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Comparative Genomic Analyses of Seventeen *Streptococcus pneumoniae* Strains: Insights into the Pneumococcal Supragenome▽†

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The distributed-genome hypothesis (DGH) states that pathogenic bacteria possess a supragenome that is much larger than the genome of any single bacterium and that these pathogens utilize genetic recombination and a large, noncore set of genes as a means of diversity generation. We sequenced the genomes of eight nasopharyngeal strains of S*treptococcus pneumoniae* isolated from pediatric patients with upper respiratory symptoms and performed quantitative genomic analyses among these and nine publicly available pneumococcal strains. Coding sequences from all strains were grouped into 3,170 orthologous gene clusters, of which 1,454 (46%) were conserved among all 17 strains. The majority of the gene clusters, 1,716 (54%), were not found in all strains. Genic differences per strain pair ranged from 35 to 629 orthologous clusters, with each strain's genome containing between 21 and 32% noncore genes. The distribution of the orthologous clusters per genome for the 17 strains was entered into the finite-supragenome model, which predicted that (i) the *S. pneumoniae* supragenome contains more than 5,000 orthologous clusters and (ii) 99% of the orthologous clusters (~3,000) that are represented in the *S. pneumoniae* population at frequencies of ≥0.1 can be identified if 33 representative genomes are sequenced. These extensive genic diversity data support the DGH and provide a basis for understanding the great differences in clinical phenotype associated with various pneumococcal strains. When these findings are taken together with previous studies that demonstrated the presence of a supragenome for *Streptococcus agalactiae* and *Haemophilus influenzae*, it appears that the possession of a distributed genome is a common host interaction strategy.

Streptococcus pneumoniae is a gram-positive bacterium commonly found in the nasopharynges of healthy persons, predominantly children. In addition to its commensal form, *S. pneumoniae* is also a major cause of morbidity and mortality worldwide. *S. pneumoniae* infection can cause meningitis and bacteremia, as well as many mucosal diseases such as pneumonia, sinusitis, and otitis media (OM). Worldwide, *S. pneumoniae* infections are estimated to result in 1.1 million deaths a year, predominantly from pneumonia, and even in the United States pneumococcus disease is one of the top 10 causes of death (21). The economic burden associated with *S. pneumoniae* infections is tremendous, because it is the causative agent for 30 to 50% of OM infections worldwide, and in the United States alone the cost of OM, which is the most prevalent infectious disease among children, is estimated at $5 billion annually (5, 12).

S. pneumoniae played a critical role in the demonstration that DNA is the hereditary genetic material. In 1944 Avery and colleagues showed that DNA is the transforming factor identified by Griffith as being capable of making avirulent *S. pneumoniae* lethal (1). Since then, *S. pneumoniae* has served as a model organism for the study of bacterial transformation. It contains an inducible system for the uptake of DNA from the environment that allows for extensive recombination (3, 29, 34). Previous work from our laboratory using genomic libraries from eight clinical strains identified novel genes not present in the TIGR4 reference strain and showed a nonuniform distribution of many *S. pneumoniae* genes, suggesting a significant degree of genomic plasticity among the isolates (37).

A large amount of intraspecies genic variation (which pertains to the absence or presence of genes and should not be confused with allelic variation) has been observed for several bacteria. Tettelin and colleagues analyzed the genomes of six *Streptococcus agalactiae* strains of multiple serotypes and showed that ~20% of the genes are not shared among all strains (43). A similar trend was found for 13 *Haemophilus influenzae* genomes, where only ~50% of the genes are conserved among all strains (18). Both of these studies support the distributed-genome hypothesis, which states that for some bacteria, the full complement of genes available to a given species exists in a "supragenome" pool, one that each member of a

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† Supplemental material for this article may be found at http://jb.asm.org/.

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TABLE 1. Bacterial strains and sources used for the genomic comparison of *S. pneumoniae* strains

Strain name	Sequence source	Serotype	Source	MLST type
CGSSp11BS70	CGS	11	Pittsburgh, PA	62
CGSSp14BS69	CGS	14	Pittsburgh, PA	124
CGSSp18BS74	CGS	6	Pittsburgh, PA	New
CGSSp19BS75	CGS	19	Pittsburgh, PA	485
CGSSp23BS72	CGS	23	Pittsburgh, PA	37
CGSSp3BS71	CGS	3	Pittsburgh, PA	180
CGSSp6BS73	CGS	6	Pittsburgh, PA	460
CGSSp9BS68	CGS	9	Pittsburgh, PA	1269
D39	TIGR	2	United States	
R6	Eli Lilly and Company	No capsule	Derivative of D39	
23F	Sanger	23	Spanish pandemic	
INV104B	Sanger	1	Oxford, United Kingdom	
INV200	Sanger	14	Oxford, United Kingdom	
OXC141	Sanger	3	Oxford, United Kingdom	
TIGR4	TIGR	4	Norway	
TIGR670-6B	TIGR	6B	Spain	
PAT6420135 (ATCC 55840)	Human Genome Sciences, Inc.	Unknown	Unknown	

population of naturally transformable bacterial strains contributes to and draws genes from, resulting in a high degree of genic diversity (7).

In this study we sequenced the genomes of eight clinical *S. pneumoniae* isolates and combined these data with sequences from nine other publicly available *S. pneumoniae* strains. We present a global comparative analysis of the genes and genomes, which demonstrates great genic diversity among the strains. In addition, we use a mathematical model to predict the number of genomes that must be sequenced to provide coverage of the *S. pneumoniae* supragenome (18).

MATERIALS AND METHODS

DNA sequencing. We obtained eight clinical *S. pneumoniae* isolates, with different serotypes and multilocus sequence typing (MLST) types, from pediatric patients participating in a Fluzone 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 GS-20 sequencer (26). Strains were sequenced to a depth of 16-fold or greater and were assembled de novo by the 454 Newbler de novo assembler to 281 contigs per genome, on average. Lander-Waterman statistics predict that more than 99.9% of each genome was sequenced. Regions of repetitive sequence caused most of the assembly gaps. Informal comparison between high-quality Sanger reads and 454 data suggest an error rate of less than 1 in 1,000 bases in each assembly. Most base call errors are single-base insertions or deletions in homo-nucleotide repeats, which can result in artifactual frameshifts.

Sequence data for the other nine strains examined came from various sources. Four isolates were sequenced by the *S. pneumoniae* Sequencing Group at the Sanger Institute: 23F, INV104B, INV200, and OXC141. The sequences of the latter three strains are incomplete and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/spn/. The published genome sequence of strain R6 (GenBank accession number AE007317) was produced by Eli Lilly & Co (19). The Institute for Genomic Research (TIGR) sequenced strains TIGR4 (GenBank accession number AE005672), TIGR670-6B (project information available at http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview) (23, 44), and strain D39 (s_pneumoniae_d39_1; GenBank accession number CP000410) (23). Unfinished sequence data for strain PAT6420135 are available from reference 22, and the strain from which the sequence was obtained is now an ATCC patent deposit bacterial strain (ATCC 55840). The Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) query "patent[TITL] AND 6420135[TITL]" retrieves the 391 DNA sequences that make up this genome assembly, which was produced by Human Genome Sciences, Inc.

Genome assembly of CGS *S. pneumoniae* sequences. The 454-assembled genomic contigs were ordered and oriented into scaffolds by alignment, using Nucmer software (6), against each of the nine non-CGS *S. pneumoniae* genomes, which indicated the closest reference strain. Using a maximum-parsimony approach, each genome was reduced to about 60 contigs by a combination of (i) Sanger sequencing of PCR amplicons targeted to fill gaps between neighboring contigs, as inferred from the scaffolding, and (ii) paired-end Sanger sequencing of clones from a library and identification of clones that spanned gaps in the 454 sequence. Gap closure experiments were designed by a custom Perl script, and PCR primers were designed by Primer3 (36). Clones and PCR amplicons were assembled along with 454 contigs by a modified Phred-Phrap-Consed pipeline where 454 contigs were converted to PHD format files and were input into Phrap as long reads (9, 10, 13, 14). Data were manipulated and visualized using CONSED.

CDS prediction. Prediction of putative coding sequences (CDSs) and gene annotation were done by NCBI using the Microbial Genome Annotation Tools and Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html). Briefly, gene predictions were performed using GeneMark, GeneMark.hmm, and Glimmer 2. To detect any genes missed by this method, a six-frame translation of slices of the nucleotide sequence was done. The predictions and the slices were then searched against the NCBI Entrez Protein Cluster database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=proteinclusters) as well as proteins from all complete microbial genomes. Annotation was supplied from both sets and supplemented with information from the Conserved Domain Database (42) and from Clusters of Orthologous Groups (25). rRNAs were predicted using BLAST to search against an RNA sequence database (4), and tRNAs were predicted using tRNAscan-SE (24).

Clustering algorithm. A complete description of the algorithms used to create the orthologous clusters is given by Hogg et al. (18). Briefly, to allocate the CDSs into core, unique, or distributed clusters, tfasty34 (Fasta package, version 3.4) was used for six-frame translation homology searches of all predicted proteins against all possible translations (33). Proprietary software designed at the CGS was used to parse this output, grouping the genes into clusters. A cluster was defined as a group of genes which share at least 70% identity, over 70% of its length, with one or more of the other genes in the group, and where at least one sequence in the cluster is equal to or longer than 120 residues. These parameters were selected because they minimize the change in the number of clusters per change in parameters and thus may represent a good estimate to distinguish orthologues from paralogues (18). Multiple genes from the same strain can be included in one cluster; therefore, in most strains the number of clusters is lower than the number of CDSs. To account for the cases where CDSs were missed in the gene-calling step, fasta34 (Fasta package, version 3.4) was used to align all predicted genes against the contigs. If an alignment with the required score was detected in the contig, the gene was considered present even if the gene was not identified.

Phylogenetic-tree building. For the genic-difference-based dendrogram, a gene possession-based phylogenetic tree of the 17 *S. pneumoniae* strains was constructed by defining the distance between a pair of genomes, *i* and *k*, to be $\sum_n |g_{n,i} - g_{n,k}|$ where $g_{n,i}$ is 1 if gene *n* is present in strain *i* and zero otherwise. The strains were clustered using tools available at http://www.let.rug.nl/~kleiweg/clustering/#cluster. "difftbl" was used for conversion of vector data into a difference table using city block as a method, and "cluster" was used for data

TABLE 2. Summary of CDSs and orthologous gene clusters for 17 *S. pneumoniae* strains

Gene category	No. of orthologous clusters (% of total)	No. of CDSs (% of genes)
Core	1,454 (46)	30,070 (73)
Distributed	1,140 (36)	9,679 (23)
Unique	576 (18)	576 (1)
Excluded by size		1,120 (3)
Total	3,170 (100)	41,445 (100)

clustering of the difference table using the group average method. For the relationship among MLST types, we used the batch allelic profile from the pneumococcal MLST database (http://spneumoniae.mlst.net/).

Nucleotide sequence accession numbers. The GenBank accession numbers for the following eight clinical isolates, sequenced in this study, are given in parentheses after the isolate designations: CGSSp3BS71 (AAZZ00000000), CGSSp6BS73 (ABAA00000000), CGSSp9BS68 (ABAB00000000), CGSSp11BS70 (ABAC00000000), CGSSp14BS69 (ABAD00000000), CGSSp18BS74 (ABAE00000000), CGSSp19BS75 (ABAF00000000), and CGSSp23BS72 (ABAG00000000).

RESULTS

Background on all publicly available *S. pneumoniae* strains and genome sequences of eight novel clinical isolates. In this analysis we compared 17 *S. pneumoniae* strains (Table 1). The genomes for eight clinical strains, each with a unique serotype and MLST type (CGSSp3BS71 [AAZZ00000000], CGSSp6BS73 [ABAA00000000], CGSSp9BS68 [ABAB00000000], CGSSp11BS70 [ABAC00000000], CGSSp14BS69 [ABAD00000000], CGSSp18BS74 [ABAE00000000], CGSSp19BS75 [ABAF00000000], and CGSSp23BS72 [ABAG00000000]), were sequenced at the CGS. The serotype for each of these strains is indicated by the number following the CGSSp prefix with the exception of CGSSp18BS74, which has recently been retyped. Each of these strains is a low-passage-number isolate obtained from the nasopharynx of a child who developed respiratory symptoms at any time over the course of a year and who was a participant in a Fluzone vaccine trial at the Children's Hospital of Pittsburgh (17, 37). Table S1 in the supplemental material shows the extent of genomic coverage and the number of contigs after assembly with Newbler and after gap closure using PCR.

The Sanger Institute sequenced four strains of serotypes 1, 14, 3, and 23F. The first three strains are from patients in the United Kingdom, while the last strain is from a multiple-antibiotic-resistant strain from a pandemic in Spain. Strain TIGR670-6B was also isolated in Spain in 1988 and is serotype 6B. TIGR4 was isolated from Norway. The clinical strain D39 was isolated about 90 years ago and gave rise to R36 (an unencapsulated, avirulent mutant that was much easier to transform), which in turn gave rise to R36A, the strain used by Avery and colleagues for their landmark experiments demonstrating that genetic information is contained in DNA (1). Strain R6 is a derivative of R36A isolated approximately 40 years ago; thus, R6 is derived from D39 (19, 23). To our knowledge, there is no publicly available information on the origins of the patented strain PAT6420135.

Identification of CDSs and core, distributed, and unique orthologous clusters. Prediction of CDSs and annotations were done using the Microbial Genome Annotation Tools and Genome Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html). The median number of CDSs per strains is 2,411, and the range is from 2,259 for strain INV200 to 2,763 for strain CGSSp14BS69. The total number of CDSs in all 17 strains is 41,445 (Tables 2 and 3). Note that all 17 genomes were submitted to the NCBI Genome Annotation Pipeline (including those with previously published CDSs) to eliminate errors that may have arisen from differences in the prediction of CDSs. A multi-fasta file with all the CDSs used in this study is available in Fig. S1 in the supplemental material and can also be downloaded from SupraGen at http://centerforgenomicsciences.org/doc_frame/index-old.html.

Together, these 17 genomes contain orthologues shared among all strains (core genes), orthologues shared only between subsets of two or more strains (distributed genes), and genes unique to one strain. Genes from all strains were grouped into orthologous clusters and divided into the three categories described above (Table S2 in the supplemental material lists the CDSs organized

TABLE 3. Numbers of CDSs and orthologous clusters for individual *S. pneumoniae* strains

Strain name	Genome size (kb)	No. of CDSs	No. of orthologous clusters	No. of unique clusters	% Noncore clusters[a]
INV200	2,200	2,259	1,850	28	21.4
PAT6420135	Unknown	2,500	1,913	10	24
R6	2,038	2,274	1,925	3	24.5
D39	2,000	2,304	1,940	3	25
CGSSp18BS74	2,033	2,354	1,955	13	25.6
CGSSp3BS71	2,027	2,331	1,960	7	25.8
CGSSp11BS70	2,044	2,336	1,986	25	26.8
TIGR4	2,160	2,410	1,993	1	27
INV104B	2,200	2,508	2,012	74	27.7
OXC141	2,200	2,663	2,014	67	27.8
CGSSp9BS68	2,090	2,397	2,021	37	28
CGSSp23BS72	2,053	2,411	2,022	41	28.1
CGSSp19BS75	2,069	2,432	2,031	40	28.4
CGSSp6BS73	2,119	2,434	2,056	55	29.3
CGSSp14BS69	2,084	2,763	2,068	61	29.7
23F	2,200	2,428	2,069	43	29.7
TIGR670-6B	2,100	2,641	2,157	68	32.6

[a] Calculated as (total clusters in the strain − 1,454 core clusters)/(total clusters in the strain).

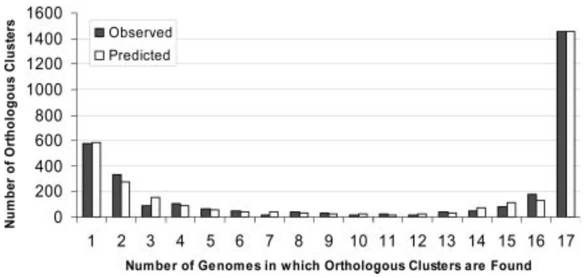


FIG. 1. Histogram of the number of observed and predicted (by the finite supragenome model) orthologous gene clusters that are present in a given number of genomes. There were 1,454 orthologous clusters observed in all strains (core); 1,140 distributed among more than one strain, but not all; and 576 in only one strain.

by orthologous clusters; this table can also be downloaded from SupraGen at http://centerforgenomicsciences.org/doc_frame/index-old.html). SupraGen software was created to identify the CDSs in these orthologous clusters for any *S. pneumoniae* sequence.

The total number of *S. pneumoniae* orthologous clusters (which includes unique genes) for all 17 strains is 3,170. Forty-six percent of the total clusters (1,454 core clusters) are found in all strains and include 73% of the total number of CDSs. Eighteen percent of the total clusters (576 clusters) are unique to a single strain, and 36% of the total clusters (1,140 distributed clusters) are present in some combination of two or more strains, but not in all strains (Table 2). Figure 1 shows a histogram plotting the number of orthologous clusters against the number of genomes in which a cluster is found, where 1 and 17 genomes correspond to the unique and core sets, respectively. Table 3 gives the numbers of CDSs, orthologous clusters, and unique genes per strain. The median number of orthologous clusters per strain is 2,012, with a range from 1,850 for INV200 to 2,157 for TIGR670-6B. Note that for each strain, 21 to 32% of the orthologous gene clusters are distributed or unique genes, i.e., not common to all strains. Considering the group of 17 strains collectively, 54% of the gene clusters are noncore.

In analyzing the correlation between the number of CDSs and orthologous clusters, a few factors must be noted. First, a gene may be included in a given orthologous cluster even if the CDS was not identified during annotation if a region with the required sequence similarity was found; this aspect of the analysis was designed to identify genes that were missed by the gene prediction programs. Second, when multiple highly similar copies of the same gene are present in one strain, they will all belong to the same orthologous cluster. Third, all orthologous clusters have a minimum size of 120 bp; thus, smaller genes may be included in a cluster only if they align to a longer sequence. Fourth, the same CDS may be intact in one strain and fragmented in another (this is most common in partially assembled genomes); in this case, the clustering algorithms will join the fragments by aligning them to an orthologue, thus converging multiple CDSs into one orthologous cluster. Since the comparisons are based on orthologous clusters, not on genes, they normalize for the effect of gene splitting often encountered in unfinished genomes. For these four reasons, there is not a good linear correlation between the numbers of CDSs and clusters ($R^2 = 0.439$).

Phylogenetic relationships among strains. To evaluate the relationships among strains, we constructed a dendrogram based on noncore genic differences (Fig. 2). As expected, strain D39 and its derivative strain, R6, are the most closely related pair. Interestingly, highly genetically diverse *S. pneumoniae* strains can be isolated from patients with the same symptoms at the same geographical location, as illustrated by the broad distribution of the eight CGS clinical isolates. Furthermore, strains OXC141 and CGSSp3BS72, which were isolated on different continents, have similar orthologous-cluster distributions. Surprisingly, the strains isolated in Norway (TIGR4) and Spain (23F) are not outliers with respect to the U.S. isolates, and the two Spanish strains (23F and TIGR670-6B) do not group together. The analysis includes two strains each of serotypes 14, 23, and 3 and three strains of serotype 6. There is great similarity between the genic contents of the two serotype 3 strains, but the same is not true for the strains with serotypes 14, 6, and 23.

An exhaustive pairwise comparison was performed among all strain pairs (Fig. 3). To provide a measure of the similarities and differences between the numerous strain pairs, we created similarity, difference, and comparison scores. The similarity score corresponds to the total number of orthologous gene

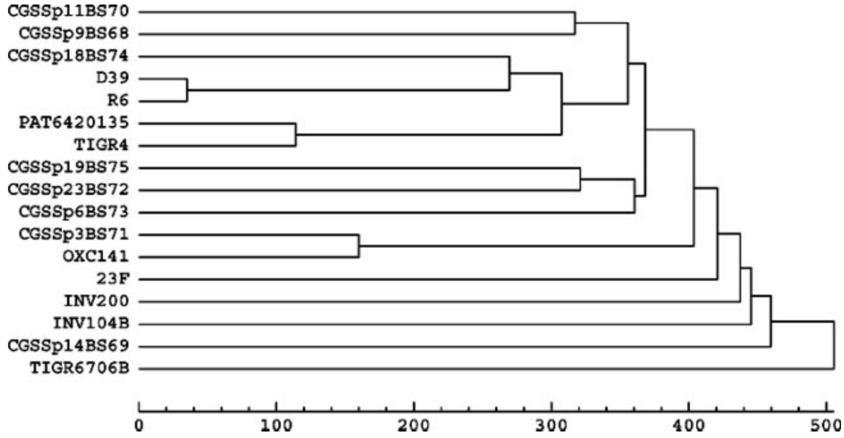
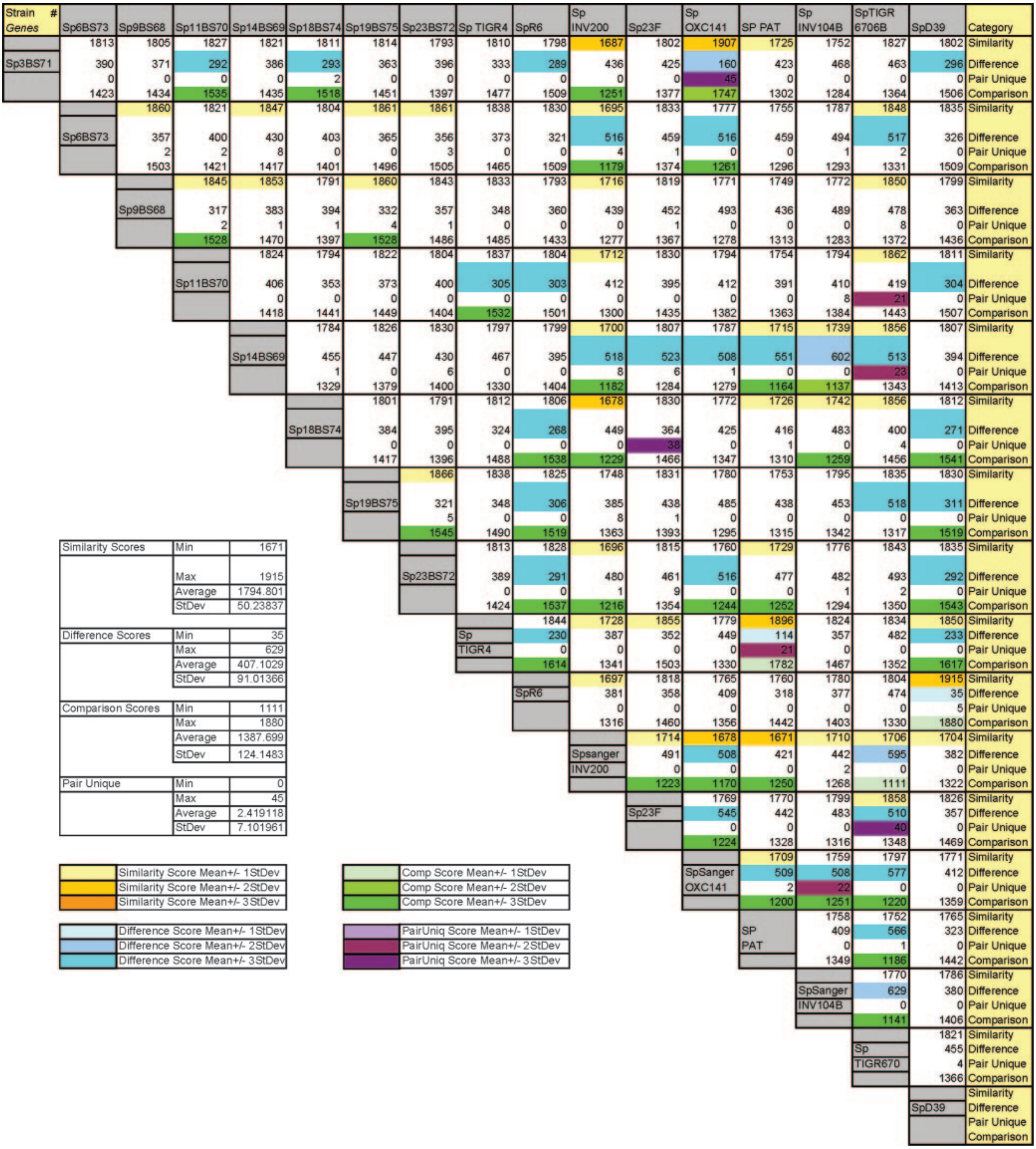


FIG. 2. Dendrogram showing the relationship among 17 *S. pneumoniae* strains based on orthologous cluster differences.



Strain # Genes	Sp6BS73	Sp9BS68	Sp11BS70	Sp14BS69	Sp18BS74	Sp19BS75	Sp23BS72	Sp TIGR4	SpR6	Sp INV200	Sp23F	Sp OXC141	SP PAT	Sp INV104B	SpTIGR 6706B	SpD39	Category
Sp3BS71	1813	1805	1827	1821	1811	1814	1793	1810	1798	1687	1802	1907	1725	1752	1827	1802	Similarity
	390	371	292	386	293	363	396	333	289	436	425	160	423	468	463	296	Difference
	0	0	0	0	2	0	0	0	0	0	0	45	0	0	0	0	Pair Unique
	1423	1434	1535	1435	1518	1451	1397	1477	1509	1251	1377	1747	1302	1284	1364	1506	Comparison
Sp6BS73		1860	1821	1847	1804	1861	1861	1838	1830	1695	1833	1777	1755	1787	1848	1835	Similarity
		357	400	430	403	365	356	373	321	516	459	516	459	494	517	326	Difference
		2	2	8	0	0	3	0	0	4	1	0	0	1	2	0	Pair Unique
		1503	1421	1417	1401	1496	1505	1465	1509	1179	1374	1261	1296	1293	1331	1509	Comparison
Sp9BS68			1845	1853	1791	1860	1843	1833	1793	1716	1819	1771	1749	1772	1850	1799	Similarity
			317	383	394	332	357	348	360	439	452	493	436	489	478	363	Difference
			2	1	1	4	1	0	0	0	1	0	0	0	8	0	Pair Unique
			1528	1470	1397	1528	1486	1485	1433	1277	1367	1278	1313	1283	1372	1436	Comparison
Sp11BS70				1824	1794	1822	1804	1837	1804	1712	1830	1794	1754	1794	1862	1811	Similarity
				406	353	373	400	305	303	412	395	412	391	410	419	304	Difference
				0	0	0	0	0	0	0	0	0	0	8	21	0	Pair Unique
				1418	1441	1449	1404	1532	1501	1300	1435	1382	1363	1384	1443	1507	Comparison
Sp14BS69					1784	1826	1830	1797	1799	1700	1807	1787	1715	1739	1856	1807	Similarity
					455	447	430	467	395	518	523	508	551	602	513	394	Difference
					1	0	6	0	0	8	6	1	0	0	23	0	Pair Unique
					1329	1379	1400	1330	1404	1182	1284	1279	1164	1137	1343	1413	Comparison
Sp18BS74						1801	1791	1812	1806	1678	1830	1772	1726	1742	1856	1812	Similarity
						384	395	324	268	449	364	425	416	483	400	271	Difference
						0	0	0	0	0	38	0	1	0	4	0	Pair Unique
						1417	1396	1488	1538	1229	1466	1347	1310	1259	1456	1541	Comparison
Sp19BS75							1866	1838	1825	1748	1831	1780	1753	1795	1835	1830	Similarity
							321	348	306	385	438	485	438	453	518	311	Difference
							5	0	0	8	1	0	0	0	0	0	Pair Unique
							1545	1490	1519	1363	1393	1295	1315	1342	1317	1519	Comparison
Sp23BS72								1813	1828	1696	1815	1760	1729	1776	1843	1835	Similarity
								389	291	480	461	516	477	482	493	292	Difference
								0	0	1	9	0	0	1	2	0	Pair Unique
								1424	1537	1216	1354	1244	1252	1294	1350	1543	Comparison
Sp TIGR4									1844	1728	1855	1779	1896	1824	1834	1850	Similarity
									230	387	352	449	114	357	482	233	Difference
									0	0	0	0	21	0	0	0	Pair Unique
									1614	1341	1503	1330	1782	1467	1352	1617	Comparison
SpR6										1697	1818	1765	1760	1780	1804	1915	Similarity
										381	358	409	318	377	474	35	Difference
										0	0	0	0	0	0	5	Pair Unique
										1316	1460	1356	1442	1403	1330	1880	Comparison
Spsanger INV200											1714	1678	1671	1710	1706	1704	Similarity
											491	508	421	442	595	382	Difference
											0	0	0	2	0	0	Pair Unique
											1223	1170	1250	1268	1111	1322	Comparison
Sp23F												1769	1770	1799	1858	1826	Similarity
												545	442	483	510	357	Difference
												0	0	0	40	0	Pair Unique
												1224	1328	1316	1348	1469	Comparison
SpSanger OXC141													1709	1759	1797	1771	Similarity
													509	508	577	412	Difference
													2	22	0	0	Pair Unique
													1200	1251	1220	1359	Comparison
SP PAT														1758	1752	1765	Similarity
														409	566	323	Difference
														0	1	0	Pair Unique
														1349	1186	1442	Comparison
SpSanger INV104B															1770	1786	Similarity
															629	380	Difference
															0	0	Pair Unique
															1141	1406	Comparison
Sp TIGR670																1821	Similarity
																455	Difference
																4	Pair Unique
																1366	Comparison
SpD39																	Similarity
																	Difference
																	Pair Unique
																	Comparison

Similarity Scores	Min	1671
	Max	1915
	Average	1794.801
	StDev	50.23837
Difference Scores	Min	35
	Max	629
	Average	407.1029
	StDev	91.01366
Comparison Scores	Min	1111
	Max	1880
	Average	1387.699
	StDev	124.1483
Pair Unique	Min	0
	Max	45
	Average	2.419118
	StDev	7.101961

🟨	Similarity Score Mean+/- 1StDev
🟨	Similarity Score Mean+/- 2StDev
🟧	Similarity Score Mean+/- 3StDev

🟩	Comp Score Mean+/- 1StDev
🟩	Comp Score Mean+/- 2StDev
🟩	Comp Score Mean+/- 3StDev

🟦	Difference Score Mean+/- 1StDev
🟦	Difference Score Mean+/- 2StDev
🟦	Difference Score Mean+/- 3StDev

🟪	PairUniq Score Mean+/- 1StDev
🟪	PairUniq Score Mean+/- 2StDev
🟪	PairUniq Score Mean+/- 3StDev

FIG. 3. Global comparison of orthologous gene clusters for 17 *S. pneumoniae* strains. The similarity score corresponds to the total number of orthologous gene clusters shared within each strain pair; the difference score corresponds to the total number of orthologous gene clusters not shared within a strain pairing; and the comparison equals the similarity score minus the difference score. In addition, the number of clusters shared only within a strain pair is noted as pair-unique. If these values are 1, 2, or 3 standard deviations away from the mean of all pairwise comparisons, the boxes are color coded as noted in the key.

clusters shared; the difference score corresponds to the total number of orthologous gene clusters not shared; and the comparison score was calculated by subtracting the difference score from the similarity score to provide a genic measure of comparison between strains. We also outlined the orthologous clusters that were shared only within a given pairing; these are designated pair-unique. The average genic difference between any two strains was 407 orthologous clusters (standard devia-

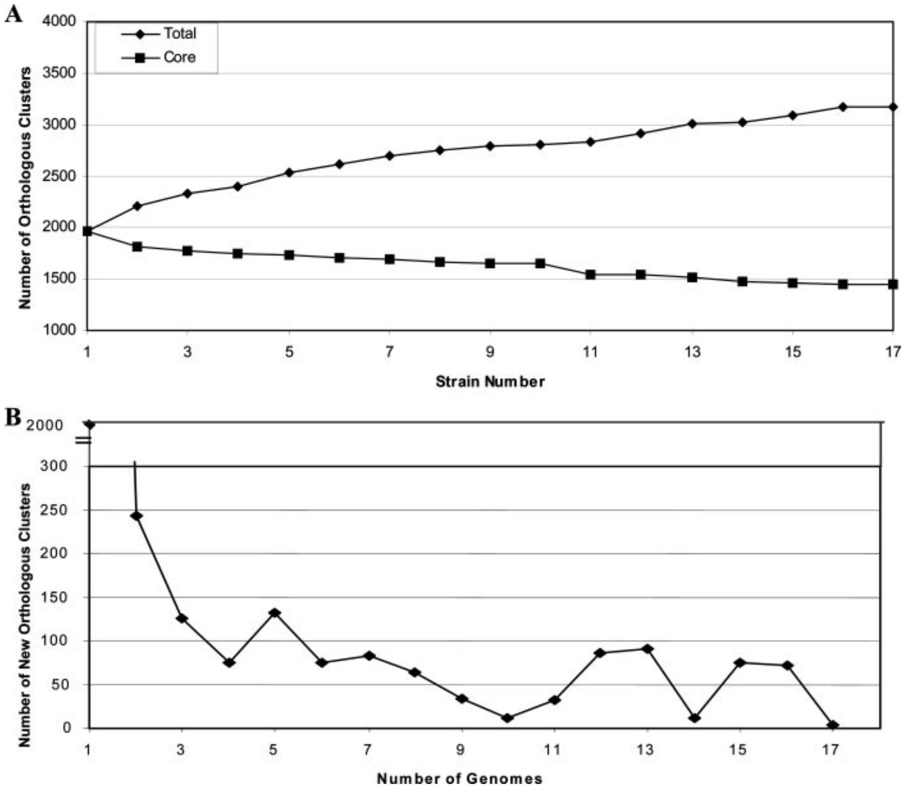


FIG. 4. (A) Plot of the numbers of total and core observed orthologous clusters as a function of the number of strains sequenced. (B) Plot of the number of new observed orthologous clusters as a function of each genome. Numbers were calculated first for two strains and then iteratively for strains added one by one.

tion, 91), while the average genic similarity was 1,794 orthologous clusters (standard deviation, 50). The three strain pairs R6–D39, CGSSp3BS71–OXC141, and PAT6420135–TIGR4 have similarity scores 3 standard deviations above the mean (these are the same pairs that group in the dendrogram). Two of these pairs also have high numbers of pair-unique orthologous clusters, as might be expected. However, more-distant strains can also share unique pairs. For example, strains 23F and CGSSp18BS7 have 38 pair-unique genes, 34 of which are genes that are physically grouped on both genomes (in a colinear fashion); 21 of these are annotated as phage related, strongly suggesting that the unique similarity between these strains results from the insertion of a phage.

We used the sum of the difference scores as a rough measure of strain diversity to investigate whether the number of unique genes serves as an indicator for gene diversity. The plot of the total number of orthologous clusters, the number of unique orthologous clusters, and the sum of the difference scores shows a modest correlation between these values (R^2 = 0.73 for unique orthologous clusters versus difference score) (see Fig. S2 in the supplemental material); however, the numbers of unique and pair-unique orthologous clusters are not sufficient to reveal the phylogenetic relationships among strains.

Supragenome size estimation. These data show that *S. pneumoniae* has a supragenome much larger than the genome of any individual strain. Our group developed a finite supragenome model based on clustering analysis of multiple *H. influenzae* genomes (18). This model accounts for the numbers of core, distributed, and unique orthologous clusters and does not assume that the distributed orthologous clusters are sampled in the population with equal probability. The absence of this assumption yields a model where almost all of the supragenome can be accurately determined after the genomes of a finite number of strains are sequenced.

To visualize how the genome sequence of each additional strain adds to our understanding of the supragenome, we calculated the numbers of total and core orthologous clusters for two strains and then recalculated these parameters on an iterative basis as we added each additional strain. These exact core and supragenome values will differ depending on the order in which the strains are added to the analysis, but the trend is always the same: decay in the number of new orthologous clusters and stabilization of the number of core orthologous clusters at ~1,400 (Fig. 4A and B). This suggests that after a finite number of genomes have been sequenced, the number of new orthologous clusters identified will be very low.

To address the question of the number of strains that must be sequenced before the vast majority of the orthologous clusters are identified, we fitted the clustering data from the 17 genomes to the finite supragenome model (18). This model predicts that the *S. pneumoniae* supragenome consists of ~5,100 orthologous clusters, where ~1,380 are core, ~2,100 are unique, and the remaining are distributed. The orthologous clusters in the supragenome are represented at different frequencies within the *S. pneumoniae* population; a cluster that is present at a population frequency lower than 0.1 presumably

TABLE 4. Predicted coverage of the *S. pneumoniae* supragenome using the finite supragenome model

Population frequency	Supragenome coverage (%)	No. of strains sequenced
≥0.1	90	11
≥0.1	95	17
≥0.1	99	33
All	90	142

does not have much impact on the population, although it may be very interesting in itself and could potentially be very important if the one strain it represents causes a pneumococcal pandemic. If all orthologous clusters are considered, the estimated supragenome size is 5,117 clusters and the core set contains 27% of the clusters. If only orthologous clusters with frequencies equal or greater then 0.1 are considered, the estimated supragenome size drops to 2,979 clusters where 46.5% are core. Accounting only for the orthologous clusters that exist in the *S. pneumoniae* supragenome at a frequency equal to 0.1 to greater than 95% of the supragenome is predicted to be identified after sequencing of 17 strains and 99% after sequencing of 33 strains. If orthologous clusters of all population frequencies are considered, 90% of the supragenome is predicted to be identified after 142 strains are sequenced (Table 4). The predicted and observed distributions are compared in Fig. 1, where the model fits the data well. Figure 5A shows the numbers of total and core orthologous clusters predicted for 50 or fewer strains, and Fig. 5B plots the predicted number of new orthologous clusters as a function of each genome sequenced. This prediction assumes that the 17 strains used in this study are representative of worldwide strains; if they are not, this prediction will underestimate the number of strains required for supragenome coverage.

Unique and core orthologous clusters. The 576 unique orthologous clusters identified in this study are distributed among all strains; the lowest number per strain is 1, for the TIGR4 strain, and the highest is 74, for INV104B (Table 3). Sixty-two percent of the unique genes are annotated as hypothetical proteins; ~5.4% have annotations related to phage, prophage, or bacteriophage; and the remaining 33% correspond to a wide range of proteins including putative transporters, transcriptional regulators and activators, lantibiotic biosynthesis and transport proteins, macrolide efflux proteins, and many other enzymes (see Table S3 in the supplemental material).

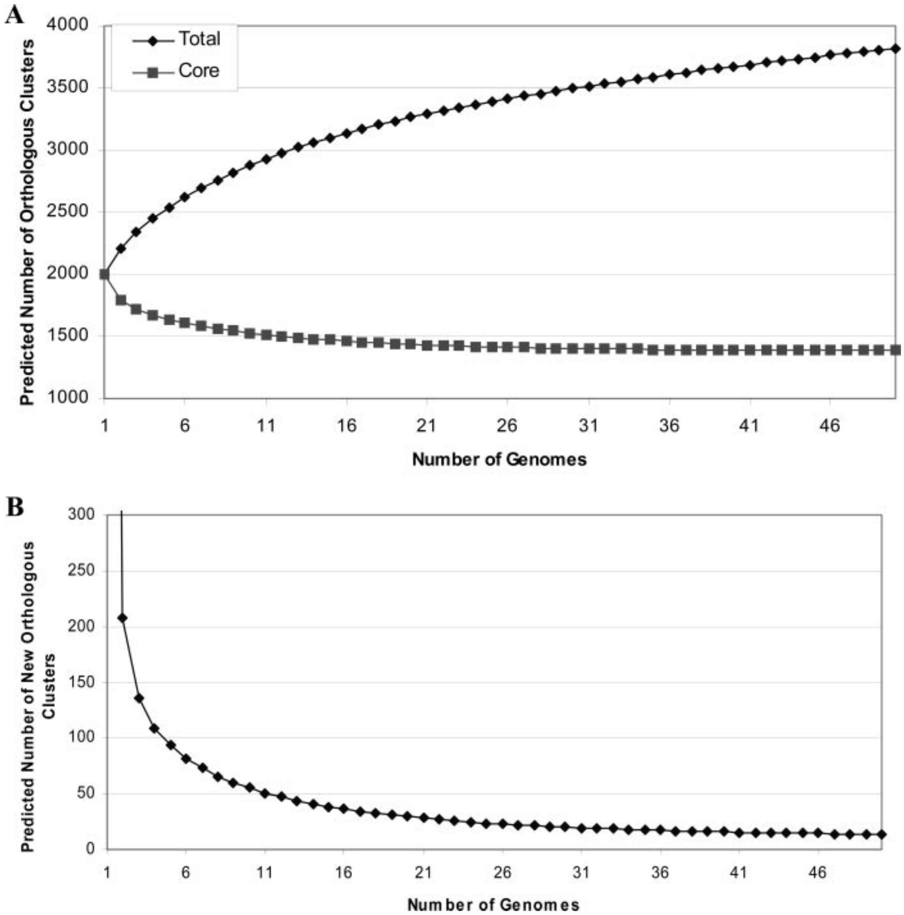


FIG. 5. Predictions using the finite supragenome model. (A) Plot of the numbers of total and core predicted orthologous clusters as functions of the number of strains sequenced. (B) Plot of the number of new predicted orthologous clusters as a function of each genome sequenced. Numbers were calculated first for two strains and then iteratively for strains added one by one.

For the most part, the genes in a given orthologous cluster have the same annotation, and somewhat surprisingly, the genes in the core orthologous clusters include a significant percentage annotated as hypothetical proteins (~30%), perhaps suggesting that many bacterial housekeeping functions remain unknown (see Table S3 in the supplemental material). Among the annotated core orthologous clusters, no phage proteins were detected, suggesting that no single phage is conserved across all strains. Interestingly, there are a few orthologous clusters with more than 100 CDSs that are annotated as transposases, often degenerate or truncated. The role of these proteins in the *S. pneumoniae* genome, if any, remains to be established. A table with the annotations for genes in the core, distributed, and unique categories is provided as Table S3 in the supplemental material.

DISCUSSION

This study compared the genic contents of 17 *S. pneumoniae* strains. Genes from all strains were organized into orthologous clusters, and these clusters were quantified for all genomes. When the genomes are analyzed together, fewer than 50% of all the orthologous clusters (corresponding to ~73% of the total CDSs) are conserved among all species. When the genomes of individual strains were evaluated, 21 to 32% of the orthologous clusters were noncore. Predictions using the finite supragenome model suggest that the total number of orthologous clusters in the *S. pneumoniae* species is around 5,100 and the total number of core orthologous clusters is around 1,380. These large strain differences illustrate the enormous genic diversity within this species, as postulated in the distributed-genome hypothesis (7, 37). The engines driving this genomic plasticity are threefold: first, it has been demonstrated that chronic infections by nasopharyngeal pathogens are generally polyclonal in nature (11, 16, 30, 31, 39–41; J. R. Gilsdorf, presented at the 9th International Symposium on Recent Advances in Otitis Media, 3 to 7 June 2007); second, the bacteria in these chronic infections adopt a biofilm mode of growth, which greatly increases the kinetics of horizontal gene transfer (8, 15, 28); and third, *S. pneumoniae* employs highly energetic fratricidal as well as autocompetence and autotransformation mechanisms for the release and uptake of pneumococcal DNA, respectively, from the surrounding environment (35). The pathological consequences of these phenomena, which collectively result in a continual reassortment of genic characters among strains within a polyclonal biofilm infection, are that the host's adaptive immune system continually encounters novel strains, making clearance very difficult, because the pathogen can generate diversity faster than the host can adapt to it, thus ensuring chronicity of infection.

In a previous study, we constructed individual genomic libraries from the eight CGS *S. pneumoniae* clinical isolates (CGSSp9BS68, CGSSp14BS69, CGSSp11BS70, CGSSp3BS71, CGSSp23BS72, CGSSp6BS73, CGSSp18BS74, and CGSSp19 BS75). Of the 4,793 clones sequenced, ~16% were not present in the TIGR4 reference strain, suggesting that many genes were not conserved across the species. In addition, the screen identified genes unrelated to any streptococcal sequences; analysis of the allocation of a subset of 58 of these found that they were not uniformly distributed across the eight strains (37). These results are in complete agreement with this study; both studies underscore the genomic plasticity of the *S. pneumoniae* species.

The use of the finite supragenome model suggests that 99% of orthologous clusters in the supragenome that have population frequencies equal or higher to 0.1 can be identified after sequencing of 33 strains and that the 17 available strains provide ~95% coverage of this set. When analyzing the *S. agalactiae* supragenome, Tettelin and colleagues presented a different mathematical model, generated using the assumption that noncore genes are sampled in the population with equal probabilities (43). Unlike the finite supragenome model, this model predicts that a constant number of new strain-specific genes will be identified with the addition of each genome, such that sequencing a limited number of strains would not provide major coverage of the supragenome.

Our analysis includes clinical strains from multiple locations including the United States, the United Kingdom, Norway, and Spain. Diversity is generated from DNA exchange among strains; thus, it is tempting to consider that strains from the same geographical location may be more similar, since they have a higher probability of exchanging genetic information (directly or indirectly, via other strains). Interestingly, we did not observe this with our limited number of strains. While it is possible that a correlation between geographical distance and genic diversity will be observed when a larger number of strains from multiple geographic regions are sequenced and compared, we must nonetheless consider that this correlation may not exist. This result would be explained if the vast majority of the orthologous clusters in the *S. pneumoniae* supragenome have been in the species for a very long time, and horizontal transfer from other species and new mutations have introduced only a minority of the supragenome's orthologous clusters, or if the extent of human population migration is now so high (at least in the West) that human pathogens are essentially homogenized around the world.

This enormous genetic diversity calls attention to the need for markers of human virulence phenotypes and highlights the potential difficulty associated with this task. *S. pneumoniae* strains are presently categorized based on capsule type and MLST. The capsular serotype is an important virulence factor and affects the ability of pneumococci to cause invasive disease (2, 38). For example, the difference in virulence between type 2 D39, which is highly virulent in the murine model of infection, and unencapsulated R6, which is avirulent, is attributed to the loss of the capsule. However, it is critical to remember that even within the same capsular type, virulence is highly related to the genetic background of the strains (20). The virulence phenotypes displayed by the eight strains isolated in Pittsburgh differ significantly in a chinchilla model of *S. pneumoniae* infection; these differences may be due to distinct serotypes, genotypes, or both (M. Forbes and J. Hayes, personal communication). Our data in Fig. 2 clearly show that the serotype cannot be correlated with the genic content, since strains of serotypes 14, 6, and 23 were not grouped based on genic differences. An analysis of sequence variation of the type 6A and 6B capsular biosynthetic loci was related to the MLST profile, yet there was also ample evidence of horizontal transfer to unrelated lineages (27). Phylogenetic trees using MLST from the seven CGS clinical strains of known MLST types did not closely resemble the phylogenies created from genic differences (data not shown). Together these data suggest that in some cases, the serotype, MLST type, and/or genetic background may correlate, but in other cases, they do not, as would be expected from strains undergoing high rates of intraspecies horizontal gene transfer. Since pathogenesis is probably a consequence not only of capsular type but also of multiple other genes, MLST type and serotype alone are not ideal markers for the disease phenotype of *S. pneumoniae* strains.

Previous work on six strains of *Streptococcus agalactiae* described a supragenome with ~80% core orthologous clusters and the remaining set consisting of partially shared and strain-specific orthologous clusters (43). In addition, these data resemble, in qualitative and quantitative terms, our comparison of 13 *H. influenzae* strains (18). The total number of *H. influenzae* orthologous clusters for the 13 strains was 2,786, of which 52% were core, 29% were distributed, and 19% were unique. Taken together, these studies suggest that a high degree of genic variation is common among multiple species. However, it may not be universal; analyses of eight *Bacillus anthracis* strains revealed substantially less variation among strains, with no new genes uncovered after analysis of only four genomes (43). It is possible that this degree of variation is intrinsic to naturally transforming bacteria such as *H. influenzae* and *S. pneumoniae*, which undergo extensive DNA recombination events. In addition, both of these bacteria exist exclusively in the human mucosa, where they form biofilms (15). Cells in a biofilm are embedded in an extracellular polymeric matrix that is rich in nucleic acids; thus, biofilms may provide ideal environments to foster such genomic plasticity (32). There is also quantitative similarity, since both species have core genomes that seem to stabilize around 1,400 proteins, or ~50% of the supragenome. This similarity suggests that similar evolutionary forces may be determining the equilibrium between core genes, noncore genes, and genome size.

This diversity suggests caution in the use of model strains to test and develop vaccines and drugs, since effective targets in one strain may be missing in a significant percentage of the other strains. It is probable that these bacteria have evolved multiple and redundant mechanisms to evade immunity and adapt to variations among hosts and their commensal microbiota.

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REFERENCES

1. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Inductions of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exp. Med. **79:**137–158.
2. **Brueggemann, A. B., D. T. Griffiths, E. Meats, T. Peto, D. W. Crook, and B. G. Spratt.** 2003. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J. Infect. Dis. **187:**1424–1432.
3. **Claverys, J. P., and L. S. Havarstein.** 2002. Extracellular-peptide control of competence for genetic transformation in *Streptococcus pneumoniae*. Front. Biosci. **7:**d1798–d1814.
4. **Daraselia, N., D. Dernovoy, Y. Tian, M. Borodovsky, R. Tatusov, and T. Tatusova.** 2003. Reannotation of *Shewanella oneidensis* genome. OMICS **7:**171–175.
5. **Del Beccaro, M. A., P. M. Mendelman, A. F. Inglis, M. A. Richardson, N. O. Duncan, C. R. Clausen, and T. L. Stull.** 1992. Bacteriology of acute otitis media: a new perspective. J. Pediatr. **120:**81–84.
6. **Delcher, A. L., S. Kasif, R. D. Fleischmann, J. Peterson, O. White, and S. L. Salzberg.** 1999. Alignment of whole genomes. Nucleic Acids Res. **27:**2369–2376.
7. **Ehrlich, G. D., F. Z. Hu, K. Shen, P. Stoodley, and J. C. Post.** 2005. Bacterial plurality as a general mechanism driving persistence in chronic infections. Clin. Orthop. Relat. Res. **2005:**20–24.
8. **Ehrlich, G. D., R. Veeh, X. Wang, J. W. Costerton, J. D. Hayes, F. Z. Hu, B. J. Daigle, M. D. Ehrlich, and J. C. Post.** 2002. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. JAMA **287:**1710–1715.
9. **Ewing, B., and P. Green.** 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. **8:**186–194.
10. **Ewing, B., L. Hillier, M. C. Wendl, and P. Green.** 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. **8:**175–185.
11. **Farjo, R. S., B. Foxman, M. J. Patel, L. Zhang, M. M. Pettigrew, S. I. McCoy, C. F. Marrs, and J. R. Gilsdorf.** 2004. Diversity and sharing of *Haemophilus influenzae* strains colonizing healthy children attending day-care centers. Pediatr. Infect. Dis. J. **23:**41–46.
12. **Gates, G. A.** 1996. Cost-effectiveness considerations in otitis media treatment. Otolaryngol. Head Neck Surg. **114:**525–530.
13. **Gordon, D.** 2004. Viewing and editing assembled sequences using Consed. John Wiley & Co., New York, NY.
14. **Gordon, D., C. Abajian, and P. Green.** 1998. Consed: a graphical tool for sequence finishing. Genome Res. **8:**195–202.
15. **Hall-Stoodley, L., F. Z. Hu, A. Gieseke, L. Nistico, D. Nguyen, J. Hayes, M. Forbes, D. P. Greenberg, B. Dice, A. Burrows, P. A. Wackym, P. Stoodley, J. C. Post, G. D. Ehrlich, and J. E. Kerschner.** 2006. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA **296:**202–211.
16. **Hiltke, T. J., A. T. Schiffmacher, A. J. Dagonese, S. Sethi, and T. F. Murphy.** 2003. Horizontal transfer of the gene encoding outer membrane protein P2 of nontypeable *Haemophilus influenzae*, in a patient with chronic obstructive pulmonary disease. J. Infect. Dis. **188:**114–117.
17. **Hoberman, A., D. P. Greenberg, J. L. Paradise, H. E. Rockette, J. R. Lave, D. H. Kearney, D. K. Colborn, M. Kurs-Lasky, M. A. Haralam, C. J. Byers, L. M. Zoffel, I. A. Fabian, B. S. Bernard, and J. D. Kerr.** 2003. Effectiveness of inactivated influenza vaccine in preventing acute otitis media in young children: a randomized controlled trial. JAMA **290:**1608–1616.
18. **Hogg, J. S., F. Z. Hu, B. Janto, R. Boissy, J. Hayes, R. Keefe, J. C. Post, and G. D. Ehrlich.** 2007. Characterization and modeling of the *Haemophilus influenzae* core and supragenomes based on the complete genomic sequences of Rd and 12 clinical nontypeable strains. Genome Biol. **8:**R103. [Epub ahead of print.]
19. **Hoskins, J., W. E. Alborn, Jr., J. Arnold, L. C. Blaszczak, S. Burgett, B. S. DeHoff, S. T. Estrem, L. Fritz, D. J. Fu, W. Fuller, C. Geringer, R. Gilmour, J. S. Glass, H. Khoja, A. R. Kraft, R. E. Lagace, D. J. LeBlanc, L. N. Lee, E. J. Lefkowitz, J. Lu, P. Matsushima, S. M. McAhren, M. McHenney, K. McLeaster, C. W. Mundy, T. I. Nicas, F. H. Norris, M. O'Gara, R. B. Peery, G. T. Robertson, P. Rockey, P. M. Sun, M. E. Winkler, Y. Yang, M. Young-Bellido, G. Zhao, C. A. Zook, R. H. Baltz, S. R. Jaskunas, P. R. Rosteck, Jr., P. L. Skatrud, and J. I. Glass.** 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. J. Bacteriol. **183:**5709–5717.
20. **Kelly, T., J. P. Dillard, and J. Yother.** 1994. Effect of genetic switching of capsular type on virulence of *Streptococcus pneumoniae*. Infect. Immun. **62:**1813–1819.
21. **Klein, J. O.** 1999. Management of acute otitis media in an era of increasing antibiotic resistance. Int. J. Pediatr. Otorhinolaryngol. **49**(Suppl. 1)**:**S15–S17.
22. **Kunsch, C. A., G. H. Choi, P. S. Dillon, C. A. Rosen, S. C. Barash, M. R. Fannon, and B. A. Dougherty.** 16 July 2002. *Streptococcus pneumoniae* polynucleotides and sequences. U.S. patent 6,420,135.
23. **Lanie, J. A., W. L. Ng, K. M. Kazmierczak, T. M. Andrzejewski, T. M. Davidsen, K. J. Wayne, H. Tettelin, J. I. Glass, and M. E. Winkler.** 2007. Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6. J. Bacteriol. **189:**38–51.
24. **Lowe, T. M., and S. R. Eddy.** 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. **25:**955–964.

25. **Marchler-Bauer, A., J. B. Anderson, M. K. Derbyshire, C. DeWeese-Scott, N. R. Gonzales, M. Gwadz, L. Hao, S. He, D. I. Hurwitz, J. D. Jackson, Z. Ke, D. Krylov, C. J. Lanczycki, C. A. Liebert, C. Liu, F. Lu, S. Lu, G. H. Marchler, M. Mullokandov, J. S. Song, N. Thanki, R. A. Yamashita, J. J. Yin, D. Zhang, and S. H. Bryant.** 2007. CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res. **35:**D237–D240.

26. **Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y. J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin, W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. Alenquer, T. P. Jarvie, K. B. Jirage, J. B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc, M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley, and J. M. Rothberg.** 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature **437:**376–380.

27. **Mavroidi, A., D. Godoy, D. M. Aanensen, D. A. Robinson, S. K. Hollingshead, and B. G. Spratt.** 2004. Evolutionary genetics of the capsular locus of serogroup 6 pneumococci. J. Bacteriol. **186:**8181–8192.

28. **Molin, S., and T. Tolker-Nielsen.** 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr. Opin. Biotechnol. **14:**255–261.

29. **Moscoso, M., and J. P. Claverys.** 2004. Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA. Mol. Microbiol. **54:**783–794.

30. **Müller-Graf, C. D., A. M. Whatmore, S. J. King, K. Trzcinski, A. P. Pickerill, N. Doherty, J. Paul, D. Griffiths, D. Crook, and C. G. Dowson.** 1999. Population biology of *Streptococcus pneumoniae* isolated from oropharyngeal carriage and invasive disease. Microbiology **145:**3283–3293.

31. **Murphy, T. F., S. Sethi, K. L. Klingman, A. B. Brueggemann, and G. V. Doern.** 1999. Simultaneous respiratory tract colonization by multiple strains of nontypeable *Haemophilus influenzae* in chronic obstructive pulmonary disease: implications for antibiotic therapy. J. Infect. Dis. **180:**404–409.

32. **Oggioni, M. R., C. Trappetti, A. Kadioglu, M. Cassone, F. Iannelli, S. Ricci, P. W. Andrew, and G. Pozzi.** 2006. Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol. Microbiol. **61:**1196–1210.

33. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.

34. **Peterson, S. N., C. K. Sung, R. Cline, B. V. Desai, E. C. Snesrud, P. Luo, J. Walling, H. Li, M. Mintz, G. Tsegaye, P. C. Burr, Y. Do, S. Ahn, J. Gilbert, R. D. Fleischmann, and D. A. Morrison.** 2004. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. Mol. Microbiol. **51:**1051–1070.

35. **Prudhomme, M., L. Attaiech, G. Sanchez, B. Martin, and J. P. Claverys.** 2006. Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. Science **313:**89–92.

36. **Rozen, S., and H. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. **132:**365–386.

37. **Shen, K., J. Gladitz, P. Antalis, B. Dice, B. Janto, R. Keefe, J. Hayes, A. Ahmed, R. Dopico, N. Ehrlich, J. Jocz, L. Kropp, S. Yu, L. Nistico, D. P. Greenberg, K. Barbadora, R. A. Preston, J. C. Post, G. D. Ehrlich, and F. Z. Hu.** 2006. Characterization, distribution, and expression of novel genes among eight clinical isolates of *Streptococcus pneumoniae*. Infect. Immun. **74:**321–330.

38. **Smith, T., D. Lehmann, J. Montgomery, M. Gratten, I. D. Riley, and M. P. Alpers.** 1993. Acquisition and invasiveness of different serotypes of *Streptococcus pneumoniae* in young children. Epidemiol. Infect. **111:**27–39.

39. **Smith-Vaughan, H. C., A. J. Leach, T. M. Shelby-James, K. Kemp, D. J. Kemp, and J. D. Mathews.** 1996. Carriage of multiple ribotypes of non-encapsulated *Haemophilus influenzae* in aboriginal infants with otitis media. Epidemiol. Infect. **116:**177–183.

40. **Smith-Vaughan, H. C., K. S. Sriprakash, J. D. Mathews, and D. J. Kemp.** 1995. Long PCR-ribotyping of nontypeable *Haemophilus influenzae*. J. Clin. Microbiol. **33:**1192–1195.

41. **Smith-Vaughan, H. C., K. S. Sriprakash, J. D. Mathews, and D. J. Kemp.** 1997. Nonencapsulated *Haemophilus influenzae* in Aboriginal infants with otitis media: prolonged carriage of P2 porin variants and evidence for horizontal P2 gene transfer. Infect. Immun. **65:**1468–1474.

42. **Tatusov, R. L., M. Y. Galperin, D. A. Natale, and E. V. Koonin.** 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res. **28:**33–36.

43. **Tettelin, H., V. Masignani, M. J. Cieslewicz, C. Donati, D. Medini, N. L. Ward, S. V. Angiuoli, J. Crabtree, A. L. Jones, A. S. Durkin, R. T. Deboy, T. M. Davidsen, M. Mora, M. Scarselli, I. Margarit y Ros, J. D. Peterson, C. R. Hauser, J. P. Sundaram, W. C. Nelson, R. Madupu, L. M. Brinkac, R. J. Dodson, M. J. Rosovitz, S. A. Sullivan, S. C. Daugherty, D. H. Haft, J. Selengut, M. L. Gwinn, L. Zhou, N. Zafar, H. Khouri, D. Radune, G. Dimitrov, K. Watkins, K. J. O'Connor, S. Smith, T. R. Utterback, O. White, C. E. Rubens, G. Grandi, L. C. Madoff, D. L. Kasper, J. L. Telford, M. R. Wessels, R. Rappuoli, and C. M. Fraser.** 2005. Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome." Proc. Natl. Acad. Sci. USA **102:**13950–13955.

44. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F. Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M. R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L. Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey, I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A. Morrison, S. K. Hollingshead, and C. M. Fraser.** 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science **293:**498–506.