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The Hpk2-Rrp2 two-component regulatory system of *Treponema denticola:* a potential regulator of environmental and adaptive responses

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SUMMARY

Treponema denticola **levels in the gingival crevice become elevated as periodontal disease develops. Oral treponemes may account for as much as 40% of the total bacterial population in the periodontal pocket. The stimuli that trigger enhanced growth of *T. denticola*, and the mechanisms associated with the transmission of these signals, remain to be defined. We hypothesize that the *T. denticola* open reading frames *tde1970* (histidine kinase) and *tde1969* (response regulator) constitute a functional two-component regulatory system that regulates, at least in part, responses to the changing environmental conditions associated with the development of periodontal disease. The results presented demonstrate that *tde1970* and *tde1969* are conserved, universal among *T. denticola* isolates and transcribed as part of a seven-gene operon in a growth-phase-dependent manner. tde1970 undergoes autophosphorylation and transfers phosphate to tde1969. Henceforth, the proteins encoded by these open reading frames are designated as Hpk2 and Rrp2 respectively. Hpk2 autophosphorylation kinetics were influenced by environmental conditions and by the presence or absence of a PAS domain. It can be concluded that Hpk2 and Rrp2 constitute a functional two-component system that contributes to environmental sensing.**

INTRODUCTION

Periodontal disease reflects an imbalance in a normally well-balanced population of bacteria in the subgingival crevice (Loesche & Grossman, 2001; Handfield *et al.*, 2008). Oral treponemes, and in particular *Treponema denticola*, are important contributors to periodontal disease (Ellen & Galimanas, 2005). *T. denticola* is a member of the red microbial complex, which consists of *T. denticola*, *Porphyromonas gingivalis* and *Tannerella forsythia* (Haffajee & Socransky, 2005; Socransky & Haffajee, 2005). The red complex is tightly associated with advanced chronic periodontitis. In healthy individuals, *T. denticola* is present in the subgingival crevice at low numbers (<1% of the total bacteria). However, as disease develops, *T. denticola* and other oral spirochetes thrive and ultimately represent as much as 40% of

the total bacterial population in the periodontal pocket (Loesche, 1988; Ellen & Galimanas, 2005).

Significant advances have been made in recent years in understanding the complex mechanisms of communication that occur between organisms in oral biofilms (Kolenbrander *et al.*, 2002, 2006; Simionato *et al.*, 2006; Handfield *et al.*, 2008). However, the communication strategies and global regulatory mechanisms of spirochetes associated with periodontal disease have been largely unexplored. In bacteria, two-component regulatory systems are key players in adaptive responses and global transcriptional regulation (Galperin, 2004). Two-component systems typically consist of a histidine kinase and a response regulator. There is considerable variation in domain architecture among histidine kinases and response regulators (Galperin, 2004, 2006). The effector mechanisms of response regulators also vary. The general paradigm for two-component systems is that external stimuli regulate the opposing autophosphorylation-phosphatase activities of the histidine kinase. Phosphorylation of the kinase activates it, allowing for the transfer of phosphate to a conserved aspartate residue in the receiver domain of the response regulator. This induces a conformational change in the output domain that activates the protein (Koretke *et al.*, 2000). The response regulator can then regulate transcription by binding to DNA. Some response regulators can also influence cellular activities through protein–protein interactions, c-di-GMP production or through the regulation of enzymatic activities (Stock *et al.*, 2000; Galperin *et al.*, 2001; Galperin, 2006; Cotter & Stibitz, 2007; Stock, 2007; Rogers *et al.*, 2009; Smith *et al.*, 2009). To date the only two-component system of an oral spirochete that has been demonstrated to be functional is the growth-phase-regulated *T. denticola* AtcR (response regulator) and AtcS (histidine kinase) system (Frederick *et al.*, 2008). AtcR is the only spirochetal response regulator identified to date that harbors a LytTR domain. This observation suggests that AtcR may play a unique role in *T. denticola* gene regulation (Frederick *et al.*, 2008).

In this report we initiate studies to test the hypothesis that the *T. denticola* open reading frames (ORFs) *tde1970* (histidine kinase) and *tde1969* (response regulator) constitute a functional two-component system that is responsive to environmental conditions. tde1970 and tde1969 are homologs of Hpk2 and Rrp2, respectively, which form a two-component system in *Borrelia burgdorferi* that is responsive to environmental stimuli (Yang *et al.*, 2003; Boardman *et al.*, 2008; Ouyang *et al.*, 2008; Blevins *et al.*, 2009). Importantly, *T. denticola* Hpk2 harbors a potential oxygen-sensing, PAS-heme binding domain (Moglich *et al.*, 2009). Oxygen concentrations in the subgingival crevice undergo significant change as periodontal disease progresses. Hpk2 and Rrp2 could prove to be critical regulators of responses to the changing environment associated with disease progression. The results presented here support the hypothesis that Hpk2-Rrp2 is a functional two-component system that functions in environmental sensing.

METHODS

Bacterial strains and culture conditions

T. denticola strains 35405, N17A1, GM1, 33521 and MS25 were cultivated in NOS media in an anaerobic chamber (5% H_2, 20% CO_2, 75% N_2; 37°C). Growth was monitored by dark-field microscopy using a microscope contained within the anaerobic chamber. All strains were obtained from the American Type Culture Collection or kindly provided by Dr. Peter Greenberg (University of Washington).

Ligation-independent cloning and generation of recombinant proteins

Ligation-independent cloning (LIC) techniques were used to generate recombinant proteins as previously described (Frederick *et al.*, 2008). Gene sequences (or portions thereof) were amplified by polymerase chain reaction (PCR) using 2 × Phusion high-fidelity *taq* (Finnzymes, Espoo, Finland) and annealed with the pET46Ek-LIC vector (Novagen, Madison, WI). All oligonucleotide primers used in this report are described in Table 1. The recombinant plasmids were propagated in *Escherichia coli* NOVABlue cells, transformed into *E. coli* BL21 (DE3) cells and protein production was induced with 1 mM isopropyl beta-D-thiogalactopyranoside (3 h; 37°C). Recombinant proteins were purified using nickel chromatography as instructed by the supplier of the resin (Novagen).

Table 1 Oligonucleotide primers used in this study[1]

Rrp2-F	GACGACGACAAGATTATGAAATTCAGTATTTTGGTTATTGATGACGAAAAAAATATTCG
Rrp2 R	GAGGAGAAGCCCGGTTTATTTTTTGCCATTTACTTTTTCCTTTTTGGATTGATTTTC
Hpk2-F	GACGACGACAAGATTATGAGAGAGTTTATGAGAAGGGGAATACAAAAATCC
Hpk2-R	GAGGAGAAGCCCGGTTCATTTTATATCCTTTACCGAATCAAAATCGAAAGTTTTATCGG
1968-69F	CGAAAACTTGATGAATATGATGCGGAG
1968-69R	AAGTTTGTATACGGAACGAGCAGG
1969-70F	GGCAGGGTATCTTACCCGAGGATATGCATAAAATATTTGAG
1969-70R	CCCATCCTCATGGCTTCAACAGCCGTTTCTACCGTCC
1970-71F	AATGAAGAATGTTCCGCTTTGGAAG
1970-71R	GGATTTTTGTATTCCCCTTCTCATAAAC
1971-72F	GTACATACGGTACAAAACATCGC
1971-72R	GGCCCCGAAAACAAAATCG
1972-73F	TTATCAATATCCAAACCTTTGTTTCATTCG
1972-73R	GCCGAACTGTTTATCATACCC
1973-74F	CAGGTGAAAAACCTGCCCTCG
1973-74R	CTCGTCTATAAAACCGGTTACGGTAACC
1974-75F	GCCTCGGATATTCTCAGGTGGAATG
1974-75R	AGGCCTCGGCAACGGCAAG
ΔPAS-F	GACGACGACAAGATTATGAAAGCCGATAAGCCTGAAGGTAAAAATAAATATATTGAAGTTTCGG

[1]Ligase independent a cloning tail sequences are indicated by underlining.

DNA sequence analysis

hpk2 and *rrp2* were amplified from several *T. denticola* strains and annealed into the pET46Ek-LIC expression vector as described above. Insert sequences, determined on a fee-for service basis (MWG Biotech, Ebersberg, Germany), were translated (Expert Protein Analysis System proteomics server) and aligned (BioEdit sequence alignment editor 7.0.9.0); percentage similarity/identity values were calculated (Matrix Global Alignment Tool).

Generation of antiserum and immunoblotting techniques

Antisera to Hpk2 and Rrp2 (derived from *T. denticola* 35405) were generated in C3H/HeJ mice using 25 μg recombinant protein (Imject Alum adjuvant; Pierce, Rockford, IL). Boosts were administered at 2, 4 and 6 weeks. The mice were sacrificed (week 7), blood was harvested and serum was prepared. To prepare cell lysates for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), *T. denticola* (with optical density at 600 nm of 0.1) was suspended in SDS sample buffer (150 μl) and boiled. The lysates (3 μl) were fractionated by SDS–PAGE (12.5% Criterion Precast SDS–PAGE gels; 200 V; 1 h), transferred to polyvinylidene fluoride (PVDF) membranes by electroblotting and screened with specific antiserum (1 : 1000 in blocking buffer; 1% phosphate-buffered saline, 0.2% Tween-20, 5% Carnation non-fat dry milk). Blots of recombinant proteins were screened with anti-His antibody (1 : 10,000 dilution). Bound antibody was detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Pierce; 1 : 40,000 dilution) using chemiluminescence.

Real-time reverse transcription PCR

Cells were cultivated for either 4 or 13 days and RNA was extracted using the RNEasy Extraction Kit (Qiagen, Valencia, CA). Reverse transcription (RT) -PCR and real-time RT-PCR analyses were conducted as previously described (Zhang *et al.*, 2005). To generate standard curves, amplicons of each gene were cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) and serial dilutions of the purified plasmid were used as the PCR template. *flaA* transcript levels, a constitutively expressed gene, served as the standardization–normalization control for real-time RT-PCR analyses.

Autophosphorylation and phosphotransfer assays

Autophosphorylation of Hpk2 was assessed using recombinant protein (20 ng μl^{-1}) under aerobic (room atmosphere) or anaerobic (5% CO_2, 10% H_2 and

85% N_2) conditions in kinase buffer (50 μl volume, 30 mM HEPES, pH 8.0, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, 40 nM γ-^{32}P ATP, 6000 Ci m mol^{-1}, at room temperature). For anaerobic assays, all reagents were equilibrated in an anaerobic chamber for 3 days. Aliquots from each reaction (0, 5, 10, 30 min) were mixed with 2× SDS sample buffer, fractionated by SDS–PAGE and transferred to PVDF membranes. The membranes were exposed to film at −80°C for 4 h with intensifying screens. To quantify autophosphorylation, the reactions were repeated as above, fractionated by SDS–PAGE, transferred to PVDF membranes, and stained with Coomassie. The bands corresponding to Hpk2 were excised, transferred to glass vials and the amount of incorporated phosphate was determined using liquid scintillation counting.

Phosphotransfer was assessed by incubating radiolabeled–phosphorylated recombinant Hpk2 (generated as described above) with recombinant Rrp2 (20 ng μl^{-1}) in kinase buffer under aerobic or anaerobic conditions. Aliquots from the reaction mixture (0, 5, 10, and 30 min) were mixed with 2× SDS sample buffer, fractionated by SDS–PAGE, electroblotted and exposed to film as above.

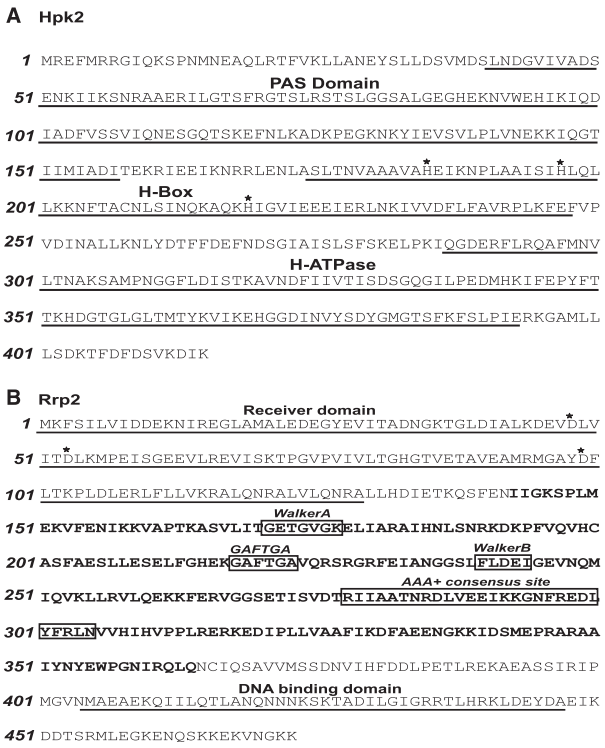

Figure 1 Sequence and functional domains of Hpk2 and Rrp2. Amino acid sequences are shown for the Hpk2 (A) and Rrp2 (B) proteins of *Treponema denticola* strain 35405. Predicted functional domains for both proteins are indicated. The amino acids of Rrp2 shown in bold type indicate the σ^{54}-interaction domain. Additional functional domains that reside within the σ^{54}-interaction domain are highlighted by boxes. Residues that may undergo autophosphorylation in Hpk2 or that serve as phosphoacceptor residues in Rrp2 are indicated by asterisks.

RESULTS

Properties, sequence conservation and distribution of tde1970 (Hpk2) and tde1969 (Rrp2) among *T. denticola* isolates

The proteins tde1970 (46 kDa) and tde1969 (53 kDa) are functionally annotated as a sensor kinase and a response regulator, respectively (Seshadri *et al.*, 2004). As a consequence of their homology with Hpk2 (Histidine protein kinase 2) and Rrp2 (Response regulator protein 2) of *B. burgdorferi* (Yang *et al.*, 2003), we designate tde1970 and tde1969 as Hpk2 and Rrp2, respectively.

The domain architecture of Hpk2 and Rrp2 is depicted in Fig. 1. Hpk2 harbors an N-terminal PAS domain with a putative heme binding pocket. The association of heme-binding PAS domains with oxygen sensing suggests that Hpk2 could play an important role in responding to changing oxygen concentrations in the subgingival crevice as disease progresses (Taylor & Zhulin, 1999; Galperin, 2004). The PAS domain is followed by an H-Box domain (with three putative His autophosphorylation sites at H185, H197 and H219) and an H-ATPase domain (ATP-Mg^{2+}-binding sites). The PCR and subsequent DNA sequence analyses of *hpk2* from a panel of *T. denticola* isolates revealed that the gene is highly conserved with amino acid identity values >96% (Table 2). All of the major functional domains and putative functionally important residues of Hpk2 are conserved (Fig. 1A). It is noteworthy that *T. denticola hpk2* sequences harbor a unique insertion within the PAS domain of 45 nucleotides/15 amino acids (indicated in Fig. 1A). This insertion is not found in other annotated PAS-domain-containing proteins, including those of other spirochetes.

Rrp2 harbors a receiver-phosphorylation domain (with three highly conserved Asp residues at positions 48, 53 and 99), a σ^{54} interaction domain, an 'AAA' ATPase domain and a helix-turn-helix

Table 2 Percentage amino acid identity and similarity values for *Treponema denticola* Hpk2 orthologs[1]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
(1) Td(35405)	–	96.6	98.8	98.6	98.8	55.9	45.5	32.7	19.5	19.3	32.0	31.5	32.0	31.7	21.6	20.2
(2) Td(N17A1)	97.8	–	95.9	95.7	95.9	57.0	46.3	33.1	17.7	19.2	33.2	32.1	32.9	32.6	22.6	18.8
(3) Td(GM1)	99.5	97.3	–	99.3	100	56.3	45.3	32.7	18.4	18.5	32.0	31.5	32.0	31.7	21.6	20.1
(4) Td(35521)	99.5	97.3	99.5	–	99.3	56.6	45.5	32.9	17.4	18.1	32.2	31.7	31.7	31.5	21.8	20.4
(5) Td(MS25)	99.5	97.3	100	99.5	–	56.3	45.3	32.7	18.4	18.5	32.0	31.5	32.0	31.7	21.6	20.1
(6) TREV10001_1211	74.7	75.6	74.9	74.9	74.9	–	49.5	34.1	18.0	19.1	34.7	34.7	34.1	33.8	23.4	18.4
(7) Tp520	65.5	66.6	65.5	65.5	65.5	67.3	–	33.3	16.5	16.8	33.6	31.9	32.3	32.6	25.9	18.2
(8) BB0764	55.7	56.1	55.7	55.7	55.7	57.9	58.9	–	18.5	18.6	74.1	73.8	72.8	73.1	23.9	18.4
(9) BG0787	33.3	35.6	34.9	34.2	34.9	37.4	33.0	38.0	–	90.9	18.2	19.2	19.4	18.6	17.3	15.5
(10) BA(PKo)0811/12	36.4	35.9	35.4	34.7	35.4	35.1	32.0	39.5	97.0	–	20.2	16.8	19.2	19.5	14.7	16.4
(11) BT0764	55.4	56.6	55.4	55.4	55.4	59.2	57.6	84.7	38.9	38.6	–	94.3	90.9	90.9	23.3	20.9
(12) BH0764	54.9	55.6	54.9	54.9	54.9	59.2	56.9	85.5	38.1	37.6	96.9	–	92.5	92.5	26.1	20.8
(13) BDU768	55.2	55.9	55.2	55.2	55.2	57.9	57.4	85.5	37.8	37.8	95.9	97.4	–	99.5	24.3	21.7
(14) BRE771	54.9	55.6	54.9	54.9	54.9	58.2	57.6	85.5	36.3	37.8	95.6	97.2	99.7	–	24.6	21.7
(15) LA2401	45.5	46.6	45.5	45.5	45.5	44.3	47.5	47.4	32.9	38.5	48.2	50.5	49.0	49.2	–	19.0
(16) LBL1667	36.4	35.7	36.4	36.6	36.4	34.1	33.4	37.8	27.2	29.5	36.2	35.7	35.4	95.4	34.2	–

[1]Identity and similarity values are presented in the upper right and lower left quadrants respectively. The *T. denticola* (Td with isolate designations in parentheses) sequences were determined as part of this report. All other sequences were obtained from the databases. The open reading frame designation/numbers are provided.
T. vincentii, TREV; *T. pallidum*, Tp; *Borrelia burgdorferi*, BB; *B. garinii*, BG; *B. afzelii*, BA; *B. turicatae*, BT; *B. hermsii*, BH; *B. duttonii*, BDU; *B. recurrentis*, BRE; *Leptospira interrogans*, LA; *L. borgpetersenii,* LBL.

DNA-binding domain (Fig. 1B). The putative functional domains of Rrp2 share significant sequence similarity with domains present in response regulators of the NtrC-fis family (σ^{54}-RNA polymerase transcriptional activators). These activators function with σ^{54}-RNA polymerase holoenzyme and stimulate isomerization of the closed promoter complex to an open complex in a reaction that requires ATP hydrolysis (Beck *et al.*, 2007). Sequence analysis of *rrp2* from a panel of *T. denticola* isolates revealed amino acid identity values >98%, respectively (Table 3).

In the *T. denticola* isolate 35405, *hpk2* and *rrp2* are surrounded by ORFs that overlap or have short intergenic spacers (Fig. 2A). This arrangement suggests that these genes may be transcribed as a polycistronic messenger RNA that initiates with tde1974 and extends through tde1968. The proteins encoded by these ORFs are described in Table 4. PCR analyses of other *T. denticola* isolates revealed that the *tde1968-tde1974* gene cluster is present and similarly oriented (data not shown). In other *Treponema* and *Borrelia* species an analogous *tde1974-tde1969* gene cluster is present but it lacks *tde1972* and *tde1968*. *tde1968* is present in the genome of these spirochetes but it is distally located from *hpk2-rrp2*.

Transcriptional analysis of *hpk2* and *rrp2*

To determine if *hpk2-rrp2* and flanking genes are co-transcribed, RT-PCR analyses were conducted using gene spanning primers. The RT-PCR analyses identified a transcriptional unit consisting of ORFs *tde1968* through *tde1974* (Fig. 2B). As a negative control, RT-PCR was performed using a primer set spanning ORFs *tde1975* and *tde1974*. A product was not obtained consistent with the opposite orientation of *tde1975*. RT-PCR of *fhbB*, a constitutively expressed membrane protein that binds to the complement regulatory protein factor H (McDowell *et al.*, 2009), served as a positive control for RT-PCR. Finally, all primers were tested using genomic DNA as template to verify that all primers were functional. Henceforth we refer to this seven-gene operon as the *hpk2-rrp2* operon.

Demonstration of growth phase-dependent expression of *hpk2* and *rrp2*

To determine if transcription of the *hpk2-rrp2* operon is influenced by growth stage, real-time RT-PCR was performed using RNA extracted from cells harvested after 4 and 13 days of cultivation. Relative to day 4, a 100-fold induction in *hpk2-rrp2* transcript levels was

Table 3 Percentage amino acid identity and similarity values for *Treponema denticola* Rrp2 orthologs[1]

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
(1) Td(35405)	–	98.7	99.6	99.8	99.6	68.7	66.6	52.0	52.0	52.0	52.2	51.6	52.4	52.2	19.3	41.9
(2) Td(N17A1)	99.6	–	99.2	98.9	99.2	68.3	66.6	51.8	51.8	51.8	52.0	51.8	52.6	52.4	18.6	41.7
(3) Td(GM1)	99.8	99.8	–	99.8	100	68.3	66.6	52.0	52.0	52.0	52.2	51.6	52.4	52.2	18.8	41.9
(4) Td(35521)	98.8	99.8	100	–	99.8	68.3	66.6	52.0	52.0	52.0	52.2	51.6	52.4	52.2	19.0	41.9
(5) Td(MS25)	98.8	99.8	100	100	–	68.3	66.6	52.0	52.0	52.0	52.2	51.6	52.4	52.2	18.8	41.9
(6) TREV10001_1211	84.1	83.9	84.1	84.1	84.1	–	64.8	52.6	53.0	52.8	53.2	53.4	54.1	53.7	16.7	39.9
(7) Tp520	81.6	81.2	81.4	81.6	81.4	83.2	–	52.6	53.2	53.2	54.1	54.3	54.3	54.1	17.8	40.3
(8) BB0764	72.5	72.1	72.3	72.5	72.3	72.4	73.4	–	96.5	97.1	84.0	86.3	84.7	84.7	17.1	39.4
(9) BG0787	72.9	72.9	72.7	72.9	72.7	73.5	74.5	98.0	–	96.9	84.2	86.5	85.2	85.2	17.5	40.1
(10) BA(PKo)0811/12	72.5	72.3	72.3	72.5	72.3	72.6	73.8	99.1	99.6	–	84.6	86.7	85.2	85.2	17.5	39.7
(11) BT0764	72.9	72.7	72.7	72.9	72.7	73.2	74.7	91.2	91.7	91.0	–	95.0	91.9	91.4	19.9	38.5
(12) BH0764	72.5	72.5	72.5	72.5	72.3	73.2	74.9	92.9	93.4	92.7	97.1	–	92.5	92.0	18.2	38.5
(13) BDU768	74.0	73.8	73.8	74.0	73.8	73.9	75.1	92.2	93.8	92.7	95.4	96.9	–	99.6	15.8	39.0
(14) BRE771	73.8	73.6	73.6	73.8	73.6	73.5	74.7	92.2	93.8	92.7	95.0	96.5	99.6	–	16.1	38.8
(15) LA2401	36.6	35.7	35.9	35.9	35.9	35.2	34.7	36.6	35.7	36.1	37.1	36.7	33.9	34.7	–	20.1
(16) LBL1667	60.9	60.9	61.1	61.1	61.1	62.5	63.1	60.8	61.1	60.8	61.2	61.9	60.5	60.3	33.7	–

[1]Identity and similarity values are presented in the upper right and lower left quadrants respectively. The *T. denticola* (Td with isolate designations in parentheses) sequences were determined as part of this report. All other sequences were obtained from the databases. The open reading frame designations/numbers are provided.
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observed at day 13 (Fig. 2C). The data were normalized against the numbers of *flaA* transcript detected and are presented as the ratio of the number of *hpk2-rrp2* transcripts to the number of *flaA* transcripts. Immunoblot analyses of *T. denticola* cell lysates obtained from cultures harvested at 4, 9 and 13 days confirmed increased production of Hpk2 and Rrp2 with growth phase (Fig. 2D). The levels of FlaA protein, a constitutively produced protein, remained unchanged.

Autophosphorylation and phosphotransfer capabilities of Hpk2 and Rrp2

Using γ-^{32}P ATP as a phosphate source, Hpk2 was demonstrated to autophosphorylate in a time-dependent manner (Fig. 3; left panel). As a control for the specificity of autophosphorylation, Rrp2, which is not expected to autophosphorylate, was incubated alone with γ-^{32}P ATP. No labeling was observed (data not shown). Phosphorylated Hpk2 was then demonstrated to transfer phosphate to Rrp2 (Fig. 3). A plateau or equilibrium in Rrp2 phosphorylation was reached by 5 min. This rapid transfer and plateau is consistent with the use of a 1 : 1 ratio of Hpk2 to Rrp2 in the reaction. Note that the 1 : 1 ratio of Hpk2 to Rrp2 was verified by immunoblotting using anti-His antiserum (Fig. 3; right panel). We noted that phosphotransfer requires preloading of Hpk2 with phosphate. When non-phosphorylated Hpk2 and Rrp2 were combined before the addition of γ-^{32}P ATP, phosphotransfer did not occur.

The Hpk2 PAS domain senses *in vitro* environmental conditions and influences the kinetics of autophosphorylation and phosphotransfer

To assess the contribution of the PAS domain in Hpk2 autophosphorylation and phosphotransfer, full-length Hpk2 and an N-terminal 122 amino acid truncation variant (Hpk2ΔPAS) were generated. The N-terminal truncation removes the PAS domain but leaves other functional domains of the protein intact. Autophosphorylation reactions were set up as detailed above except the reactions were conducted under aerobic and anaerobic conditions. Autophosphorylation was quantified by measuring the incorporation of ^{32}P into Hpk2 and Hpk2ΔPAS. Under aerobic conditions no significant difference in phosphate incorporation was observed between Hpk2 and Hpk2ΔPAS ($P > 0.05$) (Fig. 4). However, under anaerobic conditions significant differences were observed. Hpk2ΔPAS displayed

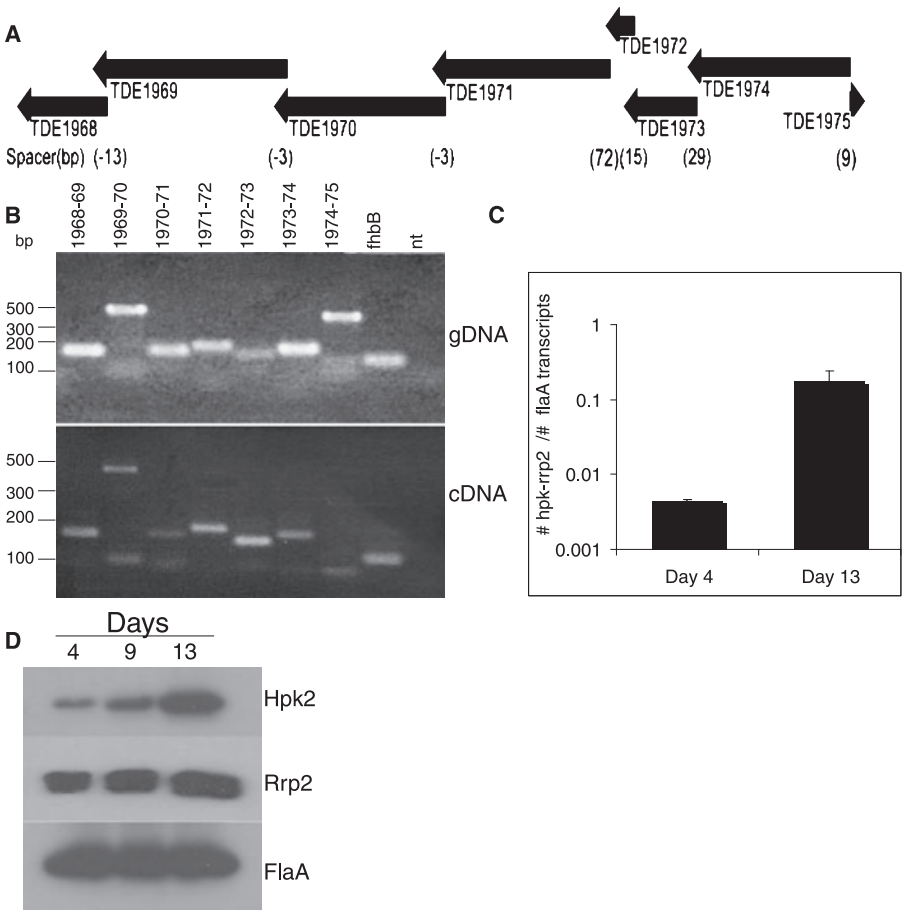

Figure 2 Schematic of the *Treponema denticola* 35405 *hpk2-rrp2* locus: demonstration of co-transcription and growth-phase-regulated expression. The organization of *hpk2*, *rrp2* and adjacent genes are shown in (A) [open reading frame (ORF) designations and the direction of transcription are indicated]. Intergenic spacer lengths (in base pairs) are listed below the schematic in parentheses with negative numbers indicating coding sequence overlap. Reverse transcription–polymerase chain reaction (RT-PCR) analyses, using primers that amplify across the intergenic spacer region, are presented in (B) (complementary DNA panel). To verify that the primers were functional, each was tested with genomic DNA (gDNA) as template. Detection of the constitutively expressed *fhbB* (Factor H binding protein B) (McDowell *et al.*, 2007) gene served as a positive control for RT-PCR. Nt indicates that no template was added (negative control). The size standards (in base pairs) are indicated to the left. The results of *hpk2-rrp2* quantitative RT-PCR analyses are presented in (C). Transcript levels were determined using RNA recovered from cultures grown for 4 or 13 days (as indicated). The data were normalized using the levels of the constitutively and highly transcribed *flaA* gene. (D) Immunoblot analyses in which the relative production of Hpk2, Rrp2 and FlaA (a constitutive control) were measured in cells cultivated for 4, 9 or 13 days. All methods are described in the text.

a reduction in phosphate incorporation relative to the full-length form of Hpk2 ($P < 0.05$) (Fig. 4; right panel). Whereas Hpk2ΔPAS retained its autophosphorylation activity (albeit at a reduced level under anaerobic conditions), it was not competent to transfer phosphate to Rrp2 (data not shown).

DISCUSSION

The 70 or more *Treponema* species that reside in the oral cavity (Paster *et al.*, 2001, 2006) represent a low percentage of the total bacterial mass of the subgingival crevice in healthy individuals. However, spirochetes become dominant in the periodontal pocket as disease progresses (Loesche, 1988; Ellen & Galimanas, 2005). The molecular basis of the adaptive responses associated with successful outgrowth of spirochetes during periodontal disease have not been delineated. The *T. denticola* genome encodes several two-component systems, orphan kinases and orphan response regulators (Seshadri *et al.*, 2004; Frederick *et al.*, 2008) that are likely to be key mediators of

Table 4 Description of genes/proteins investigated in this study

orf	Gene	Paralogs	Function and notes
tde1968	ftsJ (rrmJ)	none	23S ribosomal RNA methyltransferase; 2′-*O*-methylates residue U2552 of the A loop of 23S rRNA; methylation stabilizes the 50S subunit within the 70S ribosome (Hager *et al.*, 2002); inactivation of this gene in *Borrelia burgdorferi* impaired growth rate and morphology (Morozova *et al.*, 2005)
tde1969	rrp2	2079, 2309, 2593, 0492, 1494, 2324, 2501, 2502, 0033, 0648, 0655, 0149, 0855	σ^{54}-dependent transcriptional regulator/response regulatory protein; In *B. burgdorferi* (Bb) an Rrp2 is required for survival as deletion of *rrp2* appears to be lethal (Yang *et al.*, 2003); In Bb Rrp2 directly or indirectly controls a regulon consisting primarily of plasmid carried genes involved in virulence (Burtnick *et al.*, 2007; Caimano *et al.*, 2007; Lybecker & Samuels, 2007; Blevins *et al.*, 2009)
tde1970	hpk2	2502, 0492, 0656	Histidine kinase, possibly involved in oxygen sensing; In Bb, it is it is presumed to be the cognate kinase for Rrp2, however, this has not been directly demonstrated
tde1971	dnaX	2586	Gamma/tau subunit of DNA polymerase III, DNA replication
tde1972	HP	2397, 1131, 2094, 0692, 2223, 1235, 1005	40 amino acid peptide with a possible toxin BmKK4 domain (a member of the subfamily α-KTx17) (Zhang *et al.*, 2004a,b)
tde1973	cvpA	none	colicin V (anti-bacterial) production factor
tde1974	murG	none	A glycosyltransferase that catalyses the last intracellular step of peptidogylcan synthesis; it is required for cell growth and survival; interacts via hydrophobic interactions with the inner membrane; may be part of the divisome (Mohammadi *et al.*, 2007)

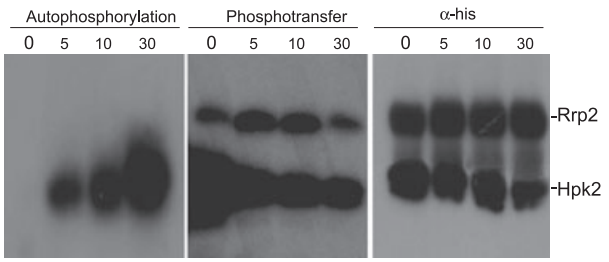

Figure 3 Demonstration of Hpk2 autophosphorylation and phosphotransfer to Rrp2. Autophosphorylation of Hpk2 (left panel) and phosphotransfer to Rrp2 (middle panel) were assessed over time (as indicated above each lane in minutes) using recombinant proteins and protocols detailed in the text. To verify that equal molar amounts of Hpk2 and Rrp2 were used in the assay, an identical blot was screened with anti-his antibody (right panel). Note that for the phosphotransfer analyses, Hpk2 was preloaded with phosphate before mixing with Rrp2 and then aliquots were removed at the time points indicated. The migration position of each protein is indicated to the right.

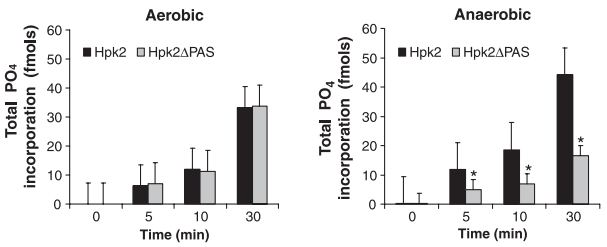

Figure 4 Measurement of Hpk2 autophosphorylation: analysis of the contribution of the PAS domain and the influence of environmental conditions. Autophosphorylation of recombinant Hpk2 and Hpk2ΔPAS under aerobic and anaerobic conditions (as indicated above each panel) was assessed as detailed in the text with incorporation of ^{32}P serving as the read out. All assays were conducted in triplicate and the variance determined. Asterisks indicate statistically significant differences between Hpk2 and Hpk2ΔPAS.

adaptive responses. However, to date, only the AtcRS system of *T. denticola* has been analysed and demonstrated to be functional in terms of autophosphorylation and phosphotransfer (Frederick *et al.*, 2008). It is our hypothesis that the *T. denticola* Hpk2 and Rrp2 proteins form a functional two-component system that plays a role in sensing changes in environmental conditions. As detailed above, the rationale for studying this particular system and for the nomenclature applied, stems from the homology of these proteins to the *B. burgdorferi* Hpk2-Rrp2 two-component system, a key transducer of environmental signals (Yang *et al.*, 2003; Burtnick *et al.*, 2007; Boardman *et al.*, 2008; Ouyang *et al.*, 2008; Blevins *et al.*, 2009). The goals of this study were to assess the sequence properties, transcriptional expression patterns, functional activity and environmental responsiveness of this previously uncharacterized two-component regulatory system.

To assess the molecular properties of Hpk2 and Rrp2, PCR, DNA sequence and database analyses were conducted. The genes are universal and the putative functional domains and residues of *hpk2* and

rrp2 are conserved among all *T. denticola* isolates that have been analysed. The sequence analyses detailed above revealed that the N-terminal PAS domain of Hpk2 harbors a 15-amino-acid insertion that is not found in the PAS domains of other bacteria (http://cmr.jcvi.org.). This insertion was detected in all *T. denticola* isolates but not in Hpk2 orthologs of other spirochetes including *T. vincentii*, *T. pallidum*, *Borrelia* spp. and *Leptospira*. It remains to be determined if this insert imparts unique biological characteristics or influences the activity of Hpk2. Conserved within the Hpk2 PAS domain is a putative heme-binding pocket. Heme-binding domains allow for the sensing of redox potential and or oxygen levels (Taylor & Zhulin, 1999; Galperin, 2004, 2006). The features of Hpk2 and Rrp2 suggest that this putative two-component system may be an important contributor to the sensing of environmental changes that occur in the subgingival crevice as periodontal disease progresses.

Analyses of a panel of *T. denticola* isolates demonstrated that the genes upstream and downstream of the *hpk2-rrp2* genes of *T. denticola* 35405 are conserved in sequence, gene order and orientation. Several of these genes have short intergenic spacers or have overlapping coding sequences. Consistent with this, transcriptional analyses revealed that *hpk2-rrp2* are co-transcribed as part of a larger polycistronic messenger RNA that includes FtsJ (23S ribosomal RNA methyltransferase), DnaK (DNA polymerase III tau/gamma subunit), CvpA (colicin V production factor), a hypothetical ORF and MurG (peptidogylcan synthesis). Transcript levels were 100-fold higher in late-stage cultures, indicating that expression of the operon responds to stimuli associated with growth phase and or cell density. The potential significance of the co-expression of these genes is discussed below.

The ability of the Hpk2-Rrp2 two-component system to function as a cognate kinase-response regulator pair was demonstrated *in vitro* using recombinant Hpk2 and Rrp2. Autophosphorylation progressed in a linear fashion out to 30 min and then reached a plateau. Phosphotransfer from Hpk2 to Rrp2 occurred rapidly but was completely dependent on the preloading of Hpk2 with phosphate. This is consistent with that previously demonstrated for the *T. denticola* AtcRS system (Frederick *et al.*, 2008). Premature interaction of Rrp2 with monomeric-unphosphorylated Hpk2 might inhibit dimerization, a necessary step for phosphotransfer. Kinase dimerization is thought to result in the presentation of a stable interaction surface for the cognate response regulator (McEvoy *et al.*, 1998; Stock *et al.*, 2000; Wright & Kadner, 2001; Ohta & Newton, 2003).

The potential contribution of the PAS domain as a whole in environmental sensing and autophosphorylation was assessed by generating a recombinant Hpk2 truncation mutant lacking the PAS domain (Hpk2ΔPAS). Precedent for assessing the function of individual domains of his-kinases was established in earlier studies (Scholten & Tommassen, 1993). As an example, the role of the PhoR PAS domain was assessed by domain deletion. Deletion of the PAS domain from this histidine kinase provided information about its functional role without abolishing the activity of the autophosphorylation or phosphotransfer-associated domains (Yamada *et al.*, 1990). As demonstrated here, autophosphorylation kinetics of Hpk2 differed significantly for full-length recombinant Hpk2 under aerobic conditions vs. under anaerobic conditions. Autophosphorylation of full-length Hpk2 occurred more rapidly and reached a higher level under anaerobic conditions. Deletion of the PAS domain significantly decreased Hpk2 autophosphorylation specifically under anaerobic conditions (see Fig. 3). These data indicate a link between the PAS domain, Hpk2 autophosphorylation efficiency and environmental conditions.

While the regulon controlled by the Hpk2-Rrp2 two-component regulatory system remains to be defined, the putative functions of the other proteins encoded by the *hpk2-rrp2* operon suggests that they may play an important role in facilitating the outgrowth of *T. denticola*. Contained within this operon are genes encoding proteins critical for DNA replication, cell wall synthesis and translational efficiency. As environmental conditions associated with the progression of periodontal disease develop, the *hpk2-rrp2* operon becomes transcriptionally activated. This presumably leads to a significant increase in the production of murG (peptidoglycan biosynthesis), DnaX (DNA replication) and FtsJ (translational efficiency) all of which could play a key role in facilitating the rapid outgrowth of *T. denticola*. The presence of CvpA (colicin V production factor) and tde1972 (a potential toxin of the α-KTx17 toxin sub-family) (Zhang *et al.*, 2004a,b) within the operon is intriguing (Fath *et al.*, 1989). These proteins are not known to contribute to core functions but it is possible that as *T. denticola* growth

becomes stimulated by physicochemical changes in the periodontal pocket, the production of CvpA and tde1972 could contribute to inhibiting the growth of competitors or inhibit host immune effector cells, allowing *T. denticola* to become a dominant organism. Future analyses will test these hypotheses. In conclusion, the results presented here, coupled with the homology of the Hpk2-Rrp2 to two-component systems of other bacteria, suggest potential involvement in regulating adaptive responses associated with changing environmental conditions.

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