but also affects the expression of other virulence genes and regulatory genes. Finally, we found that Opp is important in the virulence of GAS in mice.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and mice.** GAS strain A-20 (type M1, T1, opacity factor negative) was isolated from a patient with necrotizing fasciitis. GAS strain SW507 is a cysteine protease (*speB*) mutant and is isogenic with A-20 (45). All GAS cultures were grown in tryptic soy broth supplemented with 0.5% yeast extract (TSBY) at 35°C. *Escherichia coli* DH5 $\alpha$  (Bethesda Research Laboratories, Gaithersburg, Md.) was grown at 37°C with vigorous aeration in Luria broth, which was supplemented with 100  $\mu$ g of kanamycin per ml when the strain was carrying plasmid pSF151. Plasmid pSF151 was kindly provided by L. Tao, University of Missouri, Kansas City (43). Plasmid pDL278, used in *opp* complementation experiments, was provided by D. J. LeBlanc, formerly of the University of Texas Health Science Center, San Antonio (22). All strains were stored at –75°C in TSBY with 15% glycerol until testing. BALB/c mice were maintained with standard laboratory food and water in the laboratory animal center. The mice used in the experiments weighed about 25 g and ranged in age from 6 to 8 weeks.

**DNA manipulation and cloning of the *opp* gene.** Plasmid DNA was isolated by the alkaline lysis method as previously described (41). Chromosomal DNA was isolated from GAS as described previously (5). An integrational library of GAS DNA was constructed by partial digestion with Sau3AI; fragments of 4 to 8 kb were cloned into plasmid pSF151. A conserved region of sensor regulators in bacterial two-component system genes, 5'-ATA TCA AAT CCT AAT CCG GTT ACT-3', was used as a probe to screen the GAS A-20 library by colony

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TABLE 1. Specific PCR primer sets used in RT-PCR analysis

Gene	Primer <sup>a</sup>	Sequence (5'→3')	Start site	Reference or source <sup>b</sup>
<i>speB</i>	Fwd	GGATCCATATGGATCAAACCTTTGCTCGTAACG	81	12
	Rev	GAATTCGGATCCTAAGGTTTGATGCCTACAACAG	1175	
<i>speF</i>	Fwd	ATGGCGCAAGCAAGTACCTA	977	31
	Rev	TTTGAGTAGGTGTACCTTAT	1624	
<i>speX</i>	Fwd	TTTCTCGTCCTGTGTTTGGGA	122	10
	Rev	TTGTGATATTA AAAATTTTCTCGCC	574	
<i>speG</i>	Fwd	ACCCCATGCGATTATGAAAA	4797	8
	Rev	GGGAGACCAAAAACATCGAC	4966	
<i>speJ</i>	Fwd	TCTTTCATGGGTACGGAAGTG	41	This study
	Rev	AGCTCTCGACCTCAGAATCAA	238	
<i>sagA</i>	Fwd	CTCGCGTTCTTATCAGTTAC	140	2
	Rev	ACCTGGCGTATAACTTCC	339	
<i>sagB</i>	Fwd	TGTCCGCCAATAACTGTTGA	727	This study
	Rev	CACCGTATTCCGAAAATCT	1365	
<i>slo</i>	Fwd	ACTCTGTCACTGATAGGACC	698	This study
	Rev	GAGCTGCTTCAATCTGTG	998	
<i>csrS</i>	Fwd	TGGGTTTTCCATGACACAAA	1400	23
	Rev	AGCGCCGCGTAGTAATTAAG	1594	
<i>csrR</i>	Fwd	TGCAACATGAGGGGTATGAA	220	23
	Rev	TAAACCTGCAACCACATCCA	531	
<i>emm1</i>	Fwd	AACGGCTTCAGTAGCGGTAG	1082	11
	Rev	CTGCAACTTCCATTGCATTC	1281	
<i>sdaD</i> (DNase)	Fwd	CACAACAGCCAGGGAATTTT	925	37
	Rev	GATGGTCTTGGTCCTCCTTG	1105	
16S rRNA	Fwd	GCAGCAGTAGGGAATCTTCG	330	This study
	Rev	CGCTCGGGACCTACGTATTA	530	
<i>oppA</i>	Fwd-1	ATGAAGAAAAGTAAATGGTT	1382	36
	Fwd-2	GATCCACGGACCTACCTTGA	2901	
	Rev-1	TTATTTTCAACGTGATCAG	3352	
	Rev-2	CAGCTGCCACAACATCCTTA	3003	
<i>dacA</i>	Fwd-1	AGGCGATGACCCACTAGGGA	1015	36
	Fwd-2	TATTAGCTCCTAAAAATG	559	
<i>dppA</i>	Fwd	GAAAACCTGCAGTACTGAGGCGCATTGACA	1247	35
	Rev	GGCCGGAATTCTCAGAAGATGTCATTGCTT	2189	
<i>rofA</i>	Fwd	ATCGTCTGCTATATAGTAAG	520	This study
	Rev	ACCGTCTCAGTGCTATCAA	2031	
<i>pel</i>	Fwd	AGGAGGTAAACCTTATGTT		24
	Rev	GCTAAATAGATTATTTACCTG		
<i>oppF</i>	Rev	TTACAATTCTTTTTGATATT	7840	36

<sup>a</sup> Fwd, forward; Rev, reverse.

<sup>b</sup> Primers for which the source is listed as this study were predicted according to Primer3 software ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) calculations. The accession numbers for *speJ*, *sagB*, *slo*, 16S rRNA, and *rofA* are AF321000, AF067649, A28468, AB023575, and U01312, respectively, in the National Center for Biotechnology Information nucleotide database.

hybridization. Plasmid pMW213, containing about 1.1 kb of *oppA*, was selected and used for insertional mutagenesis of the *opp* gene to construct an *oppA* isogenic mutant. A 6.83-kb PCR product was amplified with *dacA* Fwd-1 and *oppF* Rev primers (Table 1), which contained the entire *opp* operon (6.46 kb) and a part of the *dacA* gene (encoding a penicillin-binding protein) located in the region upstream of the *oppA* gene. The linear 6.83-kb PCR fragment was transformed into the *oppA*

isogenic mutant to obtain a revertant. An *opp* complementation strain was also constructed. A 7.3-kb PCR product was amplified with *dacA* Fwd-2 and *oppF* Rev primers (Table 1), which included the full-length *opp* gene and a 0.9-kb region upstream of the *oppA* translation start site. This DNA was cloned into vector pDL278 and confirmed by the restriction enzyme digestion pattern and *oppA* and *oppF* PCR results. The resulting plasmid was designated pMW369.

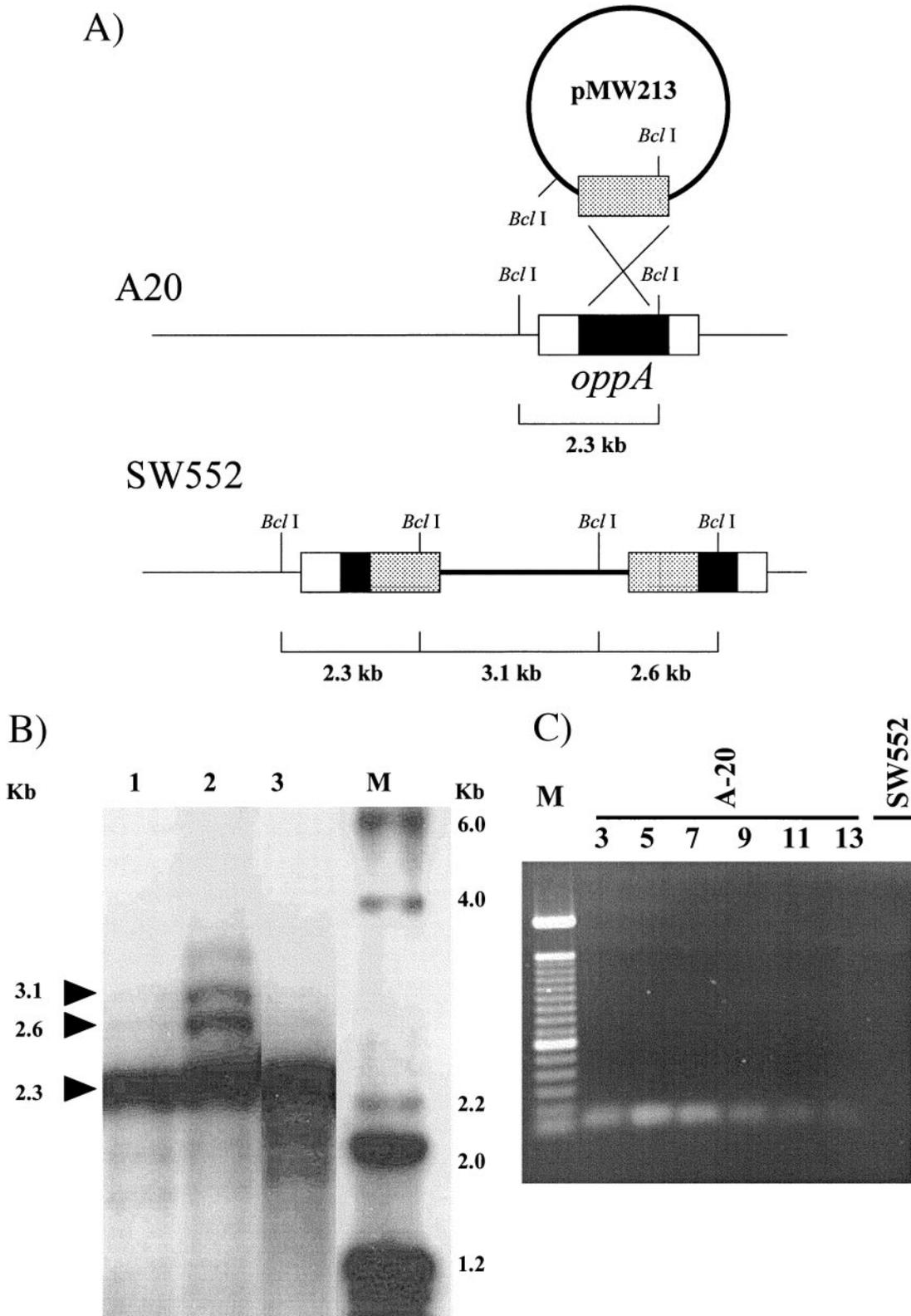


FIG. 1. (A) Map of the construction of the *oppA* isogenic mutant showing the *Bcl*I restriction sites in the *oppA* locus. The predicted hybridization fragments are also shown. (B) Southern blot assay of genomic DNA digested with *Bcl*I and extracted from GAS A-20 (lane 1), SW552 (lane 2), and SW553 (lane 3). A  $\lambda$  HindIII marker was used as a molecular size standard (lane M). The 1.97-kb probe was used as specific for the *oppA* gene and  $\lambda$  DNA. (C) Transcription of *oppA* in the wild-type strain (A-20) at various times and in its *oppA* isogenic mutant (SW552). The results of RT-PCR were amplified with *oppA* Fwd-2 and Rev-2 primers. A 100-bp marker was used as a molecular size standard (lane M).

TABLE 2. SLO activities in *oppA*-positive and *oppA*-negative mutant strains of GAS in various atmospheres

Atmosphere	Mean $\pm$ SEM % of SLO hemolysis in <sup>a</sup> :		
	A-20	SW552	SW553
20% O <sub>2</sub> , 0.5% CO <sub>2</sub>	20.00 $\pm$ 1.1 <sup>b</sup>	44.4 $\pm$ 1.1	24.2 $\pm$ 4.3
20% O <sub>2</sub> , 5% CO <sub>2</sub>	25.20 $\pm$ 3.8 <sup>b</sup>	43.5 $\pm$ 1.4	25.2 $\pm$ 1.3
5% O <sub>2</sub> , 10% CO <sub>2</sub>	10.90 $\pm$ 0.5 <sup>b</sup>	17.7 $\pm$ 1.4	12.2 $\pm$ 1.8

<sup>a</sup> The hemolytic activity of SLO was measured as described in the text. The results are from three experiments. Student's *t* test was used for statistical analysis.

<sup>b</sup> The *P* value in a comparison with SW552 was <0.05.

**Transformation and Southern blot analysis.** *E. coli* was transformed by the method of Sambrook et al. (41). For GAS electroporation, the overnight bacterial culture was collected and the pellet was suspended in 10 ml of sterile cold H<sub>2</sub>O. The bacteria were centrifuged for 10 min at 6,000 rpm and 4°C. This step was repeated twice. Finally, the GAS pellet was resuspended in 1.25 ml of sterile cold double-distilled H<sub>2</sub>O. One  $\mu$ g of target plasmid was added to 60  $\mu$ l of chilled GAS competent cells. Electroporation was performed with an ECM 600 electrocell manipulator (BTX Inc., San Diego, Calif.) at 1.8 kV and 129  $\Omega$ . Trans-

formants were selected by kanamycin resistance. Southern blot analysis was performed as described by Sambrook et al. (41).

**Growth curve assays.** The overnight culture of GAS was transferred as 1:100 dilutions to fresh TSBY. The culture was incubated at 35°C without shaking. The absorbance of the culture at 600 nm was measured. Growth curves were determined by time course measurements from 1 to 11 h.

**Protease assays.** Protease activity was detected by the method of Hynes and Tagg (15). GAS were cultured on Columbia agar base (Difco Laboratories, Detroit, Mich.) containing 3% skim milk for the detection of SpeB production (45). The zone of casein hydrolysis was observed after 24 h at 37°C.

**Preparation of anti-SpeB antibody and Western blot analysis.** The preparation of anti-SpeB antibody and Western blot analysis were carried out as described by Tsai et al. (45).

**Hemolytic activity assays.** The hemolytic activities of streptolysins was determined by the methods of Betschel et al. (2) and Limbago et al. (25). GAS were grown to late log phase (optical density at 600 nm [OD<sub>600</sub>], 1.0 to 1.2), and supernatants were collected by centrifugation at 3,500 rpm for 10 min. For testing of streptolysin O (SLO), 750  $\mu$ l of supernatant was added to 4.8  $\mu$ l of 0.4% trypan blue to inhibit streptolysin S (SLS) activity. L-Cysteine was added to a final concentration of 20 mM, and the mixture was incubated at room temperature for 10 min. For testing of SLS, supernatant was added to a final concentration of 0.5 mg/ml of cholesterol to inhibit SLO activity. Both mixtures were added to an equal volume of PBS-washed 5% sheep erythrocytes and

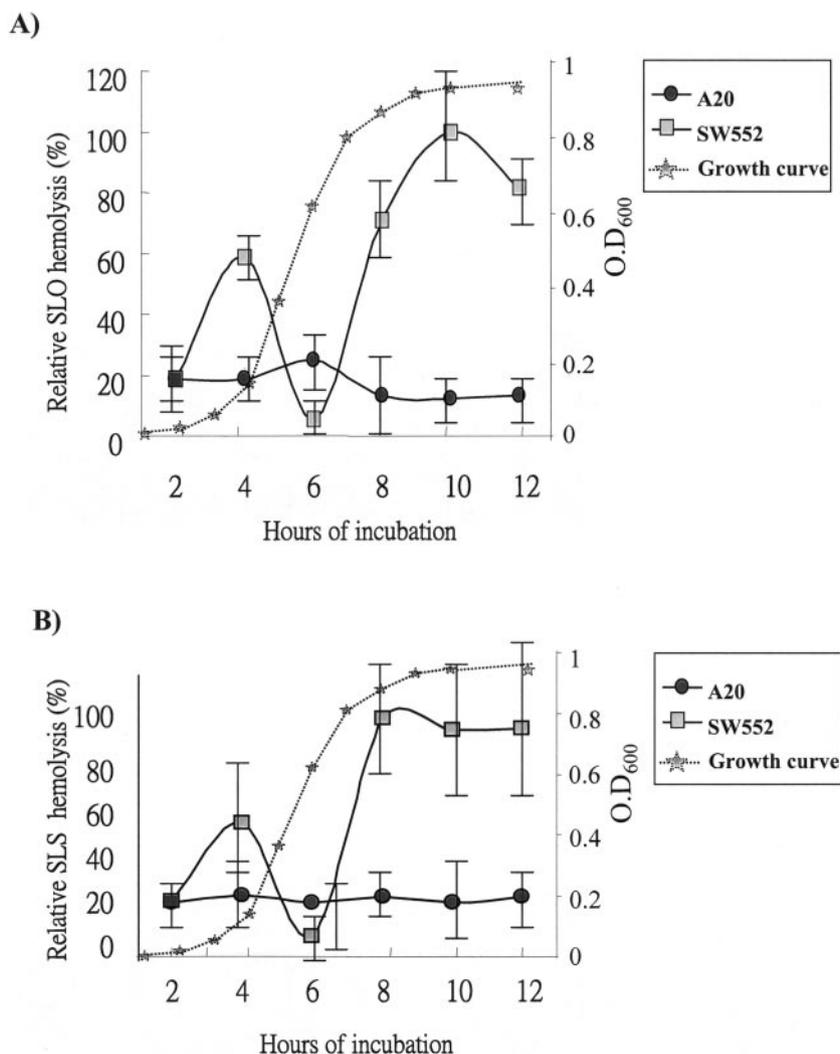


FIG. 2. Variations in hemolytic activities observed in time course assays with wild-type strain A-20 and *opp* isogenic mutant SW552. The growth curve for A-20 is also shown. (A) SLO activities of A-20 and SW552. (B) SLS activities of A-20 and SW552. The error bars indicate the standard deviations. The mean activities of SLO and SLS were calculated in three independent experiments.

incubated at 37°C for 1 h. A 5% suspension of erythrocytes lysed with deionized water served as a 100% hemolysis control. The release of hemoglobin, measured as the OD<sub>540</sub>, represented the relative hemolytic activities of SLO and SLS. Bacterial counts were measured to normalize the hemolytic activities at various time points.

**RNA preparation.** RNA was extracted by the method of Podbielski et al. (37) with modifications. Bacteria were grown in 40 ml of TSBY at 37°C for 16 h. Cells were harvested by centrifugation at 4°C and washed twice in cold 0.2 M sodium acetate. Bacteria were suspended in 10 ml of buffer (100 mM Tris [pH 7.0], 1 mM EDTA, 25% glucose), 200 µl of lysozyme (20 mg/ml; Sigma-Aldrich Co., St. Louis, Mo.), and 20 µl of mutanolysin (5,000 U/ml; Sigma-Aldrich Co.). The reaction mixture was incubated for 30 min at 37°C. The pellet was collected and resuspended in 500 µl of acetate buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]). To disrupt the cells, 1 volume of acetate buffer-saturated phenol (1:1) was added to the cells, and the mixture was kept at 60°C for 5 min. The mixture was vortexed for 5 min and then centrifuged for 10 min. The upper phase was collected. The saturated phenol extraction was repeated twice, and extraction with chloroform-isoamyl alcohol (24:1) was done twice. Acetate buffer was added to the aqueous phase, followed by 3 volumes of ice-cold ethanol. RNA was harvested by centrifugation, washed twice with 70% ethanol, and dried. The final RNA pellet was dissolved in 50 µl of 0.1% diethylpyrocarbonate-treated water and stored at -75°C. RNA was analyzed by electrophoresis to test for the quality of rRNA and by measurement of *A*<sub>260</sub> and *A*<sub>280</sub>. For further processing, RNA was incubated with RNase-free DNase and RNase inhibitor (Boehringer Mannheim, Mannheim, Germany) for 15 min at 37°C and then extracted with phenol as described above.

**RT-PCR analysis.** RNA samples were incubated at 72°C for 10 min to denature the RNA secondary structure and then were placed immediately at 4°C. The RNA template was transcribed into cDNA with a hexamer random primer and Moloney murine leukemia virus reverse transcriptase (RT) (Promega, Madison, Wis.). The first-strand cDNA amplification reaction was done at 42°C for 30 min. cDNA amplification was performed with a total volume of 50 µl of reaction mixture containing 50 pmol of each specific primer (Table 1), 25 µM deoxynucleoside triphosphate, and 5 U of *Taq* polymerase (Amersham, USB, Cleveland, Ohio). The PCR conditions were programmed for 30 cycles of 1 min at 95°C, 1 min at different annealing temperatures to optimize the binding of different primer pairs (Table 1), and elongation for 1 min 10 s at 72°C. The final products were analyzed by gel electrophoresis in 2% agarose gels stained with ethidium bromide. Triplicate assays with three independent RNAs confirmed that the transcriptional levels of 16S rRNA were not significantly different ( $P > 0.05$ ). Therefore, 16S rRNA was used as an internal control to normalize the RT-PCR results. The expression of virulence genes was normalized to the ratio of virulence gene RNA and 16S rRNA (26). Total RNA was amplified with the 16S rRNA gene to serve as a negative control to exclude DNA contamination.

**Northern blot assays.** Northern blot assays were performed as described by Sambrook et al. (41). Fifty µg of RNA was heated to 65°C for 15 min and then fractionated by electrophoresis in 1% agarose-formaldehyde gels. A vacuum blotting system was applied to accelerate RNA transfer. The immobilized RNA was hybridized with various DNA probes at 42°C overnight. The filters were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS at room temperature and then with 0.2× SSC-0.1% SDS at 42°C for 30 min. Excess solution was removed with paper towels, and the filters were ready for autoradiography. The expression of virulence genes was normalized to the ratio of virulence gene RNA and 16S rRNA in three independent experiments.

**Air pouch model of infection and LD<sub>50</sub>.** The air pouch model of infection was described previously (20). BALB/c mice were anesthetized by pentobarbital inhalation and then injected subcutaneously with 1 ml of air to form an air pouch. A bacterial suspension (10<sup>9</sup> CFU) was collected from a 16-h culture and inoculated into the air pouch. Mice injected with the wild-type strain or its *opp* mutant were monitored over 3 weeks. The 50% lethal dose (LD<sub>50</sub>) was determined by monitoring the number of surviving mice inoculated with various CFU over a 1-month period. Twenty mice were used in each LD<sub>50</sub> experiment. The data were representative of three independent experiments. The mortality effect of Opp was calculated by the Kaplan-Meier survival curve method to analyze the standardized mortality ratio in a BALB/c mouse population (32).

**Statistics.** Student's *t* test was applied as appropriate for the parametric difference. All tests of significance were two tailed; a *P* value of <0.05 was taken as significant.

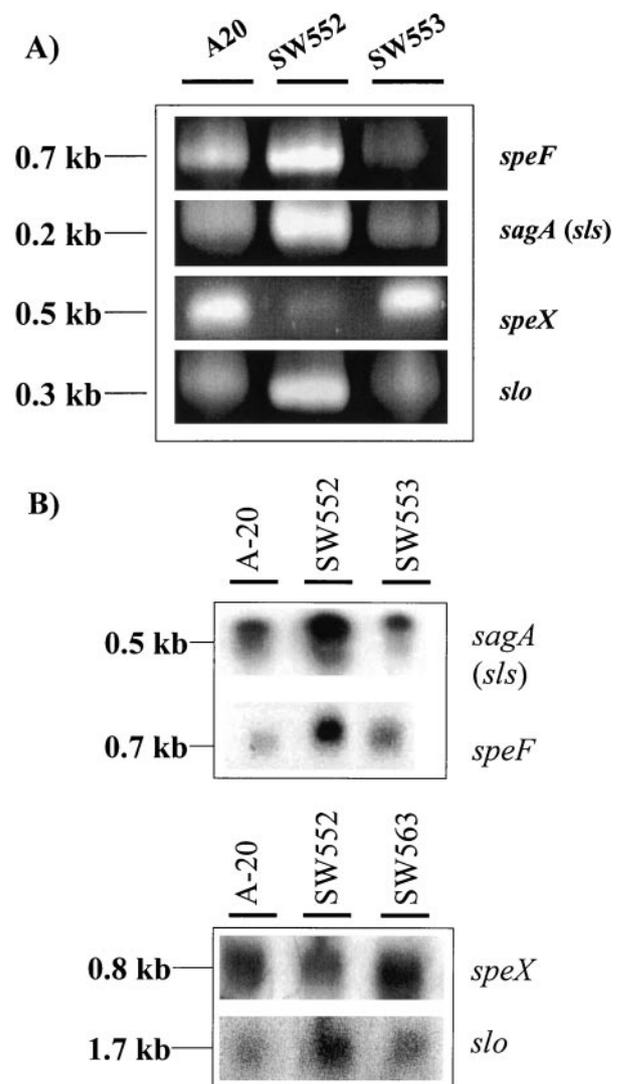


FIG. 3. Effects of Opp on streptococcal virulence factor expression. (A) RT-PCR analysis of *speF*, *sagA*, *speX*, and *slo*. (B) Northern hybridization analysis of *speF*, *sagA*, *speX*, and *slo*. Streptococcal virulence gene transcription among wild-type strain A-20, *opp* isogenic mutant SW552, *opp* revertant SW553, and *opp* complementation strain SW563 is shown.

## RESULTS

**Construction of an *opp* mutant.** The *opp* isogenic mutant in A-20 was constructed and plasmid pMW213 was used to disrupt the *oppA* gene (Fig. 1A). As analyzed by Southern hybridization, a 2.3-kb hybridization band was obtained with *Bcl*I digestion of DNA from the wild-type strain A-20 (Fig. 1B, lane 1), when a 1.97-kb *oppA* PCR product was used as a probe, whereas 2.3-, 2.6-, and 3.1-kb DNA fragments were seen in the *opp* mutant (Fig. 1B, lane 2). The 0.1-kb *oppA* RT-PCR product, amplified with *oppA* Fwd-2 and Rev-2 primers, was found in strains A-20, whereas no transcript was found in the *opp* mutant (Fig. 1C). The isogenic *opp* mutant was designated as SW552. A 6.83-kb PCR product containing the entire *opp* operon and a part of *dacA* (penicillin-binding protein) was transformed into SW552, selected for proteolytic activity on

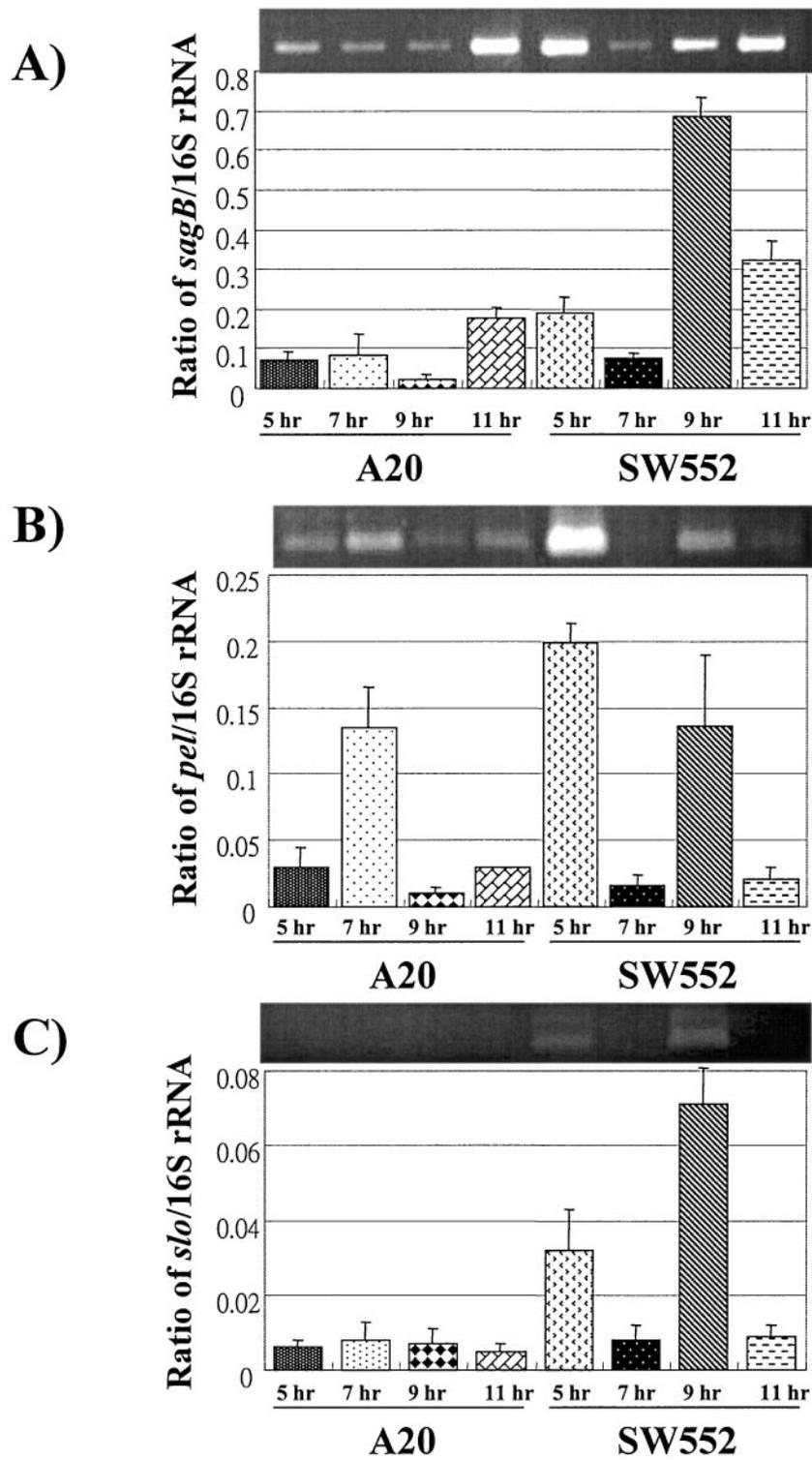


FIG. 4. RT-PCR analysis of the expression of streptococcal regulators and virulence factors during various growth periods. RT-PCR-amplified products of *sagB* (A), *pel* (B), *slo* (C), *rofA* (D), and *dppA* (E) were made from cell harvested from strains A-20 and SW552 at 5, 7, 9, and 11 h. Time zero is defined as the time when the bacterial suspension was transferred to fresh medium. The transcript of 16S rRNA was used as an internal control. The extent of expression was normalized to the ratio of virulence gene RNA and 16S rRNA. The data represent the means and standard deviations obtained from at least three independent experiments.

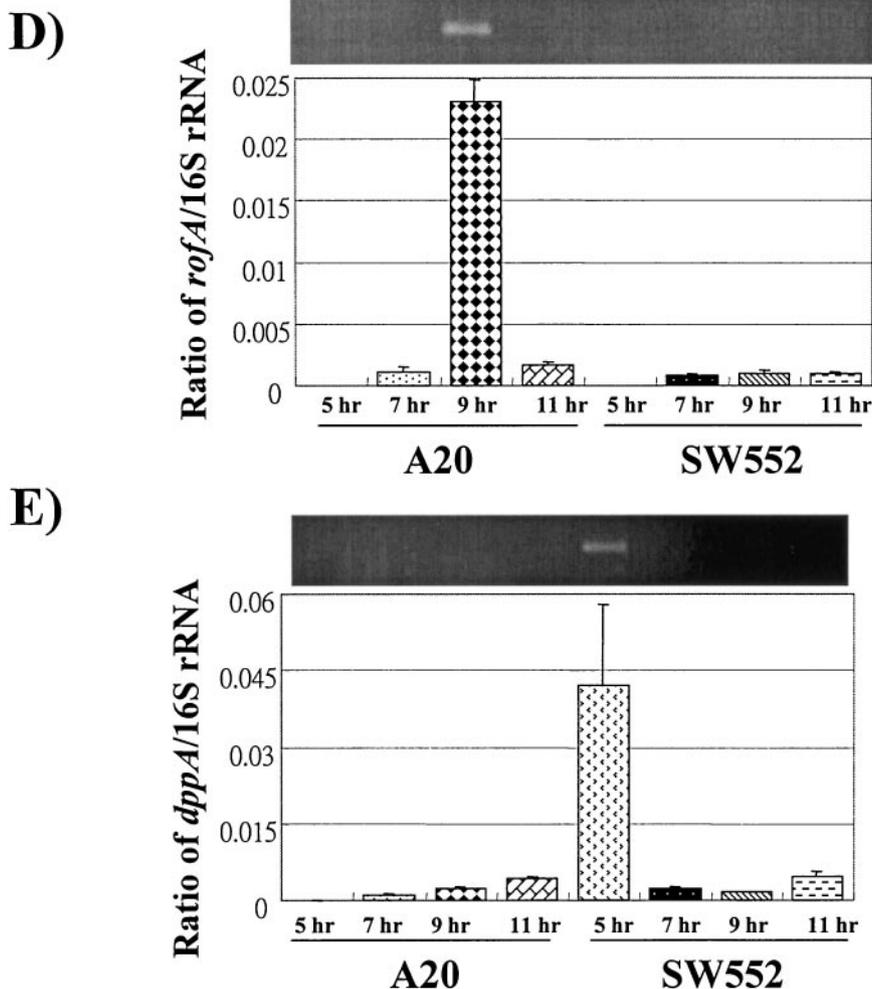


FIG. 4—Continued.

skim milk plates, and a 2.3-kb band was detected (the same size as that of the wild-type strain) in Southern hybridization (Fig. 1B, lane 3). The *oppA* revertant was designated as SW553. In addition, an *opp* complementation strain was also constructed. Plasmid pMW369 containing the entire *opp* operon was transformed into SW552, selected by spectinomycin (100 µg/ml) resistance and by a proteolytic activity assay. The *opp* complementation strain was designated as SW563. The growth curve of the *oppA* isogenic mutant (SW552) was similar to that of the wild-type strain A-20 and the *oppA* revertant (SW553) in TSBY broth (data not shown). To determine the effect of *speB* expression, a skim milk plate assay was used to detect protease activity. No protease activity was observed in SW552, whereas A-20 and SW553 showed normal proteolytic activity on the skim milk plates. The monoclonal antibody recognized the mature form of SpeB (28 kDa) in supernatants of strains A-20 and SW553, while no SpeB protein was observed in SW507 (*speB* isogenic mutant) and SW552 (data not shown).

**Effects of *opp* on SLS and SLO activities.** Regardless of the O<sub>2</sub> and CO<sub>2</sub> concentration, the SLO activity was 1 to 2-fold increased in SW552 (*opp* mutant) compared to that of A-20 (wild-type) and SW553 at a 16 h culture period (Table 2). The

SLO activity of A-20 and SW553 was not significant increased when GAS was grown in the presence of 5% CO<sub>2</sub> compared to growth at 0.5% CO<sub>2</sub>. When GAS was grown in 20% O<sub>2</sub>, however, the expression of SLO activity was 1.8 to 2.3-fold increased relative to that of GAS grown in 5% O<sub>2</sub>. A similar observation was also found in SW552 (2.5-fold increase) (Table 2). These results showed that both the *opp* operon and oxygen concentration affect the hemolytic activity of SLO. The differences of SLO activity between the wild-type strain and the *opp* mutant were due to the lack of *opp* expression.

The variation of hemolytic activity was observed by a time course assay. In the presence of 20% O<sub>2</sub>, SW552 had a fourfold increase in SLS and SLO activities compared to those of the wild-type strain (Fig. 2A and B). This was also confirmed by Northern blot and RT-PCR analyses. For *slo* (streptolysin O) and *sagA* (streptolysin S associated gene A) transcription, SW552 had a two- and threefold increase, respectively, in transcriptional activity compared to that of A-20 by the densitometer measurement (Fig. 3A and B). In SW552 (*opp* mutant), there was a sharp fall in hemolytic activity after the first hemolytic period near early log phase; the fall was then followed by a second peak of activity in late log phase (Fig. 2A and B).

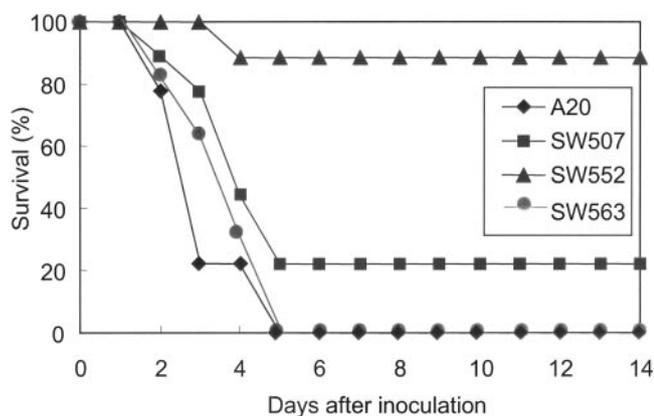


FIG. 5. Survival of GAS-infected mice after inoculation in air pouches with  $10^9$  CFU of wild-type strain A-20 ( $n = 9$ ), *speB* mutant SW507 ( $n = 9$ ), *opp* mutant SW552 ( $n = 12$ ), and *opp* complementation strain SW563 ( $n = 10$ ). These mice were injected subcutaneously with about 1 ml of air to form an air pouch, and then a bacterial suspension (0.1 ml) was inoculated into the air pouch. The mortality rates were monitored every day during the experimental period. Bacterial numbers were determined by measuring the OD<sub>600</sub> with a spectrophotometer.

The results are consistent with two periods of hemolytic activity, one starting at about  $t_4$  (where  $t_4$  is 4 h after the bacterial suspension was transferred to fresh medium) in SLO and SLS and the other at about  $t_8$  and  $t_{10}$  in SLS and SLO, respectively. The two peaks of SLO and SLS expression were not observed in the wild-type strain (A-20). Furthermore, the two-peak pattern in transcripts of *sagB* (streptolysin S associated gene B) and *slo* was also detected in  $t_5$  and  $t_9$  by the RT-PCR assay (Fig. 4A and C).

**Effects of *opp* on streptococcal erythrogenic toxin gene expression.** In addition to the *speB* transcription, the transcription of *speF* was twofold increased in SW552 relative to that of A-20, whereas transcription of *speX* in SW552 was twofold decreased compared to that of A-20 and SW553 (Fig. 3A and B). No difference was found between SW552 and A-20 in transcription of *speJ* and *speG*. The *speF* and *speX* expression was also restored either in the revertant or the *opp* complementation strain (Fig. 3A and B).

**Effects of *opp* on other virulence genes and regulatory genes.** No difference was detected by RT-PCR and Northern blot analyses between A20, SW552 and SW563 on *emm* (M protein), *sdaD* (DNase), *rgg*, and *csrR/csrS* transcription at 16 h (data not shown). However, the *rofA* (regulator of protein F) gene was transcribed at  $t_9$  in A-20 but not in SW552 (Fig. 4D). In contrast, the *dppA* (dipeptide permease) gene was transcribed at  $t_5$  in SW552 but not in A-20 (Fig. 4E). The transcriptional activity of *pel* also showed two peaks at  $t_5$  and  $t_9$  in SW552. The variation of *pel* transcription is similar to *sagB* in SW552 (Fig. 4A and B).

**Effect of *opp* in mice infected by GAS.** The LD<sub>50</sub> of A-20 and SW552 was determined as  $1.5 \times 10^8$  and  $1.1 \times 10^{11}$  CFU, respectively. To further understand the role of Opp of GAS in mice, bacteria ( $1 \times 10^9$  CFU) were inoculated into BALB/c mice via air pouch, and the mortality rates of mice inoculated with the wild-type strain (A-20), *speB* mutant (SW507), *opp* mutant (SW552) and *opp* complementation strain (SW563)

were measured. The results demonstrated that the wild-type strain and *opp* complementation strain caused 100% mortality on day 5, the *speB* mutant caused 77.8% mortality on day 5 and allowed continued survival after two weeks, whereas the *opp* mutant, SW552, caused only 8.3% mortality after two weeks (Fig. 5). Three days after infection with A-20, the skin of mice showed necrosis of the epidermis and hair loss, whereas only slight changes were observed in mice infected with SW552 (data not shown).

## DISCUSSION

In this study, the isogenic *oppA* mutant was constructed by using an integrative plasmid, and we found that the *opp* operon has dual effects on gene regulation. Opp not only positively regulates *speB*, *speX* and *rofA* gene expression but also negatively regulates the *speF*, *slo*, *saga*, *pel* and *dppA* genes.

The streptococcal pyrogenic exotoxins (Spe) are implicated as important factors in the pathogenesis of GAS infection. The Spe belong to the superantigen family and thus induce massive secretion of inflammatory cytokines (18). Overexpression of these cytokines can lead to tissue damage, organ failure, and toxic shock. At present, the *spe* family includes *speA*, *speB*, *speC*, *speF*, *speG*, *speH*, *speI*, *speJ*, *speK*, *speM*, *speL* and *speX* (8, 39, 40). *speA* is usually associated with severe diseases (46). The *speB* gene has been studied extensively as to its virulence (3). The individual effects of the *speC* and *speF* genes in the pathogenesis of invasive GAS infection are unclear. SpeF is known as a multifunctional protein that has mitogenic, superantigenic, nuclease, and vascular permeabilization activities (16, 28). SpeX (also called SMEZ3), a superantigen, preferentially stimulates V $\beta$ 8<sup>+</sup> T cells and is responsible for the mitogenic activity attributed to SpeB (4). In this study, the wild-type strain, A-20, did not contain the *speA* and *speC* genes as shown by PCR and RT-PCR (data not shown). The expression of *speG* and *speJ* was no different in the wild-type strain, A-20, and its *opp* isogenic mutant, SW552, whereas *opp* positively regulated *speB* and *speX* expression and negatively affected *speF* expression. Why *opp* could positively regulate *speB* and *speX*, and negatively regulate the expression of *speF* remains unclear. However, Nakamura et al. recently reported that the expression of virulence factors such as SpeF, Sic, SpeB and SpeX, in GAS is dependent on the various concentrations of O<sub>2</sub> and CO<sub>2</sub> (30). Although Opp can affect the hemolytic activity of SLO in different concentrations of O<sub>2</sub>, whether Opp sense the environmental changes or nutritional starvation to regulate the streptococcal pyrogenic exotoxins remains to be tested.

Both SLS and SLO, two distinct cytolysins, are important virulence factors in GAS infection (2, 25). SLO is a member of the thiol-activated pore-forming cytolysin family (34). It has been shown to exert direct toxic effects on cardiocytes and leukocytes (3), and lethal activity in experimental animals (1). SLS is the oxygen-stable and nonimmunogenic  $\beta$ -hemolysin. Various types of eukaryotic cells were lysed by SLS, including polymorphonuclear leukocytes and platelets (42). There are many regulators that have been reported to either positively (Pel and Fas) or negatively (CsrS/R, LuxS and Nra) regulate the SLS expression (19, 24, 27, 29). In this study, RT-PCR, Northern blot and phenotypic analyses confirmed that the ex-

pressions of *slo* and *sagA* increased in the *opp* mutant (SW552). It suggests that Opp may play a role as a negative regulator for both SLS and SLO transcription.

The sharp fall in SLO and SLS hemolytic activity after 4 h (the first hemolytic period), and the sharp fall in SLO and SLS after 8 h and 10 h, respectively, (the second peak of activity) (Fig. 2A and B) in the *oppA* mutant, indicates that SLO and SLS expression is unstable in the *opp* mutant. The second peak indicates a second period of transcription. The role of two peaks of expression is unclear. The appearance of two peaks might indicate two or more regulators could regulate streptolysin expression and these streptococcal regulators are active at different growth phases. The regulation of *opp* may involve one or two periods of streptolysin expression. The expression of *pel* (pleiotrophic effect locus) also had two peaks of transcription which were similar to SLS in SW552 by RT-PCR assay (Fig. 4A and B). Since the *pel* gene has been reported as a positive transcriptional regulator of SLS (24), our results indicate that *opp* can negatively regulate *pel* transcription and then *pel* positively regulated the SLS expression. However, in this study we have shown several regulators are affected by *opp* but we could not exclude the possibility that these results were posttranscriptional. What gene or genes actually control both SLO and/or SLS expression or are involved in the Opp regulation system remain subjects for further study.

It is known that Mga, Rgg, and CsrS/CsrR can affect the production of SpeB (6, 13, 36). The *rgg* gene is located in the region upstream of *speB*. CsrS/CsrR is a two-component system in GAS that could function to repress expression of the hyaluronic acid capsule, SLS, streptokinase, and SpeB (13). Since the *opp* mutant did not affect *rgg* and *csrR* transcripts, the data suggests that Rgg and Opp or CsrR and Opp may be two independent pathways regulating *speB* expression. In addition, both *opp* and *csrS/csrR* genes negatively regulate SLS activity; the data suggests these genes are independent systems to regulate SLS activity. However, we cannot rule out the possibility that Rgg or CsrR/CsrS may positively regulate the *opp* operon. Both *rofA* and *dppA* genes were reported as streptococcal regulators and expression of these genes was different between A-20 and SW552 (Fig. 5D and E), suggesting that *opp* may be involved in the streptococcal regulation network. In GAS, there are multiple systems to regulate extracellular amino acid import into bacteria, such as Opp and dipeptide permease (35). Since the *opp* gene of A-20 was expressed at  $t_5$  (Fig. 1C) and the *dpp* transcript of SW552 (*opp* mutant) was also expressed at  $t_5$  (Fig. 5E), it may indicate that a compensatory regulation system exists between the Opp and Dpp systems in the early growth phase. The *rofA* gene was previously shown to exert a direct positive control of protein F1 expression (3). The RT-PCR data showed the transcription of *rofA* was positively regulated by *opp*. Since A-20 was a protein F-deficient strain (data not shown), the role of *rofA* in A-20 strain remains to be studied.

To further confirm the role of *opp* in vivo, the air pouch model was used in BALB/c mice infected with wild-type (A-20) and mutant (SW552) strains. SW552 caused less mortality than A-20 and SW507 (*speB* mutant), whereas SW563 (*opp* complementation strain) was similar to that of A-20. The results suggest that lack of Opp also contributes to the survival of mice. Since the loss of Opp affected several virulence genes,

regulatory genes and caused less mortality in mice, the most likely model would be an indirect pathway acting through these regulators possibly influenced by amino acid starvation.

In summary, this study has demonstrated that the *opp* operon plays dual roles in the regulation of several virulence genes and regulatory genes. In addition, we also found that *opp* contributes to mortality and tissue damage in BALB/c mice. Since Opp plays multifactorial roles in regulating the virulence genes, the role of Opp in GAS infection is obviously complicated. Our results suggest that Opp plays an important role in the pathogenesis of GAS infection.

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