Generation of Genic Diversity among *Streptococcus pneumoniae* Strains via Horizontal Gene Transfer during a Chronic Polyclonal Pediatric Infection

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Abstract

Although there is tremendous interest in understanding the evolutionary roles of horizontal gene transfer (HGT) processes that occur during chronic polyclonal infections, to date there have been few studies that directly address this topic. We have characterized multiple HGT events that most likely occurred during polyclonal infection among nasopharyngeal strains of *Streptococcus pneumoniae* recovered from a child suffering from chronic upper respiratory and middle-ear infections. Whole genome sequencing and comparative genomics were performed on six isolates collected during symptomatic episodes over a period of seven months. From these comparisons we determined that five of the isolates were genetically highly similar and likely represented a dominant lineage. We analyzed all genic and allelic differences among all six isolates and found that all differences tended to occur within contiguous genomic blocks, suggestive of strain evolution by homologous recombination. From these analyses we identified three strains (two of which were recovered on two different occasions) that appear to have been derived sequentially, one from the next, each by multiple recombination events. We also identified a fourth strain that contains many of the genomic segments that differentiate the three highly related strains from one another, and have hypothesized that this fourth strain may have served as a donor multiple times in the evolution of the dominant strain line. The variations among the parent, daughter, and grand-daughter recombinant strains collectively cover greater than seven percent of the genome and are grouped into 23 chromosomal clusters. While capturing in vivo HGT, these data support the distributed genome hypothesis and suggest that a single competence event in pneumococci can result in the replacement of DNA at multiple non-adjacent loci.

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Introduction

Horizontal gene transfer (HGT) is a fundamental process in bacterial genome evolution [1]. In the context of infections it can provide pathogenic bacteria with ready access to crucial resistance determinants or virulence factors. Analysis of whole genome sequences (WGS) of multiple isolates from a single bacterial species have revealed extensive population-wide differences among strains in multiple species [2–4]. The differences among strains can occur through small-scale mutations affecting only a few base pairs (substitutions, deletions, insertions) or by HGT where DNA segments from hundreds to thousands of bases can be incorporated from another organism’s genome without the recipient being its offspring. HGT, via transformation, transduction, or conjugation, can lead to the acquisition of entirely new sequences, as well as sequences that are homologous to existing DNA. The transfer of DNA via homologous recombination (HR) leads to the replacement of a region of the genome of a recipient cell by the corresponding region from the donor cell [5]. HR can lead to differentiation by incorporation of identical genes containing single nucleotide polymorphisms (SNPs) and/or the insertion and/or deletion of entire genes and operons. In addition, HR can also increase similarity by incorporation of closely related regions [6]. Whereas only rarely are HGT events detected in *Mycobacterium* species [7], they are frequently observed in naturally transformable species such as *Streptococcus pneumoniae* where multi-locus sequence typing (MLST) and theoretical modeling have indicated that recombination rates are 3 to 10 fold higher than...
Author Summary

Bacterial infections have long been studied using Koch’s postulates wherein the paradigm is that a single clone leads to a given infection. Over the past decade, it has become clear that chronic bacterial infections often do not fit this paradigm. Instead these are associated with the presence of multiple strains or species (polyclonal) of bacteria that are organized into highly structured communities, termed biofilms, which can persist in the body and are recalcitrant to antibiotic treatment. In addition, there is extensive evidence that bacteria can incorporate genes from neighboring bacteria into their own genomes. This process can produce new strains and is known as horizontal gene transfer. In this study, we investigated for the first time, the tempo and relevance of gene transfer among bacterial strains of Streptococcus pneumoniae during a naturally occurring chronic childhood infection. We identified extensive gene transfer among multiple infecting strains, by sequencing of isolates recovered sequentially over a seven-month period. This gene transfer may serve as a counterpart to the host’s adaptive immune response and help explain the phenomenon of bacterial persistence, since, as occurs with some chronic viral and parasitic infections, the immune system may become overwhelmed by a set of related strains.

DNA polymerase mutation rates [8,9]. These highly variable HGT rates imply that the relative contributions of HGT and point mutations to the genomic diversification process vary greatly amongst species. In addition to the variability in HGT rates, there may also be extensive disparity of the sizes of genome regions that are replaced. While it is generally assumed that HR involves mainly small regions, experimental work and in silico comparison of multiple WGSs of S. agalactiae demonstrated that HR can transfer DNA segments of several hundred kilobases [10]. However, the tempo, pattern and relevance of HGT to bacterial strain evolution within natural chronic infections remains poorly understood, with there being only one published study in Helicobacter pylori [11]. In this regard, S. pneumoniae is ideal for studying the evolution of bacterial genomes in real time because it forms persistent polyclonal biofilms on the mucosal surfaces of the nasopharynx and the middle-ear. Such environments are highly conducive to HGT [12].

S. pneumoniae is a gram-positive bacterium, commonly referred to as pneumococcus, which is causatively associated with severe invasive diseases such as meningitis and bacteremia, as well as with many mucosal diseases including pneumonia, sinusitis, and otitis media (OM) [13]. Worldwide, S. pneumoniae is estimated to kill annually one million children under the age of five. In Europe and the USA, S. pneumoniae accounts for at least 30% of all cases of community-acquired pneumonia admitted to hospitals, and has a case fatality rate of 10–30% [14]. Despite its pathogenic potential, S. pneumoniae is a common natural component of the human nasopharyngeal (NP) commensal flora. In developed countries virtually every child becomes an NP carrier of S. pneumoniae during the first year of life with a recent study of European day care centers reporting that over 95% of the children were colonized by S. pneumoniae at least once during the study with many children showing evidence of polyclonal infection [15,16].

There are 91 S. pneumoniae serotypes and very significant differences with regard to genetic (gene possession) diversity and disease-inducing phenotypes both within and among serotypes [4,17–19]. Collectively WGS analyses have support the distributed genome hypothesis (DGH) that posits that there are many genetic differences that exist among the individual strains that make up a bacterial species (or infecting population). Thus, there exists a species-level supragenome (pangenome) that is much larger than the genome of any given strain [2,3] [20,21]. Previous studies have provided evidence in support of the DGH by demonstrating that fewer than 50% of the total number of S. pneumoniae genes that have been identified are found in any individual strain [4]. Both the intense intra-species competition within S. pneumoniae biofilms [22–25] and the natural capacity of S. pneumoniae to undergo transformation by the active uptake of environmental DNA embedded within the extracellular polymeric matrix of these biofilms [26], have likely driven much of the genomic diversification of this species.

The DGH postulates that the same mechanisms which promote genomic plasticity at the species level also result in the in situ creation of clouds of related S. pneumoniae strains within chronically infected individuals and that these may act as a potent counterpart to the host’s adaptive immune response [27].

Results

Selection and sequencing of S. pneumoniae strains from a pediatric patient

As part of an influenza vaccine trial, NP sampling was performed on pediatric patients presenting with flu-like respiratory symptoms. Bacteria were recovered, isolated, typed, and frozen from these samples [28]. An 8 month-old child, patient 19, enrolled in this study had 12 clinic visits due to rhinorrhea and/or ear infections over a 7-month period. These included a visit at enrollment and seven subsequent visits during which nasopharyngeal swabs were obtained for bacterial culture (Table 1). All of the bacterial strains recovered from this patient were typed as S. pneumoniae and could be divided into one of two MLST types: ST13 or ST2011. As with other bacterial species, MLST-based analyses of S. pneumoniae strains enables accurate identification using the allelic profiles of seven housekeeping genes that are strongly correlated with, and indicative of, genome-wide degrees of strain variability (ST13 and ST2011 differ in the sequence of two of these genes, specifically spt and dld) [29]. The two distinct MLSTs identified within patient 19 suggest that the child was infected with at least two divergent S. pneumoniae strains. Interestingly, half of the ST13 strains were identified as being of serotype 14, and the others were non-typeable (Table 1). The patient 19 isolates are named by their MLST type (ST13 or ST2011), followed by the visit number when they were isolated (v1 through v13).

454 Life sciences-based pyrosequencing (without paired end analysis) was used to sequence six of these isolates (Table 1). A PCR-based analysis of both of the non-sequenced isolates indicates that they are clones of sequenced isolates as their gene possession profiles are identical (data not shown). The complete genomic sequences of the six sequenced isolates have been deposited in GenBank and are also available at the Streptopneumo database http://streptopneumo-sybil.igs.umaryland.edu/. The genomes have an average size of 2,070±17 Kb and a GC content of 39% (Table 1). The Microbial Genome Annotation Tools and Genome Annotation Pipeline from NCBI were used to predict and annotate the coding sequences (CDSs) [http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html]. The average number of CDSs per strain is 2230 (Table 2).

Evidence of recombination among strains from WGS comparison

Global comparisons of the WGS of these six isolates revealed that two pairs were essentially identical (i.e. there are four strains
with two isolates each of two of the strains) despite being sampled 23 days apart (ST13v12 and ST13v13) and 5 months apart (ST13v1 and ST13v10) (Text S1 and Text S2, respectively). Thus, it can be inferred that the first ST13 strain that was isolated persisted for at least 5 months without any detectable evidence that it was an HGT recipient.

The WGS of the four genically distinct strains (ST13v1, ST2011v4, ST13v6, and ST13v12) were aligned using the progressive Mauve feature in the MAUVE genome alignment software [30]. To visualize the genomic differences, a similarity plot was generated from this alignment (Fig. 1A, white areas represent areas of low conservation). To assess the phylogenetic relationships among these strains, a maximum likelihood tree was created from the alignment using PHYML [31] as implemented in Recombination Detection Program (RDP) [32] (Fig. 1B). Both figures show that strain ST2011v4 is the most distant among the strains isolated from this patient, and that smaller differences also exist among the ST13 strains.

To investigate the role of HGT in differentiation of these strains, we created a specially modified version of RDP (RDP3) capable of comparing full-length bacterial genomic sequences and identifying recombination sequences and their breakpoints (revision 42–2; freely available from http://darwin.uvigo.es/rdp/rdp.html). RDP3 implements a variety of published recombination detection methods to determine statistical evidence of recombination [32]. To avoid any sequencing artifacts, all base pairs with a sequencing quality score of less than 40 (a probability

**Table 1.** Patient’s clinical history and clinical, sequencing and assembly information on the S. pneumoniae strains.

| Visit No | 1 | 3 | 4 | 5 | 6 | 10 | 12 | 13 |
|----------|---|---|---|---|---|----|----|----|
| **Date** | 5-Oct-99 | 8-Nov-99 | 22-Nov-99 | 9-Dec-99 | 4-Jan-00 | 23-Mar-00 | 19-Apr-00 | 12-May-00 |
| **Serotype** | 14 | 14 | NT | NT | NT | 14 | NT | NT |
| **MLST** | 13 | 13* | 2011 | 2011* | 13 | 13 | 13 | 13 |
| **Symptoms** | Enrollment | Mild rhinorrhea, conjunctivitis | Pulled ears, decreased appetite, cough, irritable, thick copious rhinorrhea | Pulled ears, fussy, irritable, thick copious rhinorrhea, Diagnoses: Right AOM | Pulled ears, fussy, irritable, thick copious rhinorrhea, Diagnoses: Right AOM | Pulled ears, fussy, irritable, thick copious rhinorrhea, Diagnoses: Right AOM | Pulled ears, fussy, irritable, thick copious rhinorrhea, Diagnoses: Right AOM | Pulled ears, fussy, irritable, thick copious rhinorrhea, Diagnoses: Right AOM |

**Table 2.** Summary of CDSs and their organization into orthologous clusters.

| Strain | CDSs | Orthologous Clusters | Distributed Clusters | Core Clusters |
|--------|------|----------------------|---------------------|--------------|
| BS292  | 2290 | 2158                 | 77                  | 2077         |
| BS457  | 2225 | 2141                 | 64                  | 2077         |
| BS458  | 2254 | N/A                  | N/A                 | N/A          |
| BS293  | 2242 | 2125                 | 44                  | 2077         |
| BS397  | 2242 | N/A                  | N/A                 | N/A          |
| BS455  | 2182 | 2196                 | 116                 | 2077         |
| All    | 13435| 2250*                | 173*                | 2077         |

*the MLST profiles for ST13v3 and ST2011v5 were determined using the Ibis T-5000 technology. ST13 and ST2011 differ in the sequence of their ddl and xpt alleles (ST is based on allelic differences of 7 housekeeping genes). AOM: acute otitis media; OME: otitis media with effusion; URI: respiratory tract infection; n/a: not available.

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The WGS of the four genically distinct strains (ST13v1, ST2011v4, ST13v6, and ST13v12) were aligned using the progressive Mauve feature in the MAUVE genome alignment software [30]. To visualize the genomic differences, a similarity plot was generated from this alignment (Fig. 1A, white areas represent areas of low conservation). To assess the phylogenetic relationships among these strains, a maximum likelihood tree was created from the alignment using PHYML [31] as implemented in Recombination Detection Program (RDP) [32] (Fig. 1B). Both figures show that strain ST2011v4 is the most distant among the strains isolated from this patient, and that smaller differences also exist among the ST13 strains.

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of >1:10^4 that they were incorrectly called) were eliminated from this and all subsequent analyses. The final RDP analysis identified evidence of 16 statistically significant recombination events among the four strains (Fig 1C, Table 3; with all detected events identified unambiguously by at least five out of seven independent recombination signal detection methods). This analysis suggests that 459 Kb of genomic sequence was exchanged among the analyzed strains by recombination. These segments vary in size from 0.4 kb to 235 Kb, with a mean size of 28 Kb and a median size of 13 Kb.

Comprehensive SNP analyses were used to further investigate the differences amongst these four strains. MAUVE was used to generate a list of all 11470 SNPs (Table S1). The majority of the SNPs (71%) are identical among the three ST13 strains but variable in relation to the ST2011v4 strain (rows 3310–11474 in Table S1). Nonetheless, 28% (3306 of 11470) of the SNPs show differences among one or more of the ST13 strain pairs (first 3308 rows in Table S1). To determine the relative positions of these 3306 ST13 SNPs, they were sorted based on their chromosomal placement. The sorted list was manually curated to group together SNPs that a) share the same distribution across strains (that is, the same strain contains the variable nucleotide) and b) are located within an area where there is high concentration of SNPs (from 4 to 68 SNPs/Kb – as opposed to isolated SNP found at levels <0.2SNPs/Kb; such groups are hereafter referred to as neighbor groups \{NG\} (Fig. 1C). The NG breakpoints were selected as areas that demark the transition between highly conserved regions (less then 0.2 SNPs/Kb) and divergent regions (more then 4 SNPs/Kb). Ninety five percent of these 3306 ST13 SNPs were organized into 23 distinct chromosomal NGs. Fourteen NGs are larger then 500 bp and nine are smaller then 500 bp (Tables 4 and 5 respectively, a detailed list of all the SNPs and their organization into NGs is illustrated in the first 3308 rows of Table S1). NG analysis suggests that HGT has led to the exchange of at least 156 Kb between strains ST13v1 and ST13v12.

The analyses with RDP3 (statistical tool package) and NG (manually curated grouping of SNPs based on pattern and localization) predict very similar recombination events (Fig 1C, Table 4). The major differences identified by these two methods are in the position of the recombination breakpoints, and thus the size of each event. The NG method is extensively curated and overall leads to the most conservative estimate (see methods and

Figure 1. Demonstration of recombination events among Streptococcus pneumoniae genomes isolated from a single patient. (A) MAUVE alignment of the WGSs of four S. pneumoniae isolates showing four distinct strains. Colored blocks highlight regions that are homologous and free from genomic rearrangement. White areas illustrate degrees of average conservation that exist within corresponding genome regions (more white = less conservation). Neighbor group (NG) numbers above the schematic denote the positions of clustered SNP groupings. (B) Maximum likelihood phylogenetic tree (given the best fit model = F84+G4) expressing the relationship between the four genomes and demonstrating that ST2011v4 is the most divergent of these strains and that differences exist among the ST13 strains. (C) Schematic showing the relative position of recombination events (gray boxes) within the ST13 genome (black), as predicted by RDP3 and NG analyses.

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The MAXCHI method used by RDP to infer breakpoint positions identifies breakpoints as the midpoint between the two phylogenetically informative SNPs bounding the borders of identified recombinant regions. As a result, the RDP estimated bounds of the recombinant regions are less conservative than the manually curated estimates because they include numerous sites on the 5’ and 3’ ends of the regions that are identical between the identified parental sequences. Regardless of the method selected for analysis, the WGS comparisons suggest that: (1) four distinct strains were isolated from one patient; (2) these strains fall into two groups, three ST13 isolates and one ST2011 isolate; (3) the differences among the ST13 strains are grouped into multiple

| Table 3. Recombination fragments predicted by RDP3 analysis, their corresponding probabilities after Bonferroni correction, and estimated size in base pairs. |
|-----------------|-----------------|-----------------|
| **RDP event**   | **RDP Probability (after Bonferroni correction)** | **Size of HGT on ST13v1 based on RDP analysis** |
| A               | 1.16E-76        | 36868           |
| B               | 2.30E-98        | 12845           |
| C               | 9.28E-47        | 3506            |
| D               | 2.31E-70        | 15544           |
| E               | 3.87E-61        | 6235            |
| F               | 2.84E-59        | 14372           |
| G               | 4.29E-121       | 34671           |
| H               | 4.30E-17        | 235426          |
| I               | 3.66E-105       | 6314            |
| J               | 1.83E-36        | 402             |
| K               | 2.50E-82        | 16599           |
| L               | 9.45E-295       | 14674           |
| M               | 9.31E-11        | 7583            |
| N               | 1.69E-123       | 4715            |
| O               | 1.03E-11        | 2044            |
| P               | 1.17E-80        | 47900           |

Table 4. Genic and allelic differences identified with large (>500 bp) regions of horizontal gene transfer among *S. pneumoniae* isolates from a single patient.

| NG# | Corresponding RDP | Start Position on ST13v1 | End Position on ST13v1 | No SNPs on WGS | No SNPs on CDSs | No Distributed Genes | Size of HGT on ST13v1 based on SNP analysis | Likely Recipient: Donor Strains | Likely Recombinant |
|-----|-----------------|-----------------|-----------------|----------------|----------------|-----------------|--------------------------------|-----------------------|-----------------|
| 1   | part of A       | 78193           | 78961           | 58             | 58             | 0               | 768 ST13v6 : ST2011v4 ST13v12        | ST13v12               |
| 2   | part of A       | 102716          | 107068          | 138            | 70             | 0               | 4352 ST13v1 : ST2011v4 ST13v6       | ST13v6                |
| 3   | B               | 165124          | 169392          | 125            | 124            | 0               | 4268 ST13v6 : ST2011v4 ST13v12       | ST13v12               |
| 4   | C               | 294136          | 295217          | 55             | 55             | 0               | 1081 ST13v6 : ST2011v4 ST13v12       | ST13v12               |
| 5   | D               | 321068          | 350885          | 630            | 525            | 17              | 29817 ST13v1 : ST2011v4 ST13v6       | ST13v6                |
| 6   | E               | 689372          | 699927          | 54             | 33             | 0               | 10555 ST13v6-like : ST2011v4 ST13v6  | ST13v6                |
| 7   | F               | 871647          | 876989          | 30             | 24             | 0               | 5342 ST13v6 : ST2011v4 ST13v12       | ST13v12               |
| 8   | part of G       | 1207132         | 1226947         | 159            | 102            | 0               | 19815 ST13v6 : ST2011v4 ST13v12      | ST13v12               |
| 9   | part of H, part of G | 1311469       | 1313700         | 16             | 6              | 0               | 956 ST13v6 : unknown ST13v12         | ST13v12               |
| 10  | I               | 1460234         | 1460971         | 58             | 0              | 0               | 737 ST13v1-like : ST2011v4 ST13v1    | ST13v1                |
| 11  | K               | 1621147         | 1627678         | 51             | 49             | 0               | 6531 ST13v6 : ST2011v4 ST13v12       | ST13v12               |
| 12  | L               | 1767032         | 1779223         | 311            | 148            | 5               | 12691 ST13v1 : ST2011v4 ST13v6       | ST13v6                |
| 13  | M               | 1841607         | 1844874         | 84             | 1              | 0               | 3267 ST13v6-like: unknown ST13v6     | ST13v6                |
| 14  | N,O,P           | 1978118         | 2034639         | 1274           | 1020           | 24              | 56521 ST13v6 : unknown ST13v12       | ST13v12               |
| Total|                  | 3043            | 2215            | 46             | 156701         |

NG = neighbor groups, i.e. contiguous genes that moved en bloc as part of a single horizontal gene transfer event. RDP = recombination blocks predicted by RDP3. Regions 1, 3, 4, 7, 8 and 11 likely were transferred during a single competence event; similarly for the regions 2, 5, and 12; both by exchanging DNA with strain ST2011v4 (or ST2011v4-like).

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chromosomal regions, (4) these regions are the result of multiple recombination events.

**Genic differences within the recombinant segments**

The RDP3 and NG WGS comparison methods are alignment based, and do not focus on DNA regions that include genic differences (presence/absence of CDSs) since these do not align to other sequences. To analyze these differences, we compared all predicted coding sequences (CDSs) from the six genomes. These CDSs were organized into 2250 orthologous gene clusters as described in [4] and further divided into 2077 core and 173 distributed gene clusters (Table 2). A distributed cluster was defined as any orthologous gene cluster not present in all strains, and as such represents one of the genic differences among strains (complete list in Table S2). There are a total of 126 distributed gene clusters differences between ST2011v4 and the other three strains. These include 37 genes present only in the ST13 strains, and 89 genes present only in the ST2011v4 strain (Table S2). Among the three ST13 isolates there are only 47 genic differences in total. ST13v1 differs from the other strains by 23 genes (18 genes present and 5 genes missing), while ST13v12 differs by 24 genes (2 genes present and 22 genes missing) (Table S2). There were no genic differences between ST13v1 versus ST13v10 or between ST13v12 versus ST13v13. This is consistent with the WGS comparisons that identified these isolate pairs as being nearly identical, thus corroborating the hypothesis that these clones have persisted in the patient without detectable HGT. A previous study showed that the number of genic differences between pairs of independently isolated *S. pneumoniae* genomes ranged from 160 to 629 [4], suggesting that all strains isolated from this patient are more closely related than most independent isolate pairs. For comparison, the difference between a clinical strain (D39) and its lab derivative (R6) was 35 genes [4], similar to the number of differences between the ST13 strain pairs. Forty six of the 47 ST13 distributed genes are grouped into three of the recombination regions; 17 belong to the type 1 capsule locus within NG5 (RDP D), 5 belong to NG12 (RDP L), and 24 belong to NG14 (RDP P) (Table 4, and Table S3 provide the annotations for these genes and their relative positions within the recombinant regions). The high degree of genic similarity shared by the CDSs within the ST13 strains, and the positioning of the genic differences within the predicted recombinant regions complements the results from the WGS comparisons, suggesting that these strains diverged from each other by multiple recombination events.

**The relative distance amongst the ST13 strains suggests they are not independent isolates**

For a population-wide perspective, we quantified the allelic and genic differences of 22 *S. pneumoniae* strains, including the 6 strains in this study. The WGS of the remaining 16 has been previously published [4]. These comparisons group strains based on either their genic or allelic content, but do not account for their phylogenetic relationships since high recombination rates can abrogate genome-wide phylogenetic signals [33]. The genic distance measured between genomes was defined as the number of distributed gene clusters shared (both strains contain the gene) or not shared (neither strain contains the gene) by a given strain pair, divided by the total number of distributed gene clusters (Fig. 2A)[34]. The allelic distance measure was based on the variation among the core gene clusters [34] (Fig. 2B). These graphs show that the ST13 strains are more closely related than most other isolates collected from independent infections. Note again that R6 is a lab derivative of D39 and therefore these strains are not independent isolates. The only other similarly closely related strains is a pair of serotype 3 ST180 strains (OXC141 and CGSSp3BS71) that show allelic distances comparable to that of the ST13 strains. Importantly, other strains from the same study, all collected in the same hospital in Pittsburgh over the same time period are highly genetically variable (asterisks in Figs. 2A and 2B), demonstrating that the similarity among the ST13 strains isolated from patient 19 is unlikely to be an effect of the isolation locale. These data strongly suggest that the ST13 strains are more similar than would be expected had they been isolated from independent infections. In contrast, the larger distance between ST2011v4 and the ST13 strains suggests that ST2011v4, or a highly related strain, was acquired during an independent infection.

**Identification of ST2011v4 as a DNA donor for ST13 strains**

The most likely donor and recombinant strains were selected based on RDP3 predictions, as well as SNP patterns from all four unique patient 19 strains where the recombinant regions between the donor and recombinant strains must be virtually identical. Fig. 3A shows the diagram generated using RDP3, with NG results
superimposed as numbers within the light gray boxes. Dark gray boxes under the WGS schematic are labeled with the name of the most likely donor strain and located under the schematic of the most likely recombinant strain. In a few cases where multiple options for the recombinant strain are probable, they are all represented. Results suggest that for at least 9 recombination events (red and orange in Fig. 3A) ST2011v4 is the most likely DNA donor, and ST13v6 and/or ST13v12 are the most likely recombinants.

ST2011v4 is identical in many of the recombination segments to one or more ST13 strains (green in Fig. 3Bi, ii). This suggests that either a) ST2011v4 served as DNA donor for these recombination segments, or b) an un-sampled strain served as a DNA donor leading to modification in one or more of the ST13 strains (blue Fig. 3Bi, ii) in a region where ST2011v4 is identical to a subset of the ST13 isolates (green Fig. 3Bi, ii). Importantly, the regions surrounding most recombination segments are virtually identical among all three ST13 strains but variable (containing many SNPs) relative to ST2011v4 (yellow versus pink in Fig. 3Bi, ii). Table S3 displays the allelic and genic differences within the recombination fragments, as well as their surrounding areas. (labeled W or S, respectively), and Table S1 shows the SNPs surrounding the recombinant region highlighted in yellow (within rows 3310–11474). Two observations provide compelling evidence for the first option where ST2011v4 acted as a DNA donor to ST13 strains. The first observation is the regional genomic similarity between ST2011v4 and subsets of the ST13 strains in the recombinant region (green Fig. 3Bi, ii). The second observation is the genomic identity among the ST13 strains but not ST2011v14 on the regions surrounding the recombinant fragments (yellow versus pink in Fig. 3Bi, ii). Moreover, the synteny among all four strains in and around the recombination breakpoints suggests that HR is the most likely operable mechanism.

The scenario involving the least number of strains and recombination events that explain the genomic sequences isolated from this patient is illustrated in Fig. 3C. Here, ST13v6 evolved from ST13v1 through the acquisition of NGs 2, 5, and 12 from ST2011v4 (ST13v6 and ST2011v4 are identical in these regions yet differ from ST13v1 - Fig. 3Bii). Using the most conservative recombination estimates, these three regions add up to 46.8 Kb, including 22 distributed genes, and differ by 1079 SNPs (strain name in orange in Fig. 3A and sizes in Table 4). Subsequently, ST13v12 evolved from ST13v6 through the acquisition of NGs 1, 3, 4, 7, 8, and 11 from ST2011v4 (ST13v12 and ST2011v4 are nearly identical in these regions yet differ from ST13v1 and ST13v6 - Fig. 3Bii). Using the most conservative recombination estimates, these six HGT regions sum to 37.8 Kb, and differ by 478 SNPs (strain name in red Fig. 3A and sizes in Table 4).

While the scenario illustrated in Fig. 3Ci is the most likely explanation for the evolution of the sequenced strains, we are unable to exclude the possibility that a different, albeit less parsimonious, pattern of HGT might have yielded the observed genetic variation (Fig. 3Cii). In this second scenario, ST13v1 may be a recombinant having arisen from transfer of DNA (NGs 2, 5, and 12) from an unknown parental donor into either ST13v6, or a highly related strain.

Collectively, these data place ST13v6 as a genomic intermediate between ST13v1 and ST13v12, since it shares 3 recombination events in common with ST13v12 (NGs 2, 5, and 12) but lacks evidence of additional events (NGs 1, 3, 4, 7, 8, and 11). Notably, this model of recombination events is consistent with the time of isolation of these strains.

Evidence for additional DNA donor(s) and mutation events

Not all of the recombination fragments can be explained by the genetic exchanges occurring among the four unique sampled strains. There are 2 recombinant regions (NGs 9 and 14- RDP H, O and P) that must have been acquired by ST13v12 from an unsampled donor, as these regions are unique with respect to all of the sequenced strains including ST2011v4 (purple in Fig. 3A and 3Bi, i). NG 14 (RDP O and P) is ~50 Kb, differs from the other ST13 strains by 24 distributed genes and ~1200 SNPs (Fig. 4). Importantly, this region is also unique with respect to the un sequenced ST13v3 and ST2011v5 strains as shown by PCR-based sequencing of the target regions, demonstrating that these strains cannot have served as donors. NG9 is also unique with respect to all of the other sequenced and unsequenced strains isolated from this patient suggesting, as for NG14, an origin from an unsequenced strain.

Further differences among the strains suggest that additional HGT events affecting a smaller number of loci have also occurred during this infection. The 5’ four Kb in NG14 (RDP N) varies between ST13v1 and ST13v6, and the 3’ end of NG8 varies between ST2011v4 and ST13v12 suggesting the occurrence of additional DNA exchange events at these regions. Also, there are an additional three regions where ST2011v4 may have served as a donor (pink in Fig 3A). In NG10 (RDP I), ST13v1 resembles ST2011v4 but not ST13v12 (data for ST13v6 is missing in this region). In NG13 (RDP M), ST13v6 differs from all strains while ST13v1 and ST13v12 resemble ST2011v4 suggesting that ST13v6 underwent an additional HGT event in this region with another donor, and therefore the direct antecedent of ST13v12 incurred an additional change creating the ST13v6 genome. Finally, for the majority of the SNPs in NG6 (RDP E), ST13v6 differs from all strains while ST13v1 and ST13v12 resemble ST2011v4 suggesting yet another HGT event from a relative of ST2011v4.

In addition to the large (>500 bp) recombinant regions, we identified an additional 9 regions that are <500 bp (Table 5). These nine small NGs (sNG) have a variety of SNP patterns (detailed SNPs in Table S1). Finally, minor differences among the strains also implicate other mutation mechanisms. Differences in the number of repeats in the gcuA binding domain of pneumococcal protein A genes is suggestive of DNA polymerase slippage, and differences in restriction endonucleases resemble DNA inversions between S subunits (Text. S1b and Fig. S1, respectively) [35].

Phenotypic differences amongst the ST13 strains

Given the polyclonal nature of infection it is not possible to correlate with a high degree of confidence the patient’s symptoms observed during any particular visit to the bacterial strain isolated from samples collected at that visit. Nonetheless, it is noteworthy that the appearance of the nontypeable (NT) strain ST2011v4 correlates with the beginning of a severe bout of acute otitis media
Figure 3. Identification of DNA donors for recombinant ST13 isolates. (A) RDP3-generated schematic of four genomes indicating evidence of recombination with neighbor group (NG) results superimposed. Light gray boxes within the whole genome sequence (WGS) schematic represent recombination events, and corresponding NG numbers have been inserted. Dark gray boxes are positioned below likely recombinant strains and are labeled with the most likely DNA donor for each transferred region. (B) Schematic illustrating the various patterns found in both the recombinant regions and surrounding areas. (i) Recombination fragments that are identical among ST2011v4, ST13v6, and ST13v12 while the surrounding regions are identical only among the ST13 strains (but contain SNPs relative to ST2011v4) (representative of NGs 5 and 12 and corresponding to RDP D and L); (ii) Recombination fragments that are identical only between ST2011v4 and ST13v12 while the surrounding regions are identical only among the

Legend for colors on strain ID:
DNA donor strain/ recombinant strain
- ST2011v4/ ST13v12
- ST2011v4/ ST13v6
- ST2011v4 or unknown / unclear
- ST2011v4 or unknown / ST13v12

Recombination fragment
Region flanking recombination fragment
Recombination breakpoint
ST13 strains (representative of NGs 1, 3, 4, 7, and 11 and corresponding to RDP part of A, B, C, F and I); (iii) Recombination fragments that differ between ST13v12 and all other strains (representative of NGs 9 and 14 and corresponding to RDP part of G and N, O and P). (C) Schematic outlining possible recombination events that may have led to the creation of the strains isolated from the patient. (i) most and (ii) next-most parsimonious. Black arrows move from the major parental strain to the recombinant, colors highlight likely DNA donor strains. The conservative estimate for the size of the recombination fragments is marked below the arrows with the corresponding percentage of the WGS in parenthesis.

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( diagnosis listed in Table 1). It is therefore conceivable that either this strain itself or sequences horizontally transferred from this strain into the ST13 strain may have had an influence on virulence. To investigate how the genetic differences amongst these strains may have affected their biology we compared the capacities of the ST13 strains to form biofilms. Biofilm-formation colonization and for the establishment and maintenance of chronic mucosal infections such as otitis media with effusion [36,37]. Confocal images of biofilms produced in vitro by the ST13 strains after 1, 3, and 5 days of growth show that the two unencapsulated strains ST13v6 and ST13v12 make much more robust biofilms when compared to ST13v1, the capsular type 14 strain (Fig. 5).

Annotation of neighboring group regions

The differences between the ST13 strains are contained within 150 genes: 46 distributed genes and 104 core genes with allelic differences that collectively contain ~2200 SNPs (labeled “W” in Table S3). The CDS that differentiate ST13v1 from both ST13v6 and ST13v12 are located within NGs 2, 5 and 12 (RDP part of A, D, I) (Table 4). NG5 (RDP D) on ST13v1 encoded the type 14 capsule genes as well as adjacent allelic core genes (Table S3). In the corresponding region, the non-typeable ST13v6, ST13v12 and ST2011v14 have lost the capsule genes yet carry two genes that are not found in ST13v1. Within NG2 (part RDP A) modifications to pneumococcal surface protein A (PspA), a virulence gene that encodes a choline-binding protein associated with resistance to fixation of complement [38] and with binding human lactoferrin [39], also differentiate ST13v1 from ST13v6/ST13v12. Of note, within ST13v12 and ST13v13 there were various nucleotide polymorphisms within PspA that were not obviously derived through HGT suggesting that this locus is under strong selective pressure (Text S1 and Table S3). Within NG12 (RDP L) there are three distributed genes in ST13v1 surrounded by 16 allelic core genes, which can be organized into four operons, two of which include only hypothetical proteins. The observed HGT event within this region resulted in ST13v6 and ST13v12 having two genes in this region that is absent in ST13v1. One of these genes has been annotated as a possible cell surface protein. A species-wide comparison demonstrated that neither of these genes is shared with any of the other 16 sequenced S. pneumoniae strains analyzed in Fig.2.

The NG14 (RDP O) region of ST13v12 is ~23 Kb smaller than the corresponding regions of ST13v1 and ST13v6 (Fig. 4). This region in these latter two strains contains 44 genes distributed over at least 4 separate operons. The annotations of these genes suggest that they function in the metabolism and/or transport of amino acids, sugars, zinc and glycerol (Table S3). The corresponding smaller region in ST13v12 is missing 22 of these genes, carries two other genes (a beta-galactosidase and a hypothetical protein), and differs from the other ST13 strains by over 1000 SNPs in their shared genes.

Discussion

S. pneumoniae has long been recognized as a major human pathogen with Sir William Osler at the turn of the 20th century referring to pneumococcal pneumonia as “the captain of the men of death”. Shortly thereafter S. pneumoniae was shown to be transformable [40] and this observation led directly to the identification of DNA as the hereditary molecule [41]. However, it has only been in the last decade that HGT has been recognized as a significant virulence trait [27]. In this study we analyzed the WGS of six S. pneumoniae isolates obtained over ~7-months from a single pediatric patient presenting with nasopharyngeal and middle-ear disease symptoms. We recovered two pairs of virtually identical genomes over the 13 visits (at visits 1 and 10 and visits 12 and 13) strongly suggesting that some strains persisted within the patient during asymptomatic periods. Moreover, the recovery of other divergent strains at visits 4 and 6 suggests that the clones isolated on visits 1 and 10 were present simultaneously with these divergent strains. Additionally, since recombination between a donor and recipient strain is much more likely if both parental strains are present simultaneously, it can be inferred that this was a polyclonal infection. This is not surprising given ample evidence for polyclonal carriage of S. pneumoniae [15,16]. Thus, while the available experimental samples (single strain isolations at each of 8 visits) did not allow us to fully survey the polyclonal nature of this S. pneumoniae infection, or extract the strains present during non-symptomatic periods, the complete genomes of the strains that we did isolate provide evidence of polyclonality and strain persistence in this patient. Collectively, these strains provide an excellent study set for characterizing in vivo HGT during polyclonal nasopharyngeal S. pneumoniae infection.

The very high degree of similarity amongst the three ST13 strains relative to all other S. pneumoniae genomes that have been sequenced suggests they had diversified mainly by HGT in amongst the strains that were present within the studied infection. The most parsimonious explanation for the nucleotide patterns within the recombinant regions suggests that ST2011v4 acted as an extensive DNA donor during both the genesis of ST13v6 from ST13v1 and the subsequent generation of ST13v12 from ST13v6 (or ST13v6-like strains).

We were however, unable to exclude the possibility that a different, albeit less parsimonious, pattern of HGT might have
yielded the observed genetic variation. Regardless of the actual recombination pathways, it was also very clear that not all of the observed exchanges could be explained by the genetic exchanges between the four sampled strains. In at least two recombination events detectable within the ST13v12 genomes sampled on visits 12 and 13 it is apparent that divergent sequences have been derived from an unsampled donor-parental lineage. Experiments with commensal populations of streptococci in the upper respiratory tract show that a few clones tend to dominate, thus it is not far fetched to suggest that there may have been multiple uncultured and undetected *S. pneumoniae* strains present within this patient during the study period [42].

The synteny in and around the recombination breakpoints, as well as the absence of phage-related sequences largely rules out transduction and suggests that HR is the most likely operative mechanism. Our detection methods are not able to differentiate between DNA acquired by conjugation or transformation. However, given that the *S. pneumoniae* are naturally competent, it is most likely that DNA from lysed bacteria enters the cells during bouts of competence, and is incorporated into the genome via homologous recombination. It is possible that each one of these 23 loci that differentiate the ST13 strains resulted from a separate HGT event and that the differences accumulated one at a time over the entire study period. Alternatively, given that at least six loci (NGs 1, 3, 4, 7, 8, and 11) were most likely all acquired by ST13v12 from ST2011v4 it is also conceivable that these regions may have been exchanged during the same competence event that supported multiple homologous recombination. The same simultaneous multiple replacement mechanism could also be used to explain the formation of ST13v6, which differs by at least three loci (NGs 2, 5, and 12) from its most likely predecessor, ST13v1. The possibility of a single competence event resulting in the replacement of multiple loci warrants further investigation as it has not been previously explored given that *S. pneumoniae* recombination studies have been limited to a subset of genes in population-wide studies [8,33]. If in fact, multiple loci were simultaneously replaced, the selection for one transferred gene or allelic difference would be enough to explain inheritance of the multiple replacements after a competence event. Ultimately, one would expect that the persistence over multiple generations of each of the replacements after a competence event. Ultimately, one would expect that the persistence over multiple generations of each of the replacements after a competence event. Ultimately, one would expect that the persistence over multiple generations of each of the replacements after a competence event. Ultimately, one would expect that the persistence over multiple generations of each of the replacements after a competence event. Ultimately, one would expect that the persistence over multiple generations of each of the replacements after a competence event.

Horizontal Gene Transfer in a Chronic Infection

Materials and Methods

**Strains and DNA sequencing, assembly, and gene prediction**

We obtained six clinical *S. pneumoniae* isolates from a pediatric patient participating in a vaccine trial at the Children’s Hospital of Pittsburgh. The genomes of these strains were sequenced at the Center for Genomic Sciences (CGS) using a 454 Life Sciences FLX sequencer. The limitation of this sequencing method is that it may overlook a frame shift mutation when it is present within a homo-nucleotide stretch. As previously described, strains were assembled by the 454 Newbler *de novo* assembler and prediction of putative coding sequences and gene annotations were done by NCBI using the Microbial Genome Annotation Tools and Genome Annotation Pipeline [4]. The final assemblies have been deposited in GenBank, the accession numbers are: ABWQ for ST13v1, ADHN for ST2011v4, ABWB for ST13v6, ABWA for ST13v10, ABWU for ST13v12, and ABWC for ST13v13. The
Gene clustering algorithm

A specially modified version of RDP3 (revision 42–2; available from http://darwin.uvigo.es/rdp/rdp.html) [32].

MLST typing

The full sequence of the seven house-keeping genes used for MLST typing were obtained from the whole genome sequences of the six sequenced strains and by Ibis T-5000 universal biosensor technology for the two unsequenced strains [56]. The internal fragments required for typing were trimmed as directed by the S. pneumoniae MLST site, and were submitted to this site to determine their ST type (http://spneumoniae.mlst.net/).

Serotyping

The strain serotypes were determined by two methods: (1) the Pneumotest-Latex kit (Statens Serum Institut), and (2) a PCR-based approach [57].

Biofilm growth

S. pneumoniae biofilms were grown and visualized as previously described [26].

Ethics statement

The study was approved by the Children’s Hospital of Pittsburgh Human Rights Committee. They recruited healthy children aged 6 to 24 months from the hospital’s primary care center and from the community at large. Research personnel informed parents in the primary care center about the study, and advertisements were placed on the radio and in the regional newspaper. Written informed consent was obtained from the parent(s) of each enrolled child. They excluded children who had been born prematurely or had a craniofacial abnormality; or who had or were living with persons who had any medical condition placing them at high risk of complications of influenza; or who had a neurologic disorder, a history of tympanostomy tube insertion, hypersensitivity to egg protein or thimerosal, or a febrile illness or severe respiratory illness within the preceding 48 hours [28].

Supporting Information

Figure S1 Example of DNA inversion within ST13 strains. (A) Mauve generated alignment where solid colored blocks represent regions that are almost identical between ST13v1 and ST13v12. The black square surrounds the region where an inversion has occurred between the strains, leading to a “switch” in the C-
terminal ends of restriction endonuclease S subunits located near each other on the chromosome. (B) Alignment of 4 genes, two from strain ST13v1 (prefix: CGSSp14BS292) and two from ST13v12 (prefix: CGSSp14BS293). Yellow, blue, red, pink, and gray highlight identical regions. Comparison between both strains suggests that sequence differences were created by site-specific DNA inversion systems, where DNA inversions occurred within the coding regions of restriction endonuclease subunits resulting in genetic polymorphism, as previously observed in S. pneumoniae [53].

Table S1 List of SNPs that differentiate strains isolated from patient 19. The first 3306 rows are loci that contain at least one SNP among the ST13 strains; the remaining rows are loci that are identical among the ST13 strains, but are variable relative to ST2011v4. QS = sequencing quality score.

Table S2 List and annotation of 173 distributed clusters that differentiate the strains isolated from patient 19. 47 of these vary among the ST13 strains (purple, pink, blue and yellow).

Table S3 List of the coding sequences within the NGs (W) as well as those in the surrounding regions (S) that contain allelic differences among ST13 strains. c = complement.

Table S4 Map of the contigs from ST13v1, ST13v6, ST13v12, and ST2011v4 onto the assembly used for whole genome alignments.

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