Nonencapsulated Haemophilus influenzae in Aboriginal infants with otitis media: prolonged carriage of P2 porin variants and evidence for horizontal P2 gene transfer.

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Aboriginal infants in the Northern Territory of Australia experience recurrent otitis media from an early age. Nonencapsulated *Haemophilus influenzae* (NCHi) colonization of the nasopharynx initially occurs within weeks of birth, persists throughout infancy and most of childhood, and contributes to otitis media. We established previously that the high carriage rates of NCHi in these infants result from concurrent and successive colonization with multiple strains, with sequential elimination of dominant strains. We have now sequenced loops 4, 5, and 6 of the NCHi P2 porin gene and characterized several strains with prolonged carriage times. Furthermore, despite a wide diversity of P2 gene sequences, we have four examples of P2 gene identity for strains with different genetic backgrounds as characterized by PCR ribotyping and randomly amplified polymorphic DNA typing, which leads us to suggest that the P2 gene has been transferred between strains. We also discuss the possibility that the paradoxical observation of cocolonization and prolonged carriage of P2-identical strains is related to immune suppression or tolerance in the host.

Aboriginal infants in the Northern Territory of Australia experience recurrent otitis media from a very early age; by 6 months of age, as many as 30% have perforated eardrums. A longitudinal study in one community has shown an association between early nasopharyngeal colonization with multiple pathogens, namely, nonencapsulated *Haemophilus influenzae* (NCHi), *Streptococcus pneumoniae*, and *Moraxella catarrhalis*, and early onset of otitis media (13).

NCHi colonization initially occurs within a few weeks of birth in these infants, persists throughout infancy (13), and can persist until 8 to 12 years of age without successful intervention (unpublished observations). We have used ribotyping (22) and PCR ribotyping (21) to identify numerous strains of NCHi and to show that individual infants were often colonized concurrently with several different strains. The persistence of NCHi colonization at rates of virtually 100% reflected successive and overlapping periods of colonization with distinct strains, with queuing of different strains in each infant (22). It was notable that although most individual NCHi types were carried for approximately 30 days in these infants, carriage for some other types apparently persisted for as long as 9 months.

We have therefore begun to explore molecular characteristics of the major outer membrane protein P2 in order to understand mechanisms that may allow the organism to persist in the host. P2 porin exhibits marked diversity among strains of NCHi. Analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis have demonstrated that strains express P2 proteins with variable molecular weights (1) that correlate with antigenic differences (16). Host antibodies to NCHi are directed to highly strain-specific immunodominant epitopes on the P2 protein (9), which suggests that the region may be subject to immune selective pressure. The P2 protein is a porin that contains eight potentially surface-exposed loops (24), of which loops 5, 6, and 8 have been studied in some detail. Duim et al. (6) demonstrated antigenic drift, predominantly in loop 6, resulting from nonsynonymous single-base mutations. These changes occurred extremely rapidly; the time scale of the appearance of variant strains of NCHi in patients was as short as 5 months. In another study, bacteriadal and therefore potentially protective) epitopes of P2 were mapped to loops 5 and 8 (10). Later, Yi and Murphy (30) subjected the eight loops, individually expressed as fusion proteins, to immunoblot assays with antisera and demonstrated a prominent antibody response exclusively to the loop 5 peptide.

This study focuses on the portion of the P2 gene corresponding to loops 4, 5, and 6. Sequencing of this region has shown marked diversity between strains differentiated by PCR ribotyping. We have characterized P2 sequences for one strain with apparent persistence for 8 months in the same infant and of another persisting for 9 months in a single infant. Furthermore, we have circumstantial evidence for four separate instances of horizontal transmission of a P2 porin gene between two distinct strains; in one case, the strains were cocolonizing.

**MATERIALS AND METHODS**

**Aboriginal subjects.** With ethical approval, 41 Aboriginal infants in a remote community, representing 80% of the appropriate age cohort, were monitored from birth to examine the relationship between bacterial colonization of the nasopharynx and the onset of otitis media (13); most infants leave the community only once or twice a year.

**Samples.** At each examination, a nasopharyngeal swab was collected, smeared for Gram staining, and frozen in 1 ml of transport medium (13). Swabs were also taken from middle ear discharge of infants with ear perforations.

**Bacterial isolates.** Strains of *H. influenzae* were isolated from nasopharyngeal and middle ear samples by culture of 10 µl of transport medium on 7% chocolate agar. Four colonies were sampled from each plate, taking care to select any colonies that were morphologically distinct. NCHi were identified by their requirements for X and V factors and by their lack of reaction with antisera against capsular antigens (Phadebas; KarlRöö Diagnostics AB). This was confirmed by failure of the isolates to generate a PCR product in reactions using capsule-specific primers (7) corresponding to the bexA- and type b-specific regions.

**NCHi strain identification.** Ribotyping (22) to differentiate NCHi strains has been superseded by PCR ribotyping (21), which is quicker and technically simpler. In addition, we used randomly amplified polymorphic DNA (RAPD) typing as an ancillary method of strain identification and used PCR amplification and DNA sequencing to characterize loops 4, 5, and 6 of the P2 gene. Our previous work in this population has shown that from a single nasopharyngeal swab, an average of 85% of colonies were of the same (dominant) NCHi strain; 13% were...
of another (subordinate) strain, and 1 to 2% were of a third (subordinate) strain (22).

**DNA preparation.** Total chromosomal DNA was initially extracted from single colonies of NCHi as described previously (22), but more recently by lysis of bacteria, cultured from single colonies, and embedded in agarose at the bottom of microtitre wells followed by extraction with proteinase K (8).

**Long PCR ribotyping.** The technique has been previously reported (21). Briefly, 70 to 270 ng of DNA was amplified in a 20-μl reaction mixture consisting of 2 μl of 10× PCR buffer, 2 μl of 2 mM deoxynucleoside triphosphates (dNTPs), 0.2 μl of 168G primer, 0.2 μl of 55 primer (20 pmol/μl), 14.48 μl of H₂O, and 0.12 μl of Taq-Pfu (15 U of Taq polymerase and 2.5 U of Pfu DNA polymerase in 16 μl). Each mixture was overlaid with 50 μl of mineral paraffin and cultivated 25 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min followed by a further 35 cycles at 94°C for 30 s, 57°C for 1 min, and 72°C for 1 min. The 25 μl reaction products were analyzed by agarose gel electrophoresis in 1× TAE buffer containing 0.5 μg of ethidium bromide/ml and photographed on Polaroid 667 film, using a 300-nm transilluminator. RAPD patterns were compared visually.

**PCR amplification.** The sequence of primer P2L4, ATYGAT of 10-mer oligonucleotides which were chosen from 34 oligonucleotides investigated as providing the best discrimination. The sequences were GAT CCA GTA C (p15), GGT ACT CCC C (p3), and GAT CGC ATT G (p22). PCR amplification was performed in 25-μl reactions containing 1 μl of DNA (approximately 70 to 270 ng), 2.5 μl of 2 mM dNTPs, 0.5 μl of 50 μM P2L5, p3, or p22 primer, 2.5 μl of 10× reaction buffer, 0.25 μl of 250 mM MgCl₂, and 0.2 μl of Taq (2 μl). Each reaction was cycled five times at 94°C for 30 s, 37°C for 2 min, and 72°C for 5 min followed by a further 35 cycles at 94°C for 30 s, 37°C for 1 min, and 72°C for 1.5 min. The 25 μl reaction products were purified from 0.8% agarose gel in 1× TAE buffer containing 0.5 μg of ethidium bromide/ml and photographed on Polaroid 667 film, using a 300-nm transilluminator. RAPD patterns were compared visually.

**DNA primers for P2 amplification.** The sequence of primer P2L4, ATYGAT GGT ACT TTA GTA TGA, corresponds to positions 484 to 503 of the H. influenzae 1479 P2 gene (19) (GenBank accession number M93268). Primer P2L6 (AGT ATC TTC CAT TAA TTC ATC) is the complement of positions 939 to 967 and P2L3 (GCC AGA AGA TAA AGA TG) corresponds to positions 399 to 418.

**P2 amplification.** The loop 4 to loop 6 region of the P2 gene was amplified in a reaction mixture containing 1 μl of template DNA (approximately 70 to 270 ng), 2.5 μl of 2 mM dNTPs, 0.5 μl of 50 μM P2L3, P2L5, or P2L6 primer, 2.5 μl of 10× reaction buffer, and 0.25 μl of 250 mM MgCl₂, in a final volume of 25 μl. The reaction mix was cycled 30 times at 95°C for 1 min, 60°C for 1 min, and 72°C for 30 s. The loop 5 to 6 region was amplified similarly, using primers P2L3 and P2L6.

**Template preparation for sequencing.** PCR products were purified from 0.8% agarose gels by using Bandpumpe (Progen) as instructed by the manufacturer.

**Determination of DNA sequence.** Cycle sequencing was performed in both directions with primers P2L4 and P2L6, using an AmpliCycle sequencing kit (Perkin-Elmer) as instructed by the manufacturer. Each sequencing reaction mix was cycled 30 times at 95°C for 1 min, 60°C for 1 min, and 72°C for 30 s in a DNA thermal cycler (Corbett). The reaction mixtures were separated on a 6% polyacrylamide gel as described by Sambrook et al. (18). Sequencing gels were dried and exposed to X-Omat AR photographic film, and the resulting sequences were read manually.

**RESULTS**

**NCHi isolates studied.** Previous work (21, 23) had shown that PCR ribotype 7 (PRT 7) colonized 8 of 10 infants investigated. Stored samples from three infants persistently colonized by PRT 7 were chosen for further study: PRT 7 was detected eight times in 8 months in infant 943, four times in 5 months in infant 932, and four times in 3.3 months in infant 946. The reproducibility of the PCR ribotyping patterns can be seen in Fig. 1, where clones of PRT 7 are typed and shown in lanes 1 to 5. PRT 12 isolates were also selected because of extended carriage, because the PRT 12 pattern differed by only one band from the PRT 7 pattern (Fig. 1, lanes 1 to 6) and because PRT 7 and PRT 12 colonized concurrently on one occasion. Isolates of PRTs 25, 38, and 6 were selected because they demonstrated extended carriage; PRT 15, 29, 32, and 41 isolates were chosen at random.

**P2 gene diversity.** The primers for the NCHi P2 porin gene amplified an approximately 360-nucleotide product which corresponded to the loop 4 to loop 6 region of the NCHi P2 porin gene (2). Isolates for 17 PRTs have been successfully amplified, and the amino acid sequences of 10 P2 types are reported here (Fig. 2A). Isolates of the same P2 type, including 23 isolates of P2 type 2, had identical DNA sequences through the region studied. When aligned to published NCHi P2 sequences (2, 6, 19, 28), the NCHi strains demonstrated a high degree of homology at the conserved interloop regions of P2 (presumed to be transmembrane regions) and marked diversity in the (extracellular) loop regions. In particular, there is marked diversity in loops 4 and 5 for local isolates (Fig. 2A), and alignment with published sequences shows no discernible relationships for loops 4 and 5 between the reference isolates and our local isolates. Loop 6 also varies between strains, with block insertions and deletions evident. We have taken P2 type 6 as a prototype for loop 6, as it demonstrates identity at the loop 6 region with the published sequences of isolates 12, 1479, and 19 (2, 19) and differs by one nucleotide from nine sequences of the 29 published P2 sequences. It can be seen that P2 types 1, 2, and 3 have up to 30 bases inserted in this region, P2 type 5 has 24 bases deleted, and P2 types 4, 6, 10 are identical or nearly identical to the prototype strain.

Isolates of PRTs 6 and 25 from different infants were typed as P2 types 8 and 7, respectively. However, one PRT 25 isolate typed as P2 type 5; it was identical in loops 4 and 5 and to other PRT 25 isolates (P2 type 7) but had a significant portion of loop 6 deleted. This observation suggests that all of loop 6 is not essential for porin function.

**Homogeneity of RAPD types within PRT 7.** As PRT 7 was found to be the most commonly colonizing strain and exhibited periods of extended carriage (23), 25 isolates of PRT 7 were analyzed by DNA fingerprinting using the RAPD method (29). All 25 PRT 7 isolates had the same RAPD profile with each of two independent primers, indicating homogeneity within the members of PRT 7. In Fig. 3A, lanes 2 to 6 show the identity of five of these PRT 7 isolates, determined by using primer p15.

**Heterogeneity of P2 type within PRT 7.** However, sequence analysis of the P2 gene across loops 4 to 6 of PRT 7 isolates showed two P2 types designated P2 types 1 and 2 (Table 1, infant 943). The two types appeared closely related but differed by three consecutive amino acids in loop 4, by three amino acids in loop 5, and by nine amino acids in loop 6, orchestrated by single-base changes and a frameshift-compensatory frameshift mutation (Fig. 2B) (17). Thus, the conservation of RAPD products with two independent primers, in contrast to the P2 heterogeneity within PRT 7, suggests that the P2 gene is evolving faster than other sites in the genome.
cocolonization of PRTs 7 and 12 in one infant (Table 1, infant 946, 28 September 1993). The P2 sequence of the PRT 12 isolate (P2 type 2) was identical to that of the PRT 7 isolate, a surprising finding with such a variable gene. However, the PRT patterns of PRTs 7 and 12 differed by only one band. It was important to confirm that the PRT 7 and 12 isolates were genetically distinct, and so we used RAPD typing as described above. The reproducibility of the RAPD technique with these primers (Fig. 3A) demonstrates that PRT 7 and 12 are indeed genetically distinct. Further, an independent technique, PCR fingerprinting, was performed as described by van Belkum et al. (26, 27) (not shown), and this analysis again confirmed that the two types were distinct.

To rule out the possibility that clones had been mislabelled, a different clone of PRT 12 obtained from the same swab was P2 typed and was again found to be identical to P2 type 2. A further trivial explanation for the apparent identity of these particular PRT 7 and 12 isolates at the P2 locus could have been cross-contamination with P2 type 2 PCR product. This, however, was ruled out by using a new set of primers spanning loops 3 through 6, which yield a longer product. The expected sequence was obtained from molecules generated on the first occasion that this longer region was ever amplified in this laboratory, a result that cannot be explained by PCR contamination. The fact that this set of primers could not have amplified contaminating loop 4 to 6 PCR products confirms the identity of PRTs 7 and 12 in this region. In the absence of evidence for any trivial explanations, we believe that horizontal transmission is the simplest explanation for the observed results. Because the evidence from RAPD typing and PCR fingerprinting suggests that the genomes of PRT 7 and PRT 12 are considerably different, we conclude that the P2 genes, and not the multiple tRNA genes, have been transferred.

Other apparent cases of horizontal transmission of the P2 gene. We observed other apparent examples of horizontal transmission of P2 type. PRT 38 was cultured from middle ear discharge approximately 3 months after first detection in the nasopharynx of infant 950. The loop 4 to 6 P2 sequence of PRT 38, designated P2 type 4, remained stable over this period and was the same P2 type as a PRT 12 isolate from the same infant 6 months later (Table 1). The distinct PRT patterns of PRTs 12 and 38 are shown in lanes 6, 8, and 9 in Fig. 1; the patterns differ by at least four bands. Further, the PRT 12 and PRT 38 isolates demonstrated different patterns upon RAPD analysis.
using the p15, p3, and p22 primers (Fig. 3B). We conclude that these isolates provide another example of different strains that have acquired the same P2 type.

A further two examples of identical or nearly identical P2 types in genetically different isolates have also been observed. Infant 938 carried PRT 25 on 1 November 1993 and PRT 29 nearly 5 months later. The PRT patterns of the two isolates differed by at least four bands, but they had identical loop 4 to 6 P2 sequences (P2 type 7). Moreover, infant 950 carried PRT 32 and PRT 41 7 months apart. These two PRTs differed by two bands whereas the P2 sequences differed by one amino acid in each of loops 4, 5, and 6; the PRT 32 and 41 isolates were designated P2 types 9 and 10, respectively. Hence, in this heterogeneous region of P2, these isolates show virtual identity, and if, as suggested above, P2 is evolving faster than other sites in the genome, these observations provide further evidence for horizontal transmission.

A single P2 type can be carried for extended periods without change. Other episodes of extended NCHi carriage were investigated. On one occasion, PRT 25 was observed to be carried for 5 months in infant 938. During this period, infant 938 was also colonized by PRT 6, this strain being carried for a minimum of 8 months. The P2 type of neither PRT 6 nor 25 changed during carriage. This observation contrasts those of others (6, 28) who demonstrated antigenic drift in the P2 region during persistent infection with NCHi over time periods as short as 5 months.

**DISCUSSION**

Evidence for horizontal transfer of the NCHi P2 gene. Horizontal gene transfer in NCHi is hardly surprising, as these organisms are naturally competent for DNA uptake (20), and cocolonization with multiple types at the same time (Table 1...
and reference 22) would provide opportunity for gene transfer. Furthermore, horizontal transfer of type-specific capsulation genes has already been invoked to explain the serotype switching leading to acquisition of the IS-bexA mutation by H. influenzae type a strains (12).

The role of horizontal gene transfer in genetic diversity is well recognized in many pathogens. For instance, horizontal transfer in Neisseria meningitidis, involving porA and other genes, leads to mosaic structures and gene replacement (15). In Streptococcus pyogenes, horizontal gene transfer leading to changes in emm, the gene for the antiphagocytic M protein, has been observed (11).

We have shown in this study that identical P2 sequences (P2 type 2), corresponding to highly variable loops of the P2 porin, were present in two genetic backgrounds that were different by RAPD and PCR ribotype analysis (Table 1). Furthermore, two different P2 sequences (P2 types 1 and 2) were observed in similar genetic backgrounds (PRT 7 and RAPD A). These observations, together with the other examples (see Results), suggest that horizontal gene transfers make a significant contribution to diversity of the P2 gene of NCHi.

Despite the consecutive carriage of closely related P2 types 1 and 2, both associated with PRT 7, in child 943, it is highly unlikely that the genetic transfer happened while the P2 type 1 strain was being carried in this child. This conclusion is supported by the multiplicity of observed changes in P2 and by the earlier detection, in June 1993, of P2 type 2 in another infant from this community (infant 946).

**Table 1. Characterization of the P2 gene in infant isolates**

<table>
<thead>
<tr>
<th>Infant no.</th>
<th>Examination date</th>
<th>Site</th>
<th>Isolate</th>
<th>PCR ribotype</th>
<th>RAPD with primer p15</th>
<th>P2 type</th>
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Shares a P2 type with an isolate of a different PCR ribotype.

ND, not done.

Immune selection and other mechanisms for genetic variation of P2. Other studies have demonstrated the stepwise accumulation of point mutations which contribute to P2 diversity (6, 28). Our NCHi strains present a complex picture of multiple insertions, deletions, and substitutions of amino acids in loops 4 to 6 of the P2 region, while horizontal transmission of P2 gene segments would offer additional mechanisms for genetic diversification.

Some P2-specific monoclonal antibodies have been shown to be potentially protective in vitro (25), so that in vivo selection of sequence variants of P2 by the immune system would help to explain the wide range of P2 variations among the NCHi population seen in this and other studies (e.g., references 2 and 19). Apparent long-term persistence of NCHi has been shown to be due to sequential carriage of different strains (14, 22, 23), considered to be immunologically distinct.

P2 variation attributed to antigenic drift has been reported in persistent NCHi infection with sequence changes in loops 4 to 6 of P2 (6, 28). However, our own data provide no evidence of antigenic drift in sequential isolates in individual children, nor were there any synonymous (third-base) changes observed in closely related P2-types. For example, between P2 types 9 and 10 there were three base changes leading to amino acid substitution but no synonymous changes in loops 4 to 6 of P2; thus, our data favor P2 evolution by selection rather than by antigenic drift.

Thus, there is strong evidence for the view that the P2 molecule is a crucial target of protective immune responses...
(10). However, the evidence for horizontal gene transfer also suggests that molecules other than P2 are also subject to immune selection. If this were not the case, there would be little advantage for a P2 variant in moving from one genetic background to another.

**Prolonged carriage times of NCHi.** We have now shown that the prolonged carriage of certain strains, of identical PCR ribotype, can be due to the sequential carriage of distinct strains which differ in P2 type. In the first instance, PRT 7 isolates, carried over at least an 8-month period, had the same RAPD type with relatively minor differences in P2 type. Yet, in the second case, PRT 12 isolates, carried over a 9-month period, differed markedly in RAPD type as well as P2 type. In addition, we observed extended carriage of a particular strain, over at least 8 months, without any change to the loop 4 to 6 region of the P2 gene. Hence, these observations suggest that the prolonged carriage of NCHi of a particular PCR ribotype can arise in various ways.

To explain the extended carriage of PRT 7, we propose that the immune response eliminating P2 type 1 from child 943 recognized at least one epitope that was not shared with P2 type 2, thus allowing P2 type 2 to colonize after the elimination of P2 type 1. It has been demonstrated elsewhere (6) that an amino acid change at the apex of the proposed loop 6 resulted in the loss of reactivity with a monoclonal antibody. Others found that changes to the NCHi P2 gene, during persistent infection, occurred predominantly in loop 6 (6) and that strains associated with chronic obstructive pulmonary disease had longer forms of loop 6 (5). P2 types 1, 2, and 3 in our study had longer forms of loop 6 compared to the prototype strain, while P2 types 4 and 6 to 10 demonstrated near identity in this region. However, it is noteworthy that the loop 6 length did not show any correlation with carriage time and clinical presentation in this population.

**Prolonged carriage and cocolonization involving identical or related P2 types.** It is of interest that identical or similar P2 types persisted for long periods in some infants despite the immunogenic nature of this molecule (9). For instance, PRT 38 and PRT 12 isolates, both of P2 type 4, colonized a single infant within 6 months of each other, while PRTs 25 and 29 (P2 type 7) were observed in infant 938 within a 5-month period. Similarly, isolates of PRT 7 and PRT 12, both P2 type 2, cocolonized an infant, and PRTs 32 and 41 (P2 types 9 and 10, which differ by three amino acids) colonized infant 950 within 7 months of each other. The phenomenon of contemporaneous cocolonization with different strains of identical or similar P2 types persisted for long periods in some infants despite the immunogenic nature of this molecule (9). For instance, PRT 38 and PRT 12 isolates, both of P2 type 4, colonized a single infant within 6 months of each other, while PRTs 25 and 29 (P2 type 7) were observed in infant 938 within a 5-month period. Similarly, isolates of PRT 7 and PRT 12, both P2 type 2, cocolonized an infant, and PRTs 32 and 41 (P2 types 9 and 10, which differ by three amino acids) colonized infant 950 within 7 months of each other. The phenomenon of contemporaneous cocolonization with different strains of identical or similar P2 types persisted for long periods in some infants despite the immunogenic nature of this molecule (9). For instance, PRT 38 and PRT 12 isolates, both of P2 type 4, colonized a single infant within 6 months of each other, while PRTs 25 and 29 (P2 type 7) were observed in infant 938 within a 5-month period. Similarly, isolates of PRT 7 and PRT 12, both P2 type 2, cocolonized an infant, and PRTs 32 and 41 (P2 types 9 and 10, which differ by three amino acids) colonized infant 950 within 7 months of each other. The phenomenon of contemporaneous cocolonization with different strains of identical or similar P2 types persisted for long periods in some infants despite the immunogenic nature of this molecule (9).

**Possible importance of immune suppression or tolerance.** In our study, the extended carriage of a single NCHi strain evident in some infants, without evidence of immune selection, suggests an inadequate immune response in these particular infants. We suggested previously that the infants in this study may be immune tolerant or immunosuppressed (13, 22); this could be due to infection at an early age, to a high antigenic load delivered to the gut (3) from swallowed pus, to nutritional deprivation, or to the limited major histocompatibility complex diversity in the Aboriginal population (4).

Immunosuppression or partial tolerance with specificity for P2 type could also help explain our unexpected observation of concurrent carriage of two different strains (PRTs 7 and 12) of identical P2 type (in child 946), as well as the sequential carriage of different strains of closely related P2 types 1 and 2 (in child 943). Tolerance could bring this about in two ways: first, type-specific tolerance would lead to prolonged carriage of the P2 type in question and provide a greater than chance opportunity for concurrent carriage of another strain of identical or similar antigenicity; second, this nonrandom aggregation of similar P2 types in an individual carrier could be perpetuated (and multiplied) by the concurrent transmission of coexisting strains to the next carrier.

**Wider implications.** As yet, we can only speculate on the rate of evolution of the P2 gene in this population. Infants and children in this community have very high carriage rates of NCHi, and concurrent carriage of multiple strains (22) in large numbers would provide an environment to favor transformation between strains. Furthermore, prolonged carriage of large numbers of bacteria in chronic infection would provide more opportunity for mutation, with selection following the (delayed) immune response, thus driving P2 evolution. This is not inconsistent with our observation that strains can persist for considerable periods without change in this population because evolutionary changes in individual carriers could still be rare events, require months or years to develop, or depend on partial sequestration of infected foci from the immune system (6). Alternatively, there may be accelerated antigenic change in older individuals, or it may be that selection acts much more slowly, which would imply that the variation observed by us could be a product of many years of evolution. Further work is in progress to help understand the evolutionary implications of these findings and to explore the biological implications that are of potential relevance for treatment, vaccine, and other disease control strategies.

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