Microarray Design. We constructed a 4,420 element *S. pneumoniae*-specific spotted DNA microarray based on the pre-annotated TIGR4 genome sequence (4). In order to minimize the potential for cross hybridization on the array, primer pairs (Illumina, Inc. San Diego, CA) were designed using the Primer3 program (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) (3) based on sequence that was determined to be unique to each ORF. This was accomplished using an algorithm that identifies the most 3' region of an ORF that contains no significant homology to any other ORF in the genome as identified by NCBI BLAST (3). In addition, primer pairs were also designed for 975 noncoding putative regulatory sequences. DNA fragments were PCR amplified using predominantly D39 genomic DNA as template, however, for 65 ORFs that are not present in this strain (4), TIGR4 genomic DNA was used as template for amplification. Amplified products were analyzed on agarose gels and ranged from 70 to 300 bp, with 210 bp as the median length and 198bp as the average length. PCR amplification methods, polylysine glass slide preparation, printing, and array postprocessing were performed as previously reported (1). The final array is comprised of 3620 elements corresponding to 98% of all TIRG4 ORFs and 975 elements corresponding to intergenic sequences. Many elements are represented by multiple spots.

Probe synthesis. RNA was converted to cDNA in 20 µl reactions by combining 0.5 µg of RNA and 0.5 µg of random hexamers (Amersham), heating to 65°C for 10 minutes, and then snap cooling the reactions on ice. The following was then added: 2 µl of 0.1 M dithiothreitol (DTT), 0.5 µl of 10 mM dNTPs, 4 µl of 5x RT buffer (Invitrogen) and 1 µl (200U) of Superscript II (Gibco BRL). This mixture was incubated at 42°C for 150 minutes. RNA was hydrolyzed with 1 µl of 1 M NaOH at 65°C for 10 minutes and neutralized with 1 µl of 1 M HCl. Samples were purified over a Qia-quick PCR column (Qiagen) according to the manufacturer's instructions and eluted with 40 µl elution buffer. Amino-allyl dUTP was incorporated into the cDNA samples as follows. For each sample, 40 µl of the eluted DNA was incubated for 5 minutes at 99°C and then for 5 minutes on ice. 5 µl of 10x random octamer buffer (NEB 1550-2), 3 µl of dNTP/dUTP mix (3 mM dGTP, dATP, dCTP; 1.8 mM aa-dUTP (Sigma-Aldrich A-0410), 1.2 mM dTTP] and 2 µl

of Exo$^-$ Klenow (NEB) were added, and the mixture was incubated for 150 minutes at 37°C and then stored at 4^{o}C overnight. Free amines were removed with a Qia-quick PCR purification kit (Qiagen), and the eluted sample was dried in a speed-vac. Samples were resuspended in 4.5 µl of dH_2O and incubated with 1µM of either Cy3 or Cy5 monofunctional reactive dye (Amersham) for 1 h at room temperature in the dark. The time point samples were incubated with Cy5, and the reference samples were incubated with Cy3. The reference sample for each time course was generated from the D39 time zero RNA. The reactions were quenched with 4.5 µl of 4 M hydroxylamine for 15 minutes at room temperature, and then each Cy5-labeled sample was mixed with a Cy3-labeled reference. Unincorporated dye was removed with a Qia-quick PCR purification kit, and probes were eluted with 40µl of EB and were dried in a speed-vac. To hybridize, the samples were resuspended in 11.3 µl of TE, pH 7.5, 1 µl of 10 mg ml^{-1} 1 yeast tRNA, 2.25 µl of 20x SSC and 0.45 µl of 10% SDS. The mixture was heated to 99°C for 2 minutes and immediately centrifuged for 2 minutes at maximum speed. The probe was applied to a microarray and incubated for at least 24 hours at 60°C.

Data Analysis. Arrays were washed and then scanned using a GenePix 4000A scanner (Axon Instruments, Foster City, CA) and images were analyzed with GENEPIX PRO software. Microarray data were stored in the Stanford Microarray (2) and are publicly available (http://genome-www.stanford.edu/microarray). The data were filtered to remove poor quality measurements and the red::green ratios were log2 transformed. The data were subsequently zero transformed for each time course by subtracting the average $t = 0$ value (n=3) for each strain from all subsequent time points measured. This allows us to identify genes whose patterns of expression differed between the two strains through the time courses relative to time 0.

References

1. **Eisen, M. B., and P. O. Brown.** 1999. DNA arrays for analysis of gene expression. Methods Enzymol **303:**179-205.
2. **Gollub, J., C. A. Ball, G. Binkley, J. Demeter, D. B. Finkelstein, J. M. Hebert, T. Hernandez-Boussard, H. Jin, M. Kaloper, J. C. Matese, M. Schroeder, P. O. Brown, D. Botstein, and G. Sherlock.** 2003. The Stanford Microarray Database: data access and quality assessment tools. Nucleic Acids Res **31:**94-6.
3. **Rozen, S., and H. Skaletsky.** 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol **132:**365-86.
4. **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.