A PorX/PorY and $\sigma^P$ Feedforward Regulatory Loop Controls Gene Expression Essential for Porphyromonas gingivalis Virulence

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ABSTRACT The PorX/PorY two-component system in the periodontal pathogen Porphyromonas gingivalis controls the expression of the por genes, encoding a type IX secretion system, and the sigP gene, encoding sigma factor $\sigma^P$. Previous results implied that PorX/PorY and $\sigma^P$ formed a regulatory cascade because the PorX/PorY-activated $\sigma^P$ enhanced the por genes, including porT, via binding to their promoters. We recently showed that PorX also binds to the por promoters, thus suggesting that an alternative mechanism is required for the PorX/PorY- and $\sigma^P$-governed expression. Here, our in vitro assays show the PorX response regulator binds to the sigP promoter at a sequence shared with the porT promoter and enhances its transcription, mediated by a reconstituted P. gingivalis RNA polymerase holoenzyme. Merely producing $\sigma^P$ in trans fails to reverse the porT transcription in a porX mutant, which further argues against the action of the proposed regulatory cascade. An in vitro transcription assay using a reconstituted RNA polymerase-$\sigma^P$ holoenzyme verifies the direct role of PorX in porT transcription, since transcription is enhanced by a pure PorX protein. Accordingly, we propose that the PorX/PorY system coordinates with $\sigma^P$ to construct a coherent regulatory mechanism, known as the feedforward loop. Specifically, PorX will not only bind to the sigP promoter to stimulate the expression of $\sigma^P$, but also bind to the porT promoter to facilitate the RNA polymerase-$\sigma^P$-dependent transcription. Importantly, mutations at the porX and sigP genes attenuate bacterial virulence in a mouse model, demonstrating that this regulatory mechanism is essential for P. gingivalis pathogenesis.

IMPORTANCE The anaerobic bacterium Porphyromonas gingivalis is not only the major etiologic agent for chronic periodontitis, but also prevalent in some common non-communicable diseases such as cardiovascular disease, Alzheimer’s disease, and rheumatoid arthritis. We present genetic, biochemical, and biological results to demonstrate that the PorX/PorY two-component system and sigma factor $\sigma^P$ build a specific regulatory network to coordinately control transcription of the genes encoding the type IX secretion system, and perhaps also other virulence factors. Results in this study verify that the response regulator PorX stimulates the expression of the genes encoding both $\sigma^P$ and the type IX secretion system by binding to their promoters. This study also provides evidence that $\sigma^P$, like the PorX/PorY system, contributes to P. gingivalis virulence in a mouse model.

KEYWORDS PorX/PorY, Porphyromonas gingivalis, extracytoplasmic function sigma factor $\sigma^P$, feedforward loop, in vitro transcription, transcription regulation, two-component system, type IX secretion system, virulence factors
The Gram-negative anaerobic bacterium Porphyromonas gingivalis is the major etiologic agent for chronic periodontitis. This pathogenic bacterium produces a repertoire of virulence factors, including specific cysteine proteases, also known as gingipains (1–3). Secretion of gingipains is mediated by a type IX secretion system (T9SS) in a manner dependent on the porX and porY gene products, PorX and PorY, which were proposed to form a two-component regulatory system (TCS) by the Nakayama laboratory (4). Particularly, their results showed that PorX, the response regulator, and PorY, the histidine kinase, were able to upregulate the expression of the T9SS-encoding genes (referred to as the por genes, herein) including porT, sov, porP, porK, porL, porM, and porN (4). Furthermore, Kadowaki et al. carried out a surface plasmon resonance analysis and showed that PorY could directly interact with, and subsequently phosphorylate, PorX (5), thus experimentally demonstrating that these two proteins should be the TCS cognate pair. However, it remains elusive whether the PorX/PorY system controls transcriptional regulation of the por genes directly and, if that is the case, how the response regulator PorX interacts with these target genes.

In accordance with the observations from Kadowaki et al. (5), the Vincent and Cascales laboratory used a bacterial two-hybrid system and confirmed the in vivo interaction between the PorX and PorY proteins (6). In contrast, their results suggested that PorX should be involved in the dynamics of the T9SS system via an interaction with the cytoplasmic domain of the T9SS component PorL (6). They further argued that PorX/PorY could not regulate the por genes since they failed to observe PorX binding to the por promoters in a P. gingivalis promoter/PorX reconstitution assay performed in Escherichia coli, in which PorX was heterologously expressed for testing its role in stimulating a plasmid-borne gfp (green fluorescent protein) gene controlled by a por promoter (6). However, we realized that the α, β, and β’ subunits of P. gingivalis RNA polymerase, which are encoded by the PGN_1841, PGN_1571, and PGN_1570 genes, respectively, merely share 38%, 46%, and 50% of identity to the corresponding subunits of the E. coli RNA polymerase. Additionally, the major sigma factor σ^70 (encoded by the PGN_0638 gene) of P. gingivalis, which is a 287-residue protein, shares just 39% identity with the C-terminal 267-aa sequence of the E. coli housekeeping sigma factor σ^70 (613 aa). Therefore, it is reasonable to postulate that P. gingivalis promoters cannot be recognize by the E. coli RNA polymerase-σ^70 holoenzyme. Accordingly, the gfp expression would very unlikely be enhanced in the P. gingivalis promoter/PorX reconstitution assay conducted by Vincent et al. (6), even if these P. gingivalis promoters could bind the heterologously expressed PorX protein in E. coli. Recently, our study confirmed that the PorX/PorY system exerted a regulatory effect on the transcription of its target genes (7). The PorX/PorY regulatory role was further verified by our electrophoretic mobility shift assay (EMSA) and DNase footprinting analyses, which provided evidence that a PorX protein was able to bind the promoter of a por gene, porT, by interacting with two DNA sequences, i.e., site I (5′-tattacttctataattgttgtg-3′) and site II (5′-gtttcggctaaatataatcttt-3′) (7).

According to the observations from Kadowaki et al. (5), the PorX/PorY system upregulates transcription of the sigP gene (i.e., PGN_0274) that encodes an extracytoplasmic function sigma factor, σ^p, and then σ^p mediates transcriptional activation of the por genes by binding to their promoters. In addition, their results suggest the function of σ^p is directly associated with PorX because these two proteins could be coimmunoprecipitated from P. gingivalis cell lysates (5). Based on these results, it seems reasonable to propose a regulatory cascade in which the PorX/PorY system stimulates σ^p and, in turn, σ^p enhances the por genes. However, it remains largely unknown whether the PorX/PorY system can directly or indirectly upregulate sigP transcription, and whether the PorX/PorY system and σ^p activate the por genes in a manner dependent on the regulatory cascade. In this study, we show that the PorX response regulator not only binds to the sigP promoter to activate transcription of this sigma factor gene, but also binds to a por promoter with a σ^p-RNA polymerase holoenzyme to initiate its transcription. Based on our observations from both in vitro and in vivo analyses, we propose a...
We conducted a phenotypic analysis to evaluate the coordinate interaction between two-component systems and their regulated sigma factors in gene regulation of \textit{P. gingivalis}. Additionally, our results demonstrate that both the PorX/PorY system and \( \sigma^P \) are virulence factors that govern transcription of the genetic loci required for \textit{P. gingivalis} virulence.

**RESULTS AND DISCUSSION**

**PorX/PorY system and sigma factor \( \sigma^P \) coordinately regulate transcription in \textit{P. gingivalis}**. The PorX/PorY two-component system and extracytoplasmic function sigma factor \( \sigma^P \) (encoded by the \textit{sigP} gene) appeared to form a regulatory cascade for upregulation of the T9SS-encoding genes (i.e., the \textit{por} genes) because PorX/PorY was shown to upregulate \( \sigma^P \) and, in turn, \( \sigma^P \) enhanced the transcription of the \textit{por} genes (5). Particularly, it was observed that the PorX/PorY-stimulated \( \sigma^P \) bound the promoters of the \textit{por} genes, including the \textit{porT} gene which encodes a T9SS component (5). Besides \( \sigma^P \), a recent result from our laboratory showed the PorX protein also bound to the \textit{porT} promoter and actually interacted with two DNA regions (7). This result not only verified the DNA-binding ability of the PorX response regulator, but also provided the possibility that both PorX and \( \sigma^P \) should directly act on the \textit{por} promoters. If PorX and \( \sigma^P \) must coordinately control but not build a regulatory cascade to regulate the \textit{por} genes, we postulate that \( \sigma^P \) expressed in \textit{trans} in the absence of PorX, or vice versa, should not stimulate \textit{por} transcription. We examined this hypothesis by determining the transcription of the \textit{porT} gene in a \textit{porX} deletion mutant (\( \Delta \text{porX} \)). As predicted, a \( \sigma^P \)-protein that was expressed in \textit{trans} from a plasmid (pT-COW-\textit{P}\(\text{sigP} \), referred to as p-\textit{sigP}) did not exert any effect on the \textit{porT} expression in the \( \Delta \text{porX} \) mutant because the \textit{porT} mRNA level in this mutant harboring p-\textit{sigP} remained similar to that in the mutant harboring the parental plasmid pT-COW (8), both of which were \( \sim \) 6.4-fold lower than that in the wild-type strain (Fig. 1A). Likewise, PorX had no effect on \textit{porT} expression in the absence of \( \sigma^P \), because a PorX protein expressed in \textit{trans} from a plasmid (pT-COW-\textit{P}\_\text{PGN}_1016\_\text{PorX}, referred to as p-\textit{porX}) did not stimulate \textit{porT} transcription in a \textit{sigP} null (\( \Delta \text{sigP} \)) mutant (Fig. 1A). In contrast, the alleviated \textit{porT} transcription was fully reversed to a wild-type level in the \( \Delta \text{sigP} \) mutant harboring p-\textit{sigP} and also in the \( \Delta \text{porX} \) mutant harboring p-\textit{porX} (Fig. 1A), indicating the \textit{trans}-expressed \( \sigma^P \) and PorX proteins were functionally active. We also determined whether this coordinate regulation was effective in controlling two other PorX/PorY- and \( \sigma^P \)-dependent genes, \textit{PGN}_0341, which encodes a predicted T9SS component (4), and \textit{PGN}_1639, which has been known as a \( \sigma^P \)-dependent gene (5) and recently identified as a PorX/PorY-dependent locus according to our transcriptomic and proteomic analyses (unpublished data). We confirmed that the transcription levels of \textit{PGN}_0341 and \textit{PGN}_1639 were upregulated by PorX and \( \sigma^P \) because their mRNA levels were significantly reduced in the \( \Delta \text{porX} \) and \( \Delta \text{sigP} \) mutants compared to those in the wild-type strain (Fig. 1B and 1C). Comparable to the \textit{porT} regulation (Fig. 1A), the alleviated transcription of \textit{PGN}_0341 and \textit{PGN}_1639 was not stimulated in \( \Delta \text{porX} \) mutant harboring p-\textit{sigP} or in \( \Delta \text{sigP} \) mutant harboring p-\textit{porX} (Fig. 1B and 1C).

It has been shown that T9SS mediates secretion of gingipains, which are required for pigmentation of \textit{P. gingivalis} on a blood plate (for review see reference 9), and consistently both \( \Delta \text{porX} \) and \( \Delta \text{sigP} \) mutants display a nonpigmented phenotype (6, 10). We conducted a phenotypic analysis to evaluate the coordinate interaction between the PorX/PorY system and \( \sigma^P \). While the \( \Delta \text{porX} \) and \( \Delta \text{sigP} \) mutants carrying pT-COW exhibited nonpigmented colonies on a brain heart infusion (BHI) blood plate, both the \( \Delta \text{porX} \) mutant harboring p-\textit{porX} and the \( \Delta \text{sigP} \) mutant harboring p-\textit{sigP} formed vigorous black-pigmented colonies (Fig. 1D). However, the \( \Delta \text{porX} \) mutant harboring p-\textit{sigP} and the \( \Delta \text{sigP} \) mutant harboring p-\textit{porX} exhibited a nonpigmented phenotype when they were grown on a BHI blood plate (Fig. 1D). Taken together, these genetic approaches suggest the PorX/PorY system and \( \sigma^P \) should govern transcription of the \textit{por} genes via a coherent regulatory network rather than a direct regulatory cascade.
PorX response regulator directly binds to the promoter of the sigma factor gene sigP. Evidence suggests that transcription of the sigP gene is activated by the PorX/PorY system (5). This is confirmed by our result derived from a reverse transcription-PCR, since the sigP mRNA level in the ΔporX mutant (lane 2, Fig. 2A) was 4.3-fold lower than that in the wild-type strain (lane 1, Fig. 2A). Our result also confirmed the plasmid p-sigP should be able to express the sigP gene in trans because it fully restored
The PorX response regulator binds to the sigP promoter region. (A) The mRNA levels of the sigP gene in the 33277 wild-type strain and the ΔporX mutant (YS19181) carrying pT-COW, p-porX, or p-sigP. Results are representative of three independent experiments. (B) EMSA analysis for binding of PorX to the sigP promoter. \[^{32}P\]-labeled sigP DNA fragment (40 fmol) was incubated with PorX-C-His\(_6\) protein at the indicated amount. Lane 5 is the same as lane 4 but supplemented with nonlabeled (cold) sigP DNA fragment (1 pmol). The PorX/DNA mixtures were subjected to 5% PAGE. The location of DNA migration was detected by autoradiography. Arrows indicate the shifted bands after DNA fragments were associated with the PorX-C-His\(_6\) protein. The experiment was repeated twice. (C) DNase footprinting analysis of the sigP promoter fragment amplified with primers \[^{32}P\]-3043 and 3044 for the coding strand and increasing amounts of PorX-C-His\(_6\) protein. Products were separated in polyacrylamide DNA sequencing electrophoresis and the bands were detected by autoradiography. The bracket indicates the region protected by the PorX-C-His\(_6\) protein. Underlined DNA sequence (right of gel) indicates the PorX-protected nucleotides in the sigP promoter. The ladder \(M\) corresponds to the same \[^{32}P\]-labeled sigP promoter fragment and degraded by the Maxam and Gilbert reaction. Results were repeated multiple times. (D) The DNA sequence of the sigP promoter region. Underlining corresponds to the PorX-protected region characterized in (C). Capital letters represent the sigP start codon. Numbering begins from (Continued on next page)
the sigP mRNA level in the ΔporX mutant (lane 4, Fig. 2A). To determine whether the PorX/PorY system can directly upregulate the sigP gene, we first characterized the sigP promoter region and investigated the PorX binding to this promoter by conducting an electrophoretic mobility shift assay (EMSA) using a 275-bp DNA fragment (marked as T1), including the 149-bp intergenic region of the sigP-PGN_0275 genes. We found that a PorX protein with a C-terminal His6 tag (referred to as PorX-c-His6) gel-shifted this DNA fragment in a concentration-dependent manner (Fig. 2B), thus suggesting this T1 fragment should contain the sigP promoter and also the sequence(s) that binds the PorX protein (i.e., the PorX-binding site). Therefore, we conducted a DNase footprinting assay to map the PorX-binding site in T1, and found that the PorX-c-His6 protein bound to an AT-rich DNA sequence (5′-tcgaaaaaaatgtttttctttgc-3′) in a concentration-dependent manner (Fig. 2C). This PorX-binding site, which is located −97 to −75 nucleotides (nt) upstream of the start codon (underlined nucleotides, Fig. 2D), shared a partial sequence with the PorX-binding site II (5′-gattcgcgcaaatacaatatcttt-3′) in the porT promoter, recently characterized by our laboratory (7). We postulate that PorX can recognize a sequence (5′-CG(A/C)AAAAA-N5-T(T/A)TCTTTGC-3′) that is conserved in these two promoters. Interestingly, the 5 nucleotides located between the conserved segments in the PorX-binding sites of the sigP and porT promoters were complementary (nucleotides labeled with arrows in Fig. 2E). Therefore, these results and our recent data (7) not only verify that PorX directly regulates transcription of the sigP gene and the por genes such as porT, but also elucidate that PorX is a DNA-binding protein and capable of recognizing specific DNA sequences in a manner similar to many other TCS response regulators.

PorX protein activates sigP transcription in vitro. To further validate the direct role of the PorX/PorY system in sigP transcription, we conducted an in vitro transcription assay using a P. gingivalis RNA polymerase holoenzyme (referred to as pg-RNAP-αβ) that was reconstructed from N-terminal His6-tagged subunit proteins, including α (PGN_1841), β (PGN_1571), β′ (PGN_1570), and the major sigma factor α5 (PGN_0638) (for details see the Materials and Methods section). When the T1 fragment, which was tested for PorX binding (Fig. 2B and C), was used as the template for the in vitro transcription reactions supplemented with 50 nM pg-RNAP-αβ, two transcripts labeled as P1 and P2, respectively, were produced (Fig. 3A). Both transcripts were stimulated by the PorX-c-His6 protein because the amount of P1 and P2 increased in a PorX concentration-dependent manner (lanes 1 to 4, Fig. 3A). These results suggest that sigP transcription is initiated from two regions that are located at 65 to 60 nt (labeled as p1) and 99 to 94 nt (p2) upstream of the start codon, respectively (illustrated in the T1 sequence, Fig. 3B). To verify whether these transcripts were produced specifically, we compared the in vitro transcripts from the wild-type T1 template and a mutated T1 template (T1-Sub) which carried 17-nt substitutions at a 103- to 87-nt sequence located upstream of the start codon. Our results showed that levels of both P1 and P2 transcripts from a reaction using the T1-Sub template were much lower than those using the T1 template (lane 2 versus lane 1, Fig. 3C). Since this substituted sequence overlaps a partial region of the PorX-binding site for the P1 transcription and the p2 region (Fig. 3B), we reasoned that these substitutions must simultaneously interfere with transcription initiated from p1 and p2 in T1-Sub. To further verify that the transcription initiation from p1 and p2 was specific, we used another template, i.e., T2, which was a longer template (291 bp) and contained an additional 16-bp sequence extending from downstream of the T1 template (275-bp). The in vitro transcription using this T2 template could still produce two transcripts, labeled as P1 ′ and P2 ′, in a PorX concentration-dependent manner (lanes 3 and 4, Fig. 3D), and both products were exactly 16-nt longer than

FIG 2 Legend (Continued)

the adenine nucleotide of the start codon. Highlighted sequences are shared by the PorX-binding site in the porT promoter (also shown in panel E). (E) The homologous sequences of the PorX-binding sites in the sigP and porT promoters. Vertical lines represent the identical nucleotides in the two sequences. Arrows represent the complementary nucleotides exhibited in the two sequences. Highlighted sequences are shared in these two promoters.
PorX promotes sigP transcription in vitro, mediated by a reconstituted P. gingivalis RNA polymerase-σ^54. (A) In vitro transcription of a 275-bp template (T₁) from the sigP promoter containing the first 29 coding nucleotides was conducted as described in the
P₁ and P₂, respectively (lanes 3 and 4 versus lane 2, Fig. 3D). Therefore, the *in vitro* transcription of the *sigP* gene must be specifically initiated from two DNA regions, p₁ and p₂, thus allowing the T₁ template to produce P₁ and P₂ transcripts and also the 16-bp longer T₂ template to produce 16-nt longer P₁’ and P₂’ transcripts.

**PorX stimulates in vitro transcription of the porT gene carried out by a reconstructed RNA polymerase-σ^D^ holoenzyme.** Since PorX directly binds to the σ^D^-dependent porT promoter (7), we postulated that it should be able to stimulate porT transcription *in vitro*. To examine this hypothesis, we conducted an *in vitro* transcription assay using a *P. gingivalis* RNA polymerase-σ^D^ holoenzyme (referred to as pg-RNAP-σ^D^) which was reconstructed from purified N-terminal His₆-tagged α, β, β’ and C-terminal His₆-tagged σ^D^ proteins (for details see the Materials and Methods section). When a 301-bp DNA fragment, including the porT promoter sequence, was used as the template, two transcripts labeled as S₁ and S₂ were produced by the reconstructed pg-RNAP-σ^D^ (at 50 nM) and both transcriptions were enhanced by PorX in a concentration-dependent manner (lanes 2 to 5, Fig. 4A). S₁ transcription was initiated from the adenosine (labeled as s₁, Fig. 4B) located 29 nucleotides downstream of PorX binding site I in the porT promoter. Thus, we postulated that PorX should bind to site I and site II and enhance the transcription initiated at s₁ and s₂, respectively. Synthesis of both S₁ and S₂ was significantly stimulated when the pg-RNAP-σ^D^ concentration was elevated from 25 nM to 50 nM (lanes 2 and 3, left panel, Fig. 4C). In contrast, the pg-RNAP-σ^D^ holoenzyme was not as efficient as pg-RNAP-σ^D^ because only S₂ could be produced to a detectable level by pg-RNAP-σ^D^ at a high concentration of 200 nM (lane 4, right panel, Fig. 4C). These observations suggest that σ^D^ should be the preferred sigma factor to mediate the porT transcription and that both PorX and σ^D^ act directly on its promoter. Interestingly, the s₁ and s₂ sites did not overlap the transcription initiation site (+1) detected from a primer extension assay using a total wild-type mRNA sample (7). This is probably because other factors in the bacterial cell might interact with PorX and RNA polymerase-σ^D^ holoenzyme to initiate the porT transcription from the +1 position.

**PorX/PorY system is essential for the virulence of *P. gingivalis* in a mouse model.** According to our previous results (7), the PorX/PorY system is a virulence regulator of *P. gingivalis* because a virulent W83 wild-type strain, but not the ΔporX mutant, could cause infection in a mouse model described previously (11). To determine whether the PorX/PorY-activated σ^D^ contributes to bacterial virulence, we compared the pathogenesis of this wild-type strain and its isogenic ΔsigP mutant in this mouse model. Six-week-old BALB/c mice were subcutaneously injected on the dorsal surface with the strains that were grown in BHI medium for 12 h, and all five mice that were challenged by W83 wild-type cells at a dose of 4.72 × 10^10^ CFU died in 48 h (Fig. 5A and 5B). On the other hand, four out of the five mice challenged with the isogenic ΔsigP mutant cells at a dose of 4.58 × 10^10^ CFU survived the 30-day observation period (Fig. 5A and 5B), thus demonstrating that the sigma factor σ^D^ is a virulence determinant. The ΔsigP mutant was highly attenuated but not as avirulent as the ΔporX mutant, which, at a

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**FIG 3 Legend (Continued)**

Materials and Methods. Left braces indicate the P₁ and P₂ transcripts synthesized by 50 nM of RNA polymerase-σ^D^ (RNAP-σ^D^) from reactions supplemented with different amounts of the PorX-C-His₆ protein. The ladder M corresponds to a PCR product generated with primers 3044 and 32P-labeled primer 3043 and degraded by the Maxam and Gilbert reaction. (B) The DNA sequence of the sigP promoter region. Underlining corresponds to the PorX-protected region. Blue dashed frames correspond to the regions labeled as p₁ and p₂, respectively, where transcription was initiated. The highlighted sequence corresponds to the wild-type sequence which was substituted by the sequence (Sub) in red capital letters. Numbering begins from the adenine nucleotide of the start codon (underlined capital letters). (C) *In vitro* transcription of the sigP templates (T₁ and T₂) with the wild-type sequence and a substituted sequence, respectively. Blue left braces indicate the transcripts, P₁ and P₂, produced from the reaction with template T₁. (D) *In vitro* transcription of the for 16 nucleotides shorter than P₁’ and P₂’ respectively. Results in A, C, and D were repeated two times.
FIG 4 PorX and $\sigma^P$ promote porT transcription in vitro. (A) In vitro transcription of a porT template containing its promoter and the first 48 coding nucleotides was conducted as described in the Materials and Methods. The left panel represents the transcripts, labeled as $S_1$ and $S_2$, respectively, synthesized in the reactions with different amounts of RNAP-$\sigma^P$ with and 100 nM PorX-C-His$_6$ protein. The right panel represents the products synthesized in the reactions with different amounts of RNAP-$\sigma^D$ and 100 nM PorX-c-His$_6$ protein. The ladder $M$ corresponds to a PCR product generated with primers 4026 and $^{32}$P-labeled primer 4025 and degraded by the Maxam and Gilbert reaction. (B) The DNA sequence of the porT promoter region. Underlined sequences correspond to the PorX-protected regions and are also labeled as I and II, respectively. Bold letters, labeled as $s_1$ and $s_2$, correspond to the transcription initiation sites detected from the in vitro transcription. Underlined capital letters present the porT start codon. (C) In vitro transcription of porT in the reactions supplemented with 50 nM RNAP-$\sigma^P$ and different amounts of PorX-C-His$_6$ protein. The ladder $M$ is the same as in A. Results in A and C were repeated two times.
A dose of $4.32 \times 10^{10}$ CFU, did not kill even one mouse in the 30-day observation period (Fig. 5A and 5B). The result of the $\Delta$porX mutant also reconfirmed that the PorX/PorY system is essential for $P. \text{gingivalis}$ virulence (7). Accordingly, we postulated that the PorX/PorY system should also be able to activate other $P. \text{gingivalis}$ virulence factors whose regulation is independent of $\sigma^P$. Based on these observations, it is reasonable to assume that the PorX/PorY system renders $P. \text{gingivalis}$ virulent in part by activating the sigP gene in this mouse model. This assumption should be further confirmed by our ongoing RNA sequencing analysis, which compares the expression of overall PorX/PorY- and $\sigma^P$-regulated genes in $P. \text{gingivalis}$ cells recovered from the animal against those grown in vitro.

In conclusion, pathogenic bacteria have developed many sophisticated mechanisms to control the expression of the genes that contribute to virulence. Growing evidence suggests that the PorX/PorY system in $P. \text{gingivalis}$ plays an essential role in the regulation of numerous virulence determinants, exemplified by the set of por genes encoding the T9SS components. This study has revealed that the PorX/PorY system and sigma factor $\sigma^P$ construct a regulatory pathway to coordinate the regulation of the por genes. We provide evidence that the PorX response regulator binds to the sigP promoter (Fig. 2B and 2C) and activates the sigP transcription in an in vitro transcription reaction system using a reconstructed RNA polymerase holoenzyme (Fig. 3A, C, and D), thus demonstrating that the PorX/PorY system directly regulates transcription of the sigP gene.

When two related regulators build a regulatory cascade, the first regulator regulates the second regulator, and then the second regulator regulates their target genes. Therefore, in the absence of the first regulator, the target genes will still be regulated by the second regulator when this regulator can be produced in trans. Although PorX/PorY activates $\sigma^P$, and then $\sigma^P$ activates the por genes, this regulatory cascade model is inapplicable to the regulation dependent on the PorX/PorY system and $\sigma^P$ because both the first regulator (PorX) and the second regulator ($\sigma^P$) are shown to bind to the por promoters (5, 7), and $\sigma^P$ produced in trans from p-sigP is unable to activate these genes in the $\Delta$porX mutant (Fig. 1A to C). We also show that the PorX protein can directly enhance in vitro porT transcription catalyzed by an RNA polymerase-$\sigma^P$ holoenzyme (Fig. 4A and 4C), which further confirms the direct action of PorX on the porT

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**FIG 5** The PorX/PorY-determined virulence of $P. \text{gingivalis}$ W83 strains. (A) Virulence test using groups of BALB/c mice ($n = 5$) that were subcutaneously injected with $P. \text{gingivalis}$ W83 wild-type, $\Delta$sigP (YS18145), and $\Delta$porX (YS19145) strains, respectively. (B) Survival curves of the results from A ($n = 5$; $P < 0.0001$). Three sets of experiments were carried out.

| Strains          | Dose of inoculum (CFU) | Survivor / total | Mean day of death |
|------------------|------------------------|------------------|-------------------|
| WT               | $4.72 \times 10^{10}$  | 0/5              | 2                 |
| $\Delta$sigP     | $4.58 \times 10^{10}$  | 4/5              | 9                 |
| $\Delta$porX     | $4.32 \times 10^{10}$  | 5/5              |                   |


Therefore, regulation of the *por* genes should be controlled coordinately by the PorX/PorY system and $\sigma^p$, in which PorX stimulates the production of $\sigma^p$, and both PorX and $\sigma^p$ regulate the *porT* transcription. We postulate that this mechanism of action of the PorX/PorY system and $\sigma^p$ should fall under the criteria of a regulatory motif, which is known as the feedforward loop (12) (Fig. 6). Our previous results have shown that the PorX/PorY system responds to hemin and enhances transcription of the *porT* gene (7). In many cases, the feedforward loop has the capability to integrate multiple signaling molecules into a gene regulation (12). It remains to be investigated whether the feedforward loop contributing to the PorX/PorY- and $\sigma^p$-governed signal transduction pathway is able to respond to signal molecules besides hemin.

It is worth noting that the transcription of the *sigP* gene in the $\Delta$porX mutant is not completely repressed (5) (Fig. 2A). According to a previous study (13), $\sigma^p$ exerts an inhibitory effect on *P. gingivalis* biofilm formation, as biofilm formation is induced in a *sigP* null mutant in an enriched BHI medium. However, the $\Delta$porX mutant grown in this BHI medium did not induce biofilm formation (unpublished result). We reason that the expression of the *sigP* gene remaining in the $\Delta$porX mutant is sufficient to inhibit biofilm formation.

The PorX/PorY system has been shown as an essential regulator for *P. gingivalis* virulence since the $\Delta$porX mutant is avirulent in mouse infection (7) (Fig. 5A and B). In this study, the murine virulence assay has demonstrated that $\sigma^p$ contributes to *P. gingivalis* virulence and the $\Delta$sigP mutant becomes attenuated. Further *in vivo* analysis will be needed to confirm the role of $\sigma^p$ in the PorX/PorY-controlled mechanism required for *P. gingivalis* pathogenesis.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media, and growth conditions.** Strains and plasmids used in this study are listed in Table 1. The *P. gingivalis* ATCC 33277 and W83 wild-type strains used in this study were

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**FIG 6** Feedforward loop model illustrating the PorX/PorY- and $\sigma^p$-dependent regulatory mechanism. In *P. gingivalis*, PorX/PorY and $\sigma^p$ build a feedforward loop. The PorY sensor kinase phosphorylates its cognate PorX response regulator. The phosphorylated PorX protein binds to the sigP promoter at the PorX-binding site and upregulates transcription of the sigP gene. The PorX/PorY-stimulated $\sigma^p$ protein and RNA polymerase core enzyme build a holoenzyme. Then, phosphorylated PorX protein and RNAP $\sigma^p$ holoenzyme coordinately activate transcription of their target genes by simultaneously binding to their promoters at the PorX-binding sites and the $\sigma^p$ recognition site, respectively. The inset illustrates the PorX/PorY $\sigma^p$ feedforward loop that modulates por expression.
obtained from Koji Nakayama (4). P. gingivalis cells were grown at 37°C in an anaerobic chamber (Model 2000, Coy Lab Products) that maintained 90% N₂/5% CO₂/5% H₂ in the atmosphere. Blood agar plates (5% sheep defibrinated blood, 1.5% agar) or brain heart infusion (BHI, purchased from BD) medium supplemented with hemin (5 μg/ml) or tetracycline (0.5 μg/ml) were used to culture P. gingivalis cells. When necessary, erythromycin (0.5 μg/ml) or tetracycline (0.5 μg/ml) was supplemented. P. gingivalis cells were harvested by centrifuging liquid cultures at 10,000 g (9,500 rpm) in a Sorvall ST 8R centrifuge with a HIGHConic III angled rotor (maximum 9,500 rpm) at 4°C for 10 min.

Construction of plasmids and strains with chromosomal mutations. All plasmids used in this study are listed in Table 1. Polymerase chain reactions (PCR) were performed using a Bio-Rad T100 thermal cycler with Taq DNA polymerase (New England BioLabs [NEB]). Custom oligonucleotides were synthesized by Integrated DNA Technologies (IDT) and are listed in Table 2. PCR products were isolated from overnight cultures of E. coli DH5α using a Qiagen plasmid minikit or midi kit (Qiagen). Restriction enzymes were purchased from New England BioLabs and used according to the manufacturer’s instructions. Digested DNA fragments were separated in 0.8 to 1% agarose gels and then isolated using a QIAquick gel extraction kit (Qiagen). Plasmids were purified from overnight cultures of E. coli DH5α in LB at 37°C using plasmid minikit or midi kit (Qiagen).

Plasmid pYS19107 for complementation assays was constructed using PCR fragments containing a 500-bp sequence of the upstream region followed by the sigP coding region amplified with primers 3160 and 3161, digested with NheI, and then ligated with NheI-digested pGEM-ERM plasmid. Plasmid pYS18943 was constructed using PCR fragments containing the sigP (PGN_1571) coding region amplified with primers 3170 and 3169 to obtain a 305-nt sigP coding region amplified with primers 3169 and 3168, digested with PstI, and then ligated with PstI-digested pGEM-ERM plasmid. Plasmid pYS18763 was constructed using PCR fragments containing the sigP (PGN_1571) coding region amplified with primers 3169 and 3168, digested with PstI, and then ligated with PstI-digested pGEM-ERM plasmid. Plasmid pYS18051 was constructed using PCR fragments containing the ropD (PGN_0638) coding region amplified with primers 3169 and 3168, digested with PstI, and then ligated with PstI-digested pGEM-ERM plasmid. Plasmid pYS18052 was constructed using PCR fragments containing the ropD (PGN_0638) coding region amplified with primers 3169 and 3168, digested with PstI, and then ligated with pET28a. Plasmid pYS18056 was constructed using PCR fragments containing the ropD (PGN_0638) coding region amplified with primers 3169 and 3168, digested with PstI, and then ligated with pET28a. Plasmid pYS18145 was constructed using PCR fragments containing the ropD (PGN_0638) coding region amplified with primers 3169 and 3168, digested with PstI, and then ligated with pET28a.
HindIII sites of plasmid pET28a. Plasmid pYS18056 was constructed using PCR fragments containing the sigP coding region amplified with primers 3148 and 3149, digested with NcoI and XhoI, and then ligated between the NcoI and XhoI sites of plasmid pET28a. All plasmids were sequenced before use. The P. gingivalis D sigP mutant was constructed by introducing suicide plasmid pYS17676 into the 33277 and W83 wild-type strains, respectively, using an electroporation procedure described previously (4). Mutated sequences in these strains were confirmed by DNA sequencing.

Quantitative real-time PCR. Bacterial cells were grown anaerobically in BHI medium at 37°C for 48 h. Total RNA was isolated from bacterial cultures using a High Pure RNA isolation kit (Roche) according to the manufacturer’s instructions. The concentration of RNA samples was determined by measuring absorbance at 260 nm using a spectrophotometer (SmartSpec Plus, BIO-RAD). The quality of RNA was evaluated in a 1.2% agarose gel. cDNAs were synthesized using random primers (IDT) and a murine leukemia virus reverse transcriptase (NEB). The amount of cDNA was quantified using PowerUp SYBR green Master Mix according to the manufacturer’s instructions with primers 3837 and 3838 for porT, 3912 and 3913 for PGN_0341, 3764 and 3765 for PGN_1639, 4193 and 4194 for sigP, and 2499 and 2500 for rpoB (Table 2) and qPCR was performed in QuantStudio 3 Real-time PCR systems (Applied Biosystems, Thermo Fisher Scientific).

Isolation of the RpoA-N-His6, RpoB-N-His6, RpoC-N-His6, sD-N-His6, sP-C-His6, and PorX-C-His6 proteins. E. coli BL21-Gold (DE3) harboring plasmids pYS18051, pYS18943, pYS18165, pYS18052, pYS18056, and pYS18456, respectively, were grown in 500 ml of LB medium by shaking at 37°C to an optical density at 600 nm (OD600) value of 0.5, then IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.4 mM, and bacterial cells were cultured for another 2 h. Bacterial cells were harvested by centrifugation at 10,000 × g for 15 min and washed with 50 ml of phosphate-buffered saline (PBS) once, suspended in 10 ml of PBS, and opened by sonication (Misonix Sonicator 3000). The cell lysate was used for purification of the RpoA-N-His6, RpoB-N-His6, RpoC-N-His6, sD-N-His6, sP-C-His6, and PorX-C-His6 proteins with Ni-NTA Affinity Gel (Qiagen) by following the manufacturer’s instructions. The purity and

| Primer no. | Sequence                  |
|------------|---------------------------|
| 2499       | gga aga gaa gac cgt agc aca agg a |
| 2500       | gag tag gcg aac cgt cca tca ggt c |
| 2741       | ccc aag ctt gac aca gca gca gga aat gc |
| 2742       | cgc gga tcc tta ctt ggg tgt cat cgt aat |
| 2768       | aaa act gca ggt ttc cac aag ctg act g |
| 2769       | aaa act gca gtt caa cct ggc tcc ttc c |
| 2827       | cgc ctc gac cat agc cga cat ggc cat a |
| 3043       | tca tca gtc agc tgt tgg            |
| 3044       | cgc agt acg ttt acc cc             |
| 3148       | cat gcc atg gca atg agc agt ttc cac aag c |
| 3149       | cgc ctc gac agc cga cat gcc cat    |
| 3158       | cat gcc atg gcc cat cat cat cat cac gca ata tta gca ttt cag |
| 3159       | cgg gat cct tat tag tct tta aat tta tac |
| 3160       | cta gct agc cat cat cat cat cac acg ccg act aca aac aac |
| 3161       | cta gct agc tta tta gtc caa aag aac act t |
| 3162       | cat gcc atg ggc cat cat cat cat cac gct ttt aga gaa gaa aat aag |
| 3163       | cgc ctc gag tta ttc ttc gca tgg tcc ttc |
| 3164       | cat gcc atg ggc cat cat cat cac agg caa ctt aat tcc |
| 3165       | ccc aag ctt tta tta gcc gag ata acc ttc cag |
| 3264       | cgg tcg gac gca gca aag tgg |
| 3470       | gtt tgt tgt cga cga atc tgc |
| 3471       | cga gga gag cat tgt tgg          |
| 3764       | cca aag cta ctc tcc tcc         |
| 3765       | tac gaa ggc atc gaa agg          |
| 3809       | cgc gga tcc cca cta ctc cta ctc tcc c |
| 3837       | atg tag gga tgc atg ccc         |
| 3838       | caa agt cgg aag caa acg         |
| 3912       | gtc agt ctt tcc act cgg         |
| 3913       | cgg gga atg gtc aga tgc         |
| 4025       | aga gag cga ctc tca acg         |
| 4026       | cac acg tcc tat att gcg          |
| 4105       | cag gcg tgt gga tcc gcg ttt ttc ttc ttc gca ata ag |
| 4106       | cgc gga tcc cag cgc ctc ttg aag cag aag c |
| 4111       | aag gct gac caa ttc atc          |
| 4193       | cgg cgc aat gcg atg tgc         |
| 4194       | agc ata ttc gcc gga aag          |

*All oligonucleotides were purchased from IDT (Integrated DNA Technologies)
concentration of protein samples were determined using a Silver Staining kit (Pierce) and BCA Protein assay kit (Pierce) by following the instructions from the manufacturer.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay (EMSA) was performed as described (15) with the following modifications. Primer 3043 was labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (PerkinElmer Life Sciences). Ten nanomoles of 32P-labeled DNA fragments containing the 275-bp sigP promoter region, amplified by PCR from 33277 chromosomal DNA with primers 3044 and [32P]-labeled 3043, were incubated at room temperature for 30 min with 0, 25, 50, or 100 pmol of PorX-C-His6 protein in 20 µl of an EMSA buffer consisting of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM dihistoethanol (DIT), 10 mM NaCl, 1 mM MgCl2, and 5% glycerol. After the addition of the DNA dye solution (40% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol), the mixture was directly subjected to 4% polyacrylamide electrophoresis. Signals were detected by autoradiography.

DNase footprinting analysis. The DNase I footprinting assay was performed as described (15) with the following modifications. 32P-labeled DNA (25 pmol, as was used for EMSA) was mixed with 0, 70, 140, or 280 pmol of the PorX-C-His6 protein in a 100 µl reaction. DNase I digestion was carried out using 0.05 units DNase I (Invitrogen) per reaction. Samples were analyzed by 6% denaturing polyacrylamide electrophoresis by comparison with a DNA sequence ladder generated by Maxam and Gilbert A + G reaction, using the same 32P-labeled PCR product. The positions of radioactive DNA fragments in the gels were detected by autoradiography.

Reconstitution of RNA polymerases from isolated subunits. A procedure for reconstitution of E. coli RNA polymerase holoenzyme developed and described in detail (16) was successfully used for reconstitution of other bacterial RNAPs. We used a modified procedure presented in a previous study (17) to carry out reconstitution of P. gingivalis RNA polymerase holoenzymes with the following modifications. Briefly, prior to the in vitro reconstitution, RNA polymerase subunits isolated from the procedure above were suspended in a denaturation buffer (6 M guanidine-HCl, 50 mM Tris-HCl [pH 7.9], 10 mM MgCl2, 10 µM ZnCl2, 10% glycerol, 1 mM EDTA, and 10 mM DTT). The mixtures were left for 30 min on ice and then spun in a 4°C microcentrifuge at 42°C for 2 h. 32P-labeled primer 4111 was used for primer extension of the transcripts derived from porT chromosomal DNA using primers 3044 and 4111. For porT transcription in vitro, the template was amplified from 33277 chromosomal DNA using primers 4025 and 4026. Transcripts in vitro were monitored after being converted into cDNAs through a primer extension performed as described (19) with the following modifications. RNA pellets derived from templates T1 and T1-sub were reverse transcribed using 2 µl of [32P]-labeled primer 3043 in a 20-µl mixture containing 25 units of M-MuLV reverse transcriptase (NEB) at 42°C for 2 h. [32P]-labeled primer 4111 was used for primer extension of the transcripts derived from template T1. Transcripts derived from the porT template were reverse transcribed by using [32P]-labeled primer 4025. The cDNA samples were precipitated with 2.5 volumes of ethanol and 0.3 M sodium acetate (pH 5.8) and resuspended with RNase-free water. For sigP transcription in vitro, the template T1 was amplified from 33277 chromosomal DNA using primers 3043 and 3044, while T1-sub with substituted PorX binding sequence (from gggtgtgaaatat to caggcgtgaaatat) was prepared with primers 3043 and 3044 and 4105 and 4106 by using an overlap extension PCR (16). The longer template T2 was amplified from 33277 chromosomal DNA using primers 4105 and 4106. For porT transcription in vitro, the template was amplified from 33277 chromosomal DNA using primers 4025 and 4026. Transcripts in vitro were monitored after being converted into cDNAs through a primer extension performed as described (19) with the following modifications. RNAP preparations were used directly in transcription assays or stored under (NH₄)₂SO₄ (65% saturation) until further use.

Transcription of sigP and porT in vitro. The in vitro transcription was conducted in a 50-µl reaction mixture containing 1× in vitro transcription buffer (80 mM HEPES-KOH [pH 7.5], 24 mM MgCl2, 2 mM spermidine, 40 mM DTT with 0.05% bromophenol blue, and 0.05% xylene cyanol), and 1 µg of linear double-stranded DNA (dsDNA) template with the desired amounts of PorX-C-His6 protein and an RNA polymerase holoenzyme. Reaction mixtures were incubated for 2 h at 37°C and transcripts were precipitated using three volumes of cold 100% ethanol and 1/10 volume of 3 M sodium acetate (pH 5.8) and resuspended with RNase-free water. For sigP transcription in vitro, the template T1 was amplified from 33277 chromosomal DNA using primers 3043 and 3044, while T1-sub with substituted PorX binding sequence (from gggtgtgaaatat to caggcgtgaaatat) was prepared with primers 3043 and 3044 and 4105 and 4106 by using an overlap extension PCR (16). The longer template T2 was amplified from 33277 chromosomal DNA using primers 4105 and 4106. For porT transcription in vitro, the template was amplified from 33277 chromosomal DNA using primers 4025 and 4026. Transcripts in vitro were monitored after being converted into cDNAs through a primer extension performed as described (19) with the following modifications. RNAP preparations were used directly in transcription assays or stored under (NH₄)₂SO₄ (65% saturation) until further use.

Virulence assay in a mouse model. All animal experiments conformed to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at our institution. Groups of 6-week-old female BALB/c mice (purchased from Charles River Laboratories) were randomly allocated into different groups. Determination of virulence of the P. gingivalis W83 and mutant strains was performed using mouse subcutaneous infection experiments, as described previously (20), with slight modifications. Briefly, bacterial cells were grown in enriched BHI broth at 37°C for 12 h. The culture was diluted 20-fold in 100 ml of fresh BHI medium and grown for the time periods indicated. The cells were harvested by centrifugation at 10,000 × g for 20 min and washed once with PBS, then adjusted to a concentration of approximately 5 × 10¹¹ CFU/ml in PBS. Resulting bacterial cultures were serially diluted and plated for bacterial CFU to determine the exact titer of all strains used for infections. Mice were challenged with subcutaneous injections of 0.1 ml at each of the two sites on the depilated dorsal surface (0.2 ml per mouse). Infected mice were examined daily for survival.
Statistics. Each in vitro experiment was conducted at least three times independently. Mice were randomly placed into different groups before tests. A Kaplan Meier curve was used for survival analysis in this study. Comparisons between two groups were performed with Student’s t test and P < 0.05 was considered significant. Statistics were calculated with GraphPad Prism version 8.0.

ACKNOWLEDGMENTS

We thank Koji Nakayama and Keiko Sato for P. gingivalis wild-type 33277 and W83 strains and plasmid pGEM-ermF, and Mary Ellen Davey for plasmid pT-COW.

This study was supported by Research Project Grant R01 DE024607 from NIDCR (Y.S. and W.K.).

W.K. and Y.S. conceived and designed the experiments. C.J., D.Y., T.H., Z.H., W.K., and Y.S. analyzed data and drafted the article. All authors analyzed the results and approved the final version of the manuscript.

We declare no conflicts of interest.

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