Supplemental Microarray Methods

RNA isolation. RNA was isolated from *S. sanguinis* using a modification of a protocol developed for *S. pneumoniae* (Peterson et al., 2000). Briefly, JFP41 was grown in TH-HS until an OD₆₆₀ of 0.068 – 0.074 was obtained. Twenty ml of culture was removed and mixed with an equal volume of hot acid phenol containing 0.1 M citrate buffer (pH 4.3, Sigma, St. Louis, MO) and 0.1% (v/v) sodium dodecyl sulfate (SDS, Ambion, Austin, TX). This was used as the “time 0” or untreated reference sample. The remainder of the culture was exposed to CSP at a final concentration of 220 ng/ml for varying durations before transfer of aliquots to hot acid phenol. Samples were incubated in a boiling water bath for 10 min, cooled in an ice bath and transferred to Phase Lock Gel™ (PLG) tubes (5 Prime Inc., Gaithersburg, MD). After centrifugation at 1500 x g for 5 min, the supernatant was removed and extracted using an equal volume of acid phenol:chloroform (1:1), then chloroform. RNA was precipitated from the supernatant by sodium acetate/isopropanol precipitation, followed by centrifugation. The resulting RNA pellet was washed with 70% ethanol, dried via tube inversion, and dissolved in 100 µl diethyl pyrocarbonate-treated water. Samples were then treated with DNase I and purified over RNeasy columns (Qiagen, Valencia, CA) according to protocols supplied by the manufacturer. Total yield was determined by examining absorbance at 260 nm. Samples with a 260/280 ratio > 2.2 or < 1.8 were discarded. Remaining samples were analyzed on an Experion™ Chip (BioRad, Hercules, CA) per the manufacturer’s protocol. RNA samples displaying a 23S/16S ratio > 1.8 were retained and stored at -80ºC until further use.

cDNA synthesis and labeling. Synthesis and labeling of cDNA was performed according to published PFGRC procedures (http://pfgrc.jcvi.org/index.php/microarray/protocols.html). Briefly, 2 µg total RNA was annealed to 6 µg random hexamers (Invitrogen, Carlsbad, CA) in a total volume of 18.5 µl and incubated at 70ºC for 10 min. The tubes were then snap frozen in ethanol at -80ºC for 30s before addition of 6 µl of 5x First Strand Buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 3 µl 0.1M dithiothreitol (DTT), and 0.6 µl of dNTP solution (25 mM each dATP, dCTP, and dGTP, 15 mM dTTP (Invitrogen) and 10 mM amino allyl-dUTP (Sigma)). The solution was then incubated overnight at 42ºC with 300 U of Superscript III (Invitrogen). The RNA template was then hydrolyzed via the addition of 10 µl 0.5 M EDTA and 10 µl 1 M NaOH and incubation at 65ºC for 15 min. The mixture was neutralized with 25 µl 1 M Tris pH 7.0 and 10 µl 3 M sodium acetate, and purified using MiniElute® PCR Purification Kit (Qiagen) replacing the Tris buffers with phosphate buffers (5 mM KPO₄, pH 8, 80% EtOH) and (4 mM KPO₄, pH 8.5) for the wash and elution steps respectively. The reaction was then dried in a Speed-Vac centrifuge before being resuspended in 4.5 µl 0.1 M Na₂HCO₃, pH 9.3 and 4.5 µl of the ester dye (Cy3 or Cy5 (Amersham) for the control and experimental samples respectively). The coupling reaction proceeded for 1.5 – 2 h at room temperature in the dark before being stopped via the addition of 35 µl 100 mM sodium acetate and then purified over Qiagen MiniElute PCR purification columns per manufacturer’s protocol and supplied buffers. Samples with > 200 pmol of dye incorporation and a nucleotide to dye incorporation ratio < 40 were retained. The probes derived from an untreated sample and a CSP-treated sample from the same culture were then combined and dried to completion.

Microarray hybridization. Microarray slides for *S. sanguinis* were obtained from the J. Craig Venter Institute (JCVI), and contained unique 70-base oligonucleotides representing internal regions of all 2274 ORFs predicted from the genome sequence of *S. sanguinis* (Xu et al., 2007). All oligonucleotides were printed in quadruplet. Slides were pretreated for 2 – 3 h at 42ºC in 50 µl of prehybridization solution (5x SSC, 0.1% SDS, 1% BSA) in hybridization cassettes (ArrayIt®, Sunnyvale, CA). The slides were then washed three times with MilliQ water in a High Throughput Wash Station (ArrayIt®) followed with one wash in isopropanol and dried using a
mini-slide centrifuge (Labnet International, Edison, NJ). Dried probes were then resuspended in 50 µl hybridization buffer (40% formamide, 5x SSC, 0.1% SDS, 0.6 µg/µl salmon sperm DNA). The solution was heated at 95°C for 10 min while vortexing intermittently and then transferred onto pretreated slides under a LifterSlip™ (Erie Scientific, Portsmouth, NH). The probes were allowed to hybridize for 16 – 20 h at 42°C in a hybridization cassette. Humidity was maintained with the addition of 15 µl of unused hybridization buffer in each well. Slides were washed twice in 2x SSC, 0.1% SDS, 0.1 mM DTT at 55°C for 10 min, twice at room temperature in 0.1x SSC, 0.1% SDS, 0.1 mM DTT for 5 min, and twice at room temperature in 0.1x SSC, 0.1 mM DTT for 5 min. The slides were then dipped in dH2O before being dried in a mini-slide centrifuge. Microarrays were immediately scanned in an Axon GenePix® 4200 scanner (Molecular Devices, Sunnyvale, CA).

Array analysis. Data were collected and analyzed using GenePix Pro 6.0 (Molecular Devices). Each spot on the array was individually checked and flagged if it contained one of the following problems: intensity of the signal < two-fold that of the local background level; high background streaks; or if > 25% of the spot contained poor hybridization where signal was undetectable. Once all values were collected, they were submitted to the Ramhorn Array Database (RAD) (http://ramhorn.csbc.vcu.edu/ilat/), where the spots were filtered based on three user-defined parameters. Firstly, the regression correlation for each spot had to be greater than 0.5. Secondly, the mean of the red channel (experimental values) normalized to the green channel (control values) had to be 1.8-fold greater than the normalized median values of the background for the red channel. Lastly, the mean values of the green channel had to be 1.8-fold greater than the median values of the background. This allows a more unbiased method of filtering out elements that passed through the initial user-based filtering process as described above. Next, genes were deleted from the analysis if ≥25% of the spots for that gene across all time points were filtered out. In order to determine the significance of apparent changes of expression, the Significance Analysis of Microarray (SAM) method (Tusher et al., 2001) was applied for each remaining gene. For this study, a threshold was used so that the false discovery rate (FDR) was < 0.05. Once genes with significant changes in expression were identified for each time point, genes with expression changes that were < 2-fold at all time points were deleted. The remaining genes were analyzed using Cluster 3.0 (Eisen et al., 1998) and visualized using TreeView (Eisen et al., 1998).

References

