A Eukaryotic-Type Serine/Threonine Protein Kinase Is Required for Biofilm Formation, Genetic Competence, and Acid Resistance in *Streptococcus mutans*

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We report an operon encoding a eukaryotic-type serine/threonine protein kinase (STPK) and its cognate phosphatase (STPP) in *Streptococcus mutans*. Mutation of the gene encoding the STPK produced defects in biofilm formation, genetic competence, and acid resistance, determinants important in caries pathogenesis.

Streptococcus mutans is the principal etiological agent of human dental caries (7). Virulence properties include its ability to grow in the mixed-species biofilm known as dental plaque on tooth surfaces (14, 33) and its ability to tolerate low pH (29). Genetic competence is also thought to be important for survival in the oral cavity, as it promotes genome plasticity and therefore adaptation to changing environments.

Several genes with regulatory roles in biofilm formation, acid tolerance, and genetic competence are known, including the HK11/RR11 and VicRK two-component regulatory systems (17, 30), the histidine kinase CiaH (27), the response regulator TarC (11), trigger factor RopA (32), and the ComCDE quorum-sensing system (5, 15, 16, 18).

Protein phosphorylation is an important mechanism used to translate extracellular signals into cellular responses and is carried out by coupled protein kinases and phosphatases. Although serine/threonine protein kinases (STPK) and their associated phosphatases (STPP) have been known for some time to play major regulatory roles in eukaryotes, their discovery in prokaryotes is relatively recent (13, 22, 31). Eukaryotic-type STPK and STPP have been identified in several bacteria, and some possess multiple kinases and/or phosphatases (3, 12, 25). In gram-positive bacteria, STPK and/or STPP has been found in *Bacillus subtilis* (20), *Listeria monocytogenes* (2), *Streptococcus pneumoniae* (6, 24), *Streptococcus agalactiae* (28), and *Streptococcus pyogenes* (4). The *S. pneumoniae* and *S. agalactiae* STPK are required for virulence in animal models, and the *S. pneumoniae* enzyme is required for genetic competence (6, 28).

Identification of STPK- and STPP-encoding genes. Analysis of the genome sequence (1) identified *pknB* (NC_004350; GeneID 1029535), encoding a homologue of the eukaryotic-like STPK family. The highest similarity is with STPK of *S. pyogenes* (NCBI accession no. AAM79976; 63% identity), *S. agalactiae* (AAM99225; 59% identity), and *S. pneumoniae*

(AAM47530; 51% identity) and is most pronounced in the N-terminal region containing the consensus Hanks' subdomains that comprise the catalytic domain (8, 9). Downstream of the kinase domain is a hydrophobic region that may be a transmembrane domain. Topology analysis (http://www.ch.embnet.org/software/TMPRED_form.html) predicts that PknB is an N-in, C-out membrane protein with the catalytic domain in the cytoplasm and the C-terminal, presumably sensory, domain located extracellularly. The SMART software (<http://smart.embl-heidelberg.de/>) predicts three tandem PASTA (penicillin-binding protein- and serine/threonine kinase-associated) domains (34) in the C terminus. The PASTA domain is thought to bind β -lactam antibiotics and their peptidoglycan analogues, suggesting that PknB may sense unlinked peptidoglycan (34).

pknB is located downstream of, and overlaps with, *pppL* (GeneID 1027981), a gene predicted to encode an STPP of the 2C subfamily (Prosite entry PDOC00792) that is most closely related to the *S. pyogenes* enzyme (AAM79977; 73% identity). The presence of a consensus promoter sequence (Fig. 1) within the gene preceding *pppL* and a transcriptional terminator following *pknB* suggested that *pknB* and *pppL* are cotranscribed, and this was confirmed by reverse transcription-PCR (data not shown). The proximity of the *hk11/rr11* gene pair encoding a two-component regulatory system involved in biofilm formation and acid resistance (17) is interesting, as STPK genes are sometimes adjacent to genes encoding their substrates (21, 23).

Construction and complementation of a *pknB* mutant. *pknB* and flanking DNA were PCR amplified from strain UA159 and cloned into pGEM-T-Easy (Table 1). A 1.7-kb fragment of *pknB* was deleted, and a unique SmaI site was introduced by inverse PCR (Table 1) and used to clone a kanamycin resistance gene (*aphA3*), producing pEA72. The insert was released from pEA72 and transformed into UA159 as a linear fragment. Allelic replacement was confirmed by PCR using primers flanking *pknB* (data not shown).

pVA838 (19) was used to make two constructs for complementation: pEA78, containing *pknB* regulated by the erythromycin resistance gene promoter (P_{erm}) from pVA838, and pEA74, identical to pEA78 except that it lacked a ribosome binding site for *pknB*. Transformation (10) of the *pknB* mutant

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FIG. 1. Physical map of the *pppL/pknB* locus. Thick arrows denote open reading frames. Putative promoter and terminator regions are represented as P and T, respectively. Genes were assigned putative functions based on high BLAST scores with known proteins (accession numbers of the closest orthologues in parentheses): 1, guanylate kinase (AAM79982); 2, RNA polymerase omega subunit (AAM79981); 3, primosomal replication factor Y (AAK34400); 4, methionyl tRNA formyltransferase (AAK34399); 5, rRNA methyltransferase (AAT87518); 6, conserved hypothetical protein (AAM79975); 7, peptidyl-prolyl *cis-trans* isomerase (AAK75626); 8, polyribonucleotide nucleotidyltransferase (general stress protein 13) (AAK34392).

(PKNB) with pEA74 and pEA78 yielded a single *Erm*^r colony in each case.

The *pknB* mutant has a reduced early growth rate. Comparison of the growth of strains PKNB and UA159 in brain heart infusion (BHI) broth showed that PKNB had a reduced growth rate early in the growth cycle, but by exponential phase the growth rate of PKNB was not significantly different from that of UA159 (data not shown). As the mutant had a tendency to clump, cultures were vortexed before removal of samples for optical density (OD) measurement. After overnight culture in tryptone soy broth containing 0.5% (wt/vol) yeast extract, UA159 grew as a uniformly turbid suspension, whereas PKNB accumulated at the bottom of the culture vessel (data not shown). Interestingly, aggregation of PKNB was less apparent in BHI broth (data not shown). Both defects were partially restored by complementation in strain PKNBC1, whereas

PKNBC2 exhibited similar growth kinetics and a propensity to aggregate in the same way as PKNB.

The *pknB* mutant has a transformation defect. pVA838 (19) or genomic DNA from a spontaneous streptomycin-resistant (*Str*^r) strain of UA159, each at 1 µg/ml, was used to measure transformation (10) (Table 2). To eliminate the possibility that the transformation defect was a result of the reduced growth rate of PKNB, assays were carried out that ensured that PKNB and UA159 had reached the same density (OD at 600 nm [OD₆₀₀], 0.2) before DNA addition. These assays gave results similar to those in Table 2 (not shown). Addition of synthetic competence-stimulating peptide (500 ng/ml; Sigma-Genosys Ltd.) (18) did not improve the transformation efficiency of PKNB, whereas UA159 showed a 10-fold increase (data not shown). Reintroduction of *pknB* on a plasmid in PKNBC1 resulted in a transformation frequency 100-fold greater than

TABLE 1. Oligonucleotides, strains, and plasmids used^a

Oligonucleotide, strain, or plasmid	Sequence (5'-3') or relevant characteristic(s)	Amplicon/purpose or source/reference
EA71F	AATGAATTAGTTAAGGC	<i>pknB</i> + flanking
EA71R	TTCAAATATCAAAGACG	<i>pknB</i> + flanking
EA72MF	GCCCCCGGGTGTTCACATCAGG	Inverse PCR
EA72MR	GCCCCCGGGAGCAAATAATTTGCC	Inverse PCR
EA80FF	CAGTCGACGTGAGGGAGATCAATGATTC	<i>pknB</i> with RBS ^b
EA80R	CTGCATGCAAGGATTGAGAAGATTC	<i>pknB</i> with/without RBS
EA80F	CAGTCGACTCAATGATTCAGATTGGC	<i>pknB</i> without RBS
EA62F	GCCGGATCCATAAAAATCGAAACAGC	<i>P_{erm}</i>
EA78R	GCCTCTAGAATTTTATATTTTGTTC	<i>P_{erm}</i>
Strains and plasmids		
<i>Escherichia coli</i>		
XL1 Blue	<i>recA1 endA1 lac</i> [F' <i>proAB lacI</i> ^q Tn10 (Tet ^r)]	Stratagene
DH5α	F ⁻ <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1 supE44 λ⁻ gyrA96 relA1</i>	Gibco-BRL
<i>S. mutans</i>		
UA159	Wild type	1
PKNB	UA159 Δ <i>pknB</i> :: <i>aphA3</i> , Km ^r	This work
PKNBC1	PKNB carrying pEA78	This work
PKNBC2	PKNB pEA74	This work
pGEM-3Z	Cloning vector; Ap ^r	Promega
pGEM-T-Easy	TA cloning vector; Ap ^r Km ^r	Invitrogen
pVA838	<i>E. coli-Streptococcus</i> shuttle vector; <i>Erm</i> ^r	19
pEA71	pGEM-T-Easy derived; carries <i>pknB</i> and flanking DNA as a 2.5-kb amplicon; Ap ^r	This work
pEA72	pEA71 derived, with 1.7-kb deletion in <i>pknB</i> and inserted 1.5-kb <i>aphA3</i> ; Ap ^r Km ^r	This work
pEA74	Identical to pEA78 but lacks RBS	This work
pEA75	pGEM-3Z derived; 180-bp fragment containing <i>P_{erm}</i> promoter cloned in BamHI/XbaI sites	This work
pEA77	pEA75 derived; 1.8-kb fragment containing <i>pknB</i> cloned in Sal/Sph sites of pEA75	This work
pEA78	pVA838 derived; 2-kb fragment of pEA77 containing <i>P_{erm}</i> and <i>pknB</i> cloned in BamHI/SphI sites	This work

^a Restriction sites are underlined. A ribosome binding site in primer EA80FF is in bold.

^b RBS, ribosome binding site.

TABLE 2. Transformability of strains tested using pVA838 or Str^r genomic DNA

DNA source	Strain and experiment no.	Total count (CFU/ml)	Transformants (CFU/ml)
pVA838 ^a	UA159		
	1	7.0×10^{10}	9.1×10^3
	2	3.8×10^9	3.0×10^3
	3	1.2×10^{10}	3.5×10^3
	4	4.2×10^{10}	3.5×10^3
	PKNB		
	1	8.2×10^8	1
	2	7.9×10^8	0
Genomic ^b	UA159		
	1	1.8×10^9	3.8×10^3
	2	1.3×10^{11}	9.6×10^3
	3	1.0×10^{11}	1.3×10^4
	PKNB		
	1	7.8×10^8	<10
	2	8.4×10^{10}	40
	3	5.4×10^{10}	20
	PKNBC1		
	1	3.2×10^8	3.3×10^5
	2	7.6×10^{10}	3.8×10^6
	3	4.8×10^{10}	4.2×10^6
	PKNBC2		
	1	ND ^c	ND
	2	4.8×10^{10}	20
	3	7.5×10^9	180

^a Results are expressed as the median counts from experiments performed in triplicate.

^b Results are expressed as the mean counts from experiments performed in duplicate.

^c ND, not determined.

that of the parent (Table 2), presumably as a result of the increased gene dosage. PKNBC2 gave similar levels of transformation as PKNB.

Mutation of *pknB* results in defects in biofilm formation.

Biofilms were grown on hydroxyapatite disks in 24-well culture clusters (Corning Inc., from Fisher Scientific, Loughborough, United Kingdom). Two milliliters of undiluted and diluted (1:50) exponential-phase culture (OD_{600} , ~0.5) in BHI broth was added to the wells containing the hydroxyapatite disks. After incubation for 48 h the disks were rinsed, treated with *BacLight LIVE/DEAD* stain (Molecular Probes), and visualized by confocal laser scanning microscopy (35). Marked differences in biofilm depth and coverage were apparent between the mutant and parent biofilms (Fig. 2). The parent strain produced a thicker biofilm (typically around 28 μ m) than the mutant (typically around 13 μ m) and produced more biomass. The structure of the parent biofilm is typical of a mature oral biofilm (26), comprising areas covered by stacks and areas with little or no coverage that form channels between the regions of biomass. The mutant produced smaller stacks and clusters with less coverage and more intercellular gaps, more typical of a biofilm during early development. The reduced biofilm density in PKNB is not due to its reduced early growth rate, as prolonged incubation did not result in an increase in biofilm density. In PKNBC1, biofilm density was restored to approximately 70% that of the parent biofilm (data not shown). The fact that PKNBC1 showed only a partial restoration of the biofilm defect whereas it gave levels of transformation greater than the parent is presumably due to perturbation of the STPK-controlled signal transduction pathways that results from overexpression of *pknB* on a multicopy plasmid.

FIG. 2. Confocal laser scanning microscopy images of biofilm that developed on hydroxyapatite disks by *S. mutans* UA159 (A) and *S. mutans* PKNB (B). The intersecting lines indicate the position of the sagittal section (side panels). Dimensions of the region displayed are 167 by 167 μ m (x-y perspective; square panel) and 167 by 28 μ m (x-z perspective; rectangular panel). The images shown are representative of the biofilms after 24 h of growth.

FIG. 3. Effect of pH on the growth of *S. mutans* strains UA159, PKNB, and PKNBC1.

The *pknB* mutant is sensitive to low pH. The abilities of UA159 and its derivatives to grow at low pH were examined by growing them on agar adjusted to pH 5.0 (17). Compared to the parent, PKNB showed reduced growth on pH 5.0 agar, whereas it grew as well as the parent on agar at pH 7.0. PKNBC1 grew as well as the parent at both pH 5.0 and pH 7.0 (Fig. 3).

Summary. Disruption of *pknB*, encoding a eukaryotic-type STPK in *S. mutans*, causes defects in biofilm formation, acid resistance, and genetic competence. Other genes with regulatory roles in these phenotypes are known, raising the possibility that the defects in the *pknB* mutant are the result of altered expression or activity of one or more of these proteins.

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