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Sequence Conservation of Glycerophosphodiester Phosphodiesterase among *Treponema pallidum* Strains

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Previous investigations have demonstrated that immunization with *Treponema pallidum* subsp. *pallidum* glycerophosphodiester phosphodiesterase significantly protects rabbits from subsequent treponeme challenge. In this report, we show that the glycerophosphodiester phosphodiesterase amino acid sequence is conserved among 12 strains from a total of five pathogenic treponemes. The invariant nature of this immunoprotective antigen makes it an attractive candidate for inclusion in a universal subunit vaccine against *T. pallidum* infection. In addition, these studies show a silent nucleotide substitution at position 579 of the *gpd* open reading frame which is consistently observed in the non-*T. pallidum* subsp. *pallidum* strains. This sequence alteration introduces a *Ple*I restriction site in the nonsyphilis strains and thus allows genetic differentiation from *T. pallidum* subsp. *pallidum* strains.

The human-infective *Treponema pallidum* subsp. *pallidum*, *pertenue*, and *endemicum* are the causative agents of syphilis, yaws, and bejel, respectively. Two highly related treponemes that are naturally infectious for animals have also been identified; these are the rabbit-infective species *T. paraluiscuniculi* and the Simian isolate obtained from skin lesions of a monkey (12). Although the human-infective pathogenic treponemes cause clinically distinct diseases, differentiation between *T. pallidum* subsp. *pallidum* and the other *T. pallidum* subspecies on the genetic level has only recently been described (6).

Syphilis remains a public health concern worldwide, with an estimated 3.5 million cases occurring annually (16). Successful control of syphilis depends on the development of an effective vaccine that demonstrates cross-protection between *T. pallidum* subsp. *pallidum* strains. To date, complete protection has been demonstrated only in experimental animals using impractical immunization protocols involving gamma-irradiated treponemes (15). Partial protection has been achieved in experimental animals by immunizing with treponemes that were antiformin treated (21) or aged at 4°C (14), as well as with several recombinant or native *T. pallidum* subsp. *pallidum* proteins, including protein 4D (1), purified endoflagella (8), and TmpB (23). Recent investigations conducted in our laboratory have identified three additional recombinant antigens that provide significant protection against experimental syphilis infections; these are TprK (5), Tp92 (3), and glycerophosphodiester phosphodiesterase (Gpd) (2, 20). Antigens that confer complete protection against infection have yet to be discovered, and successful vaccination regimens against syphilis may involve concurrent vaccination with promising immunoprotective antigens as part of a vaccine cocktail.

In this study, we further extend our investigations into the suitability of Gpd as a potential vaccine candidate by determining the degree of Gpd sequence conservation among pathogenic treponemes.

Bacterial species. The Gpd coding sequence was PCR amplified from genomic DNAs isolated from a variety of treponemal strains. All strains were propagated in New Zealand White rabbits as previously described (13). *T. pallidum* subsp. *pallidum* Nichols was originally sent to the University of Washington by James N. Miller (University of California, Los Angeles) in 1979, and *T. pallidum* subsp. *pertenue* Gauthier was supplied by Peter Perine (Centers for Disease Control and Prevention, Atlanta, Ga.) in 1981. *T. pallidum* subsp. *pallidum* Bal-3, Bal-7, and Bal 73-1; *T. paraluiscuniculi* Cuniculi A; *T. pallidum* subsp. *pertenue* Haiti B; *T. pallidum* subsp. *endemicum* Iraq B; and the Simian isolate were supplied by Paul Hardy (Johns Hopkins University, Baltimore, Md.). *T. pallidum* subsp. *pallidum* Sea 81-3 and Sea 83-1 were isolated by Sheila A. Lukehart from the cerebrospinal fluid of untreated syphilis patients.

PCR amplifications. To obtain the entire *gpd* open reading frame, primers were designed from the 5′ (5′-TGCACGGTG ACGATCTGTGC-3′) and 3′ (5′-GGTACCAGGCGACACT GAAC-3′) noncoding regions flanking the *gpd* gene (11). These primers are located 48 bp upstream and 51 bp downstream, respectively, of the *gpd* open reading frame. PCR amplification of the *gpd* gene was performed by using a 100-μl reaction mixture containing 200 μM deoxynucleoside triphosphates, each primer at 0.25 μM, 1× *Taq* polymerase buffer (50 mM Tris-HCl [pH 9.0] at 20°C, 1.5 mM $MgCl_2$, 20 mM NH_4SO_4), and 1 μl of genomic DNA containing 5,000 to 10,000 treponeme equivalents for each strain. The PCR conditions were 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of extension at 74°C. For each reaction, a hot-start PCR (9) was performed by adding 2.5 U of *Taq* polymerase after the initial denaturation step. Following the PCR, the amplification products were cloned into the pGEM-T vector (Promega, Madison, Wis.) and each insert was sequenced in its entirety in both directions. To reduce the possibility of PCR- or sequencing-induced errors, two clones derived from independent PCR amplifications were sequenced for each strain.

Sequence analysis. Double-stranded plasmid DNA was extracted by using the Qiagen Plasmid Mini Kit (Qiagen, Chatsworth, Calif.), and both strands of insert DNA were sequenced by using the Applied Biosystems dye terminator sequencing kit (PE Applied Biosystems, Foster City, Calif.) and the ABI 373A DNA sequencer in accordance with the manufacturer's in-

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TABLE 1. Summary of Gpd sequence conservation between *T. pallidum* subsp. *pallidum* Nichols and various pathogenic treponeme strains

T. pallidum subspecies	Strain	Sequence divergence from strain Nichols	
		Nucleotides	Amino acids
pallidum	Bal-3	None	None
pallidum	Bal-7	None	None
pallidum	Bal-73-1	None	None
pallidum	Sea 81-3	None	None
pallidum	Sea 83-1	None	None
pallidum	Mexico A	None	None
pertenue (?)	Haiti B	None	None
pertenue	Gauthier	Bp 579, A to G	None
endemicum	Iraq B	Bp 579, A to G	None
?	Simian	Bp 579, A to G	None
paraluiscuniculi	Cuniculi A	Bp 263, G to A	Residue 88, R to H
		Bp 459, A to G	None
		Bp 579, A to G	None
		Bp 711, A to G	None
		Bp 960, C to T	None
		Bp 999, G to C	None

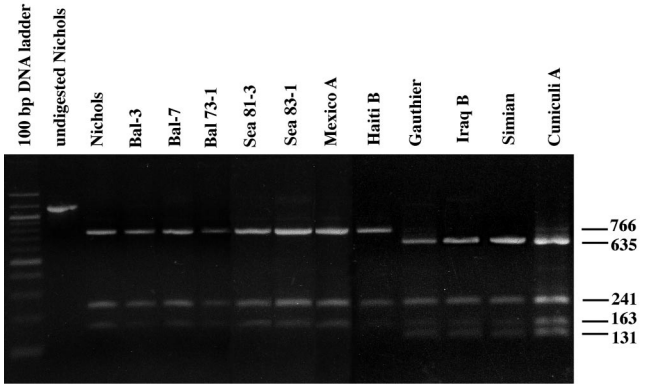

FIG. 1. RFLP analysis of the *gpd* amplicons from various pathogenic treponeme strains. The *gpd* open reading frame was amplified from each of the specified strains, digested with *Ple*I, and subjected to agarose gel electrophoresis and ethidium bromide staining. The left lane shows the 100-bp DNA ladder (New England Biolabs). Shown also is the undigested *gpd* amplicon from the Nichols strain. The sizes, in base pairs, of the DNA fragments generated by *Ple*I digestion of the *gpd* amplicons from the various strains are shown on the right.

structions. In all cases, both universal sequencing primers and internal primers designed from the insert sequence were used. Nucleotide sequences were translated and analyzed by using the Sequencher Version 3.1RC4 sequence analysis software (Gene Codes Corporation, Ann Arbor, Mich.). Alignment of protein and DNA sequences was performed by using the Clustal W general-purpose multiple-alignment program (22).

RFLP analysis. RFLP analysis was performed on the *gpd* open reading frame amplified from each treponeme strain. One microgram of each of the amplified templates was digested with *Ple*I (New England Biolabs, Beverly, Mass.) for 4 h at 37°C prior to electrophoresis on a 1.5% NuSieve (FMC BioProducts, Rockland, Maine) agarose gel.

As shown in Table 1, all six strains of *T. pallidum* subsp. *pallidum* have identical *gpd* gene sequences, while the other human subspecies (*pertenue* and *endemicum*) and the animal pathogens (Simian strain and *T. paraluiscuniculi*) have a silent A-to-G change at bp 579. Interestingly, *T. paraluiscuniculi* (the only different species represented) has five additional base pair changes, one of which (bp 263) results in a conservative amino acid substitution at residue 88. This demonstrates genetic divergence of the nonvenereal treponemal strains and the rabbit pathogen away from the syphilis strains, consistent with their different clinical diseases and host ranges. The Simian strain has been thought to be very closely related (or identical) to the human *pertenue* subspecies (10, 18), and this study supports this hypothesis.

The base pair change at position 579 in the nonsyphilis strains introduces a *Ple*I restriction site that creates different restriction fragment length polymorphism (RFLP) patterns between the *T. pallidum* subsp. *pallidum* strains and the other human and animal pathogens. As shown in Fig. 1, *Ple*I digestion of the *T. pallidum* subsp. *pallidum* strains generates three restriction fragments with sizes of 766, 241, and 163 bp. The presence of the additional *Ple*I site in the nonsyphilis strains generates four restriction fragments with sizes of 635, 241, 163, and 131 bp. These characteristic RFLP patterns provide a means of genetically differentiating between infections caused by the *pallidum* subspecies and those caused by the various other pathogenic treponemes.

The finding that the Haiti B strain, which is reportedly a *T. pallidum* subsp. *pertenue* strain, shows sequence identity with the *pallidum* subspecies and not with the nonsyphilis strains supports the proposal by Centurion-Lara et al. (6) that this strain was misidentified and should be classified as a *T. pallidum* subsp. *pallidum* strain. Similar sequence analyses performed on the *tprK* (7) and *tp92* (4) sequences from the Haiti B strain further support its identification as a *T. pallidum* subsp. *pallidum* strain.

Homologues of Gpd from other bacterial species also demonstrate remarkable conservation of the amino acid sequence. The enzyme from *Haemophilus influenzae*, designated protein D, is 98% conserved among eight strains (19). The corresponding molecule from the relapsing-fever spirochete *Borrelia hermsii*, GlpQ, exhibits a range of 96.5 to 100% amino acid sequence similarity among 26 *B. hermsii* isolates (17). Similarly, results reported here show that Gpd is highly conserved among 12 strains that encompass a total of five pathogenic treponemes. The invariant nature of Gpd, combined with the immunoprotective capability previously described for this molecule in the experimental syphilis model (2), makes it an attractive candidate for inclusion in a universal subunit vaccine against *T. pallidum* infection.

Nucleotide sequence accession numbers. The nucleotide sequences of the *gpd* genes from the Nichols, Bal-3, Bal-7, Bal 73-1, Sea 81-3, Sea 83-1, Mexico A, Haiti B, Gauthier, Iraq B, Simian, and Cuniculi A strains have been assigned GenBank accession no. AF004286 and AF127415 to AF127425, respectively.

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