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ORIGINAL ARTICLE

Gene expression profile of early *in vitro* biofilms of *Streptococcus pneumoniae*

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ABSTRACT

In this study, the gene expression profile of early *in vitro Streptococcus pneumoniae* biofilm with respect to planktonic cells in cDNA microarray analysis is reported. Microarray analysis with respect to planktonic cells was performed on total RNA extracted from biofilms grown in 24-well microtiter plates. To validate the microarray results, real-time RT-PCR was performed on 13 differentially expressed genes and one constitutively expressed gene. The cDNA-microarray analyses identified 89 genes that were significantly differentially expressed in biofilm and planktonic cells. Genes involved in isoprenoid biosynthesis, cell wall biosynthesis, translation and purine and pyrimidine nucleotide metabolic pathways were exclusively expressed in the biofilms, whereas transcription regulator genes were exclusively expressed in planktonic cells. The real-time RT-PCR results of 13 differentially regulated genes were completely in agreement with the microarray data. The exclusive up regulation in biofilms of genes involved in the mevalonate pathway, cell wall biosynthesis, translation and purine and pyrimidine nucleotide metabolic pathways suggests that expression of these genes may be required for initial biofilm formation, and growth and survival of bacteria in biofilms. The up regulation of related genes suggests that cells in biofilms may be under stress conditions and possibly actively involved in the protein synthesis required to adapt to a new environment.

Key words biofilm; gene expression; microarray; *Streptococcus pneumoniae*.

World-wide, otitis media is the commonest illness for which children visit a physician, receive antibiotics, or undergo surgery (1, 2). The bacteria most often associated with OM are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (3). *S. pneumoniae* asymptomatically colonizes up to 50% of children (4, 5). Colonization of the upper respiratory tract is the first step in infection; even transient colonization provides an opportunity for *S. pneumoniae* to invade the middle ear space.

In humans, *S. pneumoniae* biofilm formation has been shown to occur during nasopharyngeal colonization and recurrent OM. Indeed, direct detection of biofilms in the nasopharyngeal cavity, the middle ear mucosa of children with recurrent or chronic OM, and in animal studies, and their indirect detection in pneumonia and meningitis, has been reported (6–11). Moreover, biofilm resembling that formed by other OM pathogens is reportedly present in *S. pneumoniae* infections in chinchillas (12). Biofilm pneumococci display gene expression profiles similar to

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List of Abbreviations: ABC, ATP binding cassette; CcpA, catabolite control protein A; DMAPP, dimethylallyl diphosphate; IMP, inosine monophosphate biosynthesis; IPP, isopentenyl diphosphate; OM; otitis media; *S. pneumoniae, Streptococcus pneumoniae;* SSC, saline-sodium citrate; TE, Tris-EDTA; TSB, tryptic soy broth.

those of bacteria isolated from the lungs of mice; these profiles are distinct from planktonic bacteria isolated from either blood or culture media (13).

Studies by Muñoz-Elías *et al.*, Parker *et al.* and Trappetti *et al.* indicate that the genes required for robust biofilm formation *in vitro* are important for nasopharyngeal colonization and, in some instances, progression towards lung disease (14–16). In contrast, studies by Tapianen *et al.*, Camilli *et al.*, Lizcano *et al.*, and Sanchez *et al.*, reported no correlation between the ability of isolates to form robust biofilms *in vitro* and their virulence potential in humans and mice (17–20).

Furthermore, Lizcano *et al.* reported that the ability to form early biofilms *in vitro* does not reflect virulence potential (18). Therefore, investigation of the gene expression profile of early *in vitro* biofilms is an important subject. To find out more about pneumococcal genes that are associated with adherence in early biofilms, we utilized a microarray approach to study pneumococcal gene expression changes in biofilm and planktonic cells. Here, we report development of a *S. pneumoniae* cDNA microarray of early biofilm formation in a static model.

MATERIALS AND METHODS

Bacteria strains

Streptococcus pneumoniae R6 strain (BAA-255), which was obtained from the American Type Culture Collection (Manassas, VA, USA) is an unencapsulated and avirulent strain derived from encapsulated serotype 2 pathogenic strain D39. The bacteria were grown routinely in TSB or on blood agar plates supplemented with 5% v/v sheep blood at 37°C in an atmosphere of 5% CO_2.

In vitro biofilm formation

In vitro biofilm formation was carried out in 24-well, flat-bottom polystyrene microtiter plates (BD Falcon, Sparks, MD, USA) in a static model by a procedure described previously (13, 21). Briefly, *S. pneumoniae* grown up to mid-logarithmic phase in TSB medium was diluted 1:100 with fresh sterile TSB medium supplied with 1% glucose, inoculated in 1.5 mL aliquots in a 24-well microtiter plate and incubated for 15 hr at 37°C in 5% CO_2. After incubation, the medium was discarded, and the plates gently washed three times with 1.5 mL sterile, cold PBS. Adherent cells were scraped off and immediately processed for RNA extraction. For RNA extraction from planktonic cells, 5 mL of mid-logarithmic phase cell suspension was pelleted by centrifugation, washed three times with sterile PBS, and immediately processed for RNA extraction.

RNA extraction and cDNA synthesis

Cell-lysis was carried by incubation of cell pellets in 100 uL (3 mg/mL in TE) lysozyme (Sigma-Aldrich, St. Louis, MO, USA) for 4 mins. Total RNA extraction of pneumococcal biofilm cells and planktonic cells were carried out by using a RNeasy Total RNA Isolation System kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions with few modifications. On column RNAase-free DNAse (Qiagen) treatment was performed for 10 mins at 20–25°C. The integrity of bacterial total RNA was checked by capillary electrophoresis Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). cDNA synthesis was carried out using a ImProm-II Reverse Transcriptase kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, tailing of RNA with a random hexamer primer was performed at 70°C for 5 mins, annealing at 25°C for 5 mins, extension at 37°C for 1 hr and inactivation of samples at 70°C for 15 mins.

Development of *Streptococcus pneumoniae* cDNA microarray

In situ synthesis microarray was used for analysis of 2025 genes of *S. pneumoniae* R6 strain in 21 replicate spots. The cDNA probes for microarray analysis were prepared by reverse-transcription of total RNA (25 μg) in the presence of aminoallyl-dUTP and 6 μg of random primers (Invitrogen, Carlsbad, CA, USA) for 3 hrs. The cDNA probes were cleaned using a Microcon YM-30 column (Millipore, Bedford, MA, USA) followed by coupling of Cy3 dye (planktonic samples) or Cy5 dye (biofilm samples) (Amersham Pharmacia, Uppsala, Sweden). The Cy3 or Cy5-labeled cDNA probes were purified with a QIAquick PCR Purification kit (Qiagen). Dried Cy3 or Cy5-labeled cDNA probes were resuspended in hybridization buffer containing 30% formamide, 5X SSC, 0.1% sodium dodecyl sulfate, and 0.1 mg/mL salmon sperm DNA. The Cy3 or Cy5-labeled cDNA probes were mixed together and hybridized to a microarray slide (MYcroarray.com, Ann Arbor, MI, USA). After overnight incubation at 42°C, the slide was washed twice with washing solution 1 containing 2X SSC and 0.1% SDS for 5 mins at 42°C, once with washing solution 2 containing 0.1X SSC and 0.1% SDS for 10 mins at room temperature and finally four times with 0.1X SSC for 1 min at room temperature. The slide was dried by centrifugation at 225 *g* for 5 mins. The hybridization image on the slide was scanned by 4000B apparatus (Axon Instruments, Union City, CA, USA).

Microarray data analysis

Hybridization image was analyzed by GenePix Pro 3.0 software (Axon Instruments) to obtain gene expression

Table 1. Gene specific primers used for real-time RT-PCR analysis

Gene locus and genes	Forward primer sequences	Reverse primer sequences	Amplicon size (bp)
spr1703- *amiF*	5′ -GCTGAAAGCAAGGCTTGAGTA-3′	5′-ACTTGTCGGTTGTTCGCTTT-3′	130
spr1123- *ftsY*	5′-TCGAAAATTCTTTGGCCTGT-3′	5′-ATCAAACGTGTTGTGCCAGA-3′	97
spr1571- *mvaS*	5′-ATAGGGCAGTTCGTTGGTTG-3′	5′-AGTGGAGCTGTGGCTGAGTT-3′	103
spr0516- *pnpA*	5′-TCGAGAAATTTGGTGCCTTT-3′	5′-ACGAGTCCAAGCCATCTCAG-3′	80
spr0102- *argG*	5′-AAATCGCTTGGTTGGGATTA-3′	5′-CACAAGCGTCAAGTCCTCAA-3′	100
spr039- *mvd1*	5′-GGCTATGCATGCTACGACAA-3′	5′-CGAACAAAGTCCATAGCCTCA-3′	84
spr0049- *purN*	5′-TCCAGCCTACTTGCCAGAAT-3′	5′-CCAGTGAATGGTCACACCAG-3′	91
spr0588- *miaA*	5′-GAGCGTATCAACCACCGAGT-3′	5′-TAGGGGAATGGTCAAACAGC-3′	82
spr1823- *pbp2a*	5′-GGTGCAATTCCATTTTGCTT-3′	5′-CGGTGCAGCTCATGTCTTTA-3′	105
spr0584- *glcK*	5′-TGAAAAAGGAGAATATGATGAGTCAA-3′	5′-TGCAAATTTGATAGAAGTTCCAC-3′	71
spr1480- *marR*	5′-TGACCCCAAACAAAGAAGAC-3′	5′-CCGCAATTTCCTTGTTGGTA-3′	84
spr1608- *prmA*	5′- ATGGAAACATGGCAAGAGTT-3′	5′- GCTCCCAGCTCAATCAAGAG-3′	83
spr1813- *ccpA*	5′- GACAGGAAAAGGAATGAATGC-3′	5′- GGAAACACCTGCTTCACGAG-3′	70
spr0715- *gyrB*	5′-GATTTGGCGCAGAATTTGAT–3′	5′-GTACGAATGTGGGCTCCATC-3′	91

ratios (planktonic vs biofilm samples). Microarray data analysis was carried out by Genowiz 4.0 (Ocimum Biosolutions, Hyderabad, India). Global Lowess was used for data analysis with normalization. The benchmarks for up regulated and down regulated genes in each hybridization were + 1.5-fold and - 1.56-fold, respectively. The microarray experiment was performed with three biological replicates samples and statistical significance assessed with Student's *t*-test, $P < 0.05$ being classified as significant. The functional annotation clustering tool of the Database for Annotation, Visualization, and Integrated Discovery (available from URL: http://david.abcc.ncifcrf.gov) and UniPortKB database, (available from URL: http://www.uniprot.org/uniprot/P0A4M0) was used to search for clusters of biological processes of the gene ontology database within two sets of differentially expressed genes, namely biofilm versus planktonic. To comply with the minimum information about a microarray experiments requirement, microarray data have been deposited in the NCBI gene expression omnibus database (available from URL: http://www.ncbi.nlm.nih.gov/geo) under accession number GSE34751.

Real-time reverse transcriptase polymerase chain reaction analysis

To confirm microarray data by real-time RT-PCR, 13 differentially expressed genes in biofilm along with *gyrB* as a control gene were chosen. Primers were designed by standard procedures from nucleotide sequence of *S. pneumoniae* R6 strain (obtainable from URL: http://www.ncbi.nlm.nih.gov/nuccore/NC˙003098) (Table 1). Real-time RT-PCR was carried out in a total volume of 20 μL, consisting of 10 μL 2X SYBR Green PCR Master Mix (Roche Applied Science, Indianapolis, IN, USA), 5 pmol of forward and reverse primers, and 2 μL cDNA. PCR conditions included initial denaturation at 95°C for 10 mins, followed by 45 cycles of denaturation (95°C for 15 s), annealing (57°C for 10 s), extension (72°C for 15 s) and final extension (72°C for 5 mins) followed by melting curve analysis from 60–95°C. Negative controls containing nuclease-free water instead of RNA were run concomitantly to confirm that the samples were free from contamination. To verify the absence of contaminating genomic DNA, each RT-PCR experiment included a no reverse transcriptase control. Relative gene expression was analyzed using the $2^{-\Delta\Delta CT}$ method (22). The reference gene was *gyrB* and the standard condition was planktonic cells grown up to exponential phase in TSB medium supplied with 1% glucose.

Statistical analysis

Statistical analysis was carried out with Student's *t* test and *P* value < 0.05 was classified as significant.

RESULTS

Microarray analysis

cDNA-microarray analysis identified 89 genes that were significantly differentially expressed in biofilm and planktonic cells (Table 2 and 3). Functional group analysis categorized 43 (almost 50%) of these genes as uncharacterized and hypothetical. Of the 46 protein coding genes, 34 showed an increase in expression (Table 2) and 16 showed a decrease in expression in biofilm (Table 3). Among 39 hypothetical and conserved genes, 22 were up

Table 2. Genes up regulated in pneumococcal biofilms detected by microarray and real-time RT-PCR

Gene locus and gene	Annotation	Fold changes in biofilm (detected by microarray)	#*P*-value	Fold changes in biofilm (detected by real-time RT PCR)	#*P*-value
spr0102- *argG*	Arginine biosynthetic process	2.00	0.0223	2.0	0.03
spr0588- *miaA*	tRNA processing	1.92	0.0052	2.8	0.02
spr1703- *amiF*	Protein transport	1.92	0.0267	2.1	0.03
spr0517- *cysE*	Transferase activity	1.88	0.0082		
spr1397- *asnS*	Translation	1.88	0.0105		
spr0139- *ugd*	UDP-glucose dehydrogenase	1.88	0.0317		
spr0023- *dut*	dUTP metabolic process	1.75	0.0431		
spr0878 -*rnr*	RNA metabolic process	1.72	0.0287		
spr1789 -*acyP*	Pyruvate metabolism	1.72	0.0440		
spr1891- *IS1381*	Transposition	1.72	0.0134		
spr1123 -*ftsY*	SRP-dependent cotranslational protein targeting to membrane	1.72	0.0072	2.4	0.04
spr1947 -*IS1381*	Transposition	1.70	0.0098		
spr1823 -*pbp2a*	Cellular wall organization	1.64	0.0054	2.3	0.03
spr1311 -*trmH*	RNA binding	1.63	0.0167		
spr0516- *pnpA*	RNA processing	1.62	0.0374	2.0	0.02
spr0049- *purN*	'de novo' IMP biosynthetic process	1.61	0.0155	2.2	0.01
spr0208 -*rplO*	Translation	1.61	0.0494		
spr1573 -*IS1381*	Transposition	1.61	0.0232		
spr0064 -*agaS*	Carbohydrate metabolic process	1.60	0.0241		
spr0245 -*glmS*	Glutamine metabolic process	1.60	0.0064		
spr0713- *thiJ*	4-methyl-5(B-hydroxyethyl)-thiazole monophosphate biosynthesis protein	1.60	0.0294		
spr0762 -*pcp1*	Proteolysis	1.57	0.0350		
spr1396- *rpsF*	Translation	1.57	0.0183		
spr1704- *amiE*	Protein transport	1.57	0.0219		
spr0215 -*rpoA*	DNA binding	1.55	0.0388		
spr0527 -*pep27*	Secreted peptide, which is the signal sensed by VncR/S	1.55	0.0178		
spr1861 -*cglD*	Competence	1.55	0.0150		
spr1648 -*galT*	Galactose metabolic process	1.55	0.0129		
spr0914- *hemH*	Heme biosynthetic process	1.54	0.0195		
spr0339 -*mvd1*	Isoprenoid biosynthetic process	1.54	0.0465	3.1	0.01
spr0403 -*ilvC*	Branched chain family amino acid biosynthetic process	1.52	0.0266		
spr1909 -*pbp1b*	Cellular wall organization	1.52	0.0364		
spr1571-*mvaS*	Isoprenoid biosynthetic process	1.52	0.0196	2.9	0.01

#Statistical analysis was carried out by Student's *t*-test from the findings on three independent RNA samples. *P* value < 0.05 was classified as significant.

regulated in biofilm and 17 down regulated. Genes associated with many functional categories were differentially regulated in biofilm and planktonic cells (Fig. 1). These included genes involved in purine and pyrimidine nucleotide metabolism, RNA/DNA metabolism, amino acid transport and metabolism, translation, transporter protein, carbohydrate transport and metabolism, cell wall biosynthesis, isoprenoid biosynthesis, transcription regulation, and cellular process (Table 2 and 3). Functional annotation showed that the genes of certain functional categories such as isoprenoid biosynthesis, cell wall biosynthesis, translation and purine and pyrimidine nucleotide metabolic pathways gene were exclusively expressed in biofilm, whereas the transcription regulator genes were exclusively expressed in planktonic cells (Table 2 and 3).

Cell wall biosynthesis protein coding genes

Microarray and real-time RT-PCR detected up regulation of the two cell wall protein coding genes, *pbp2a* and *pbp1b,* in biofilms. These two genes encode enzymes required for the biosynthesis of peptidoglycan through an amino sugar metabolic pathway.

Table 3. Genes down regulated in pneumococcal biofilm detected by microarray and real time RT-PCR

Gene locus and gene	Annotation	Fold change in biofilm (detected by microarray)	#*P*-value	Fold change in biofilm (detected by real-time RT PCR)	#*P*-value
spr1556 -*dnaB*	DNA replication	−2.00	0.042339		
spr0584- *glcK*	Kinase activity	−1.91	0.014745	−2.1	0.02
spr1505 –*divIVA*	Cell division	−1.90	0.036878		
spr1813 -*ccpA*	Sequence-specific DNA binding transcription factor activity	−1.90	0.010418	−2.7	0.01
spr1608 -*prmA*	Protein methyltransferase activity	−1.80	0.025147	−2.2	0.02
spr1578- *pppL*	Hydrolase	−1.79	0.046817		
spr1971- *fucU*	Monosaccharide metabolic process	−1.75	0.024642		
spr0665- *prfB*	Translation release factor activity, codon specific	−1.73	0.016231		
spr1425-*aldR*	Transcription regulator	−1.68	0.042948		
spr1040- *rexA*	Double-strand break repair exonuclease activity	−1.67	0.027213		
spr1958 -*arcC*	Carbamate kinase activity	−1.62	0.033791		
spr1480 -*marR*	Sequence-specific DNA binding transcription factor activity	−1.61	0.004306	−2	0.04
spr0623 -*glnP*	Integral to membrane transporter activity	−1.57	0.015959		
spr0463 -*rr13*	two-component response regulator activity	−1.56	0.018633		
spr0842 -*IS1381*	Transposition	−1.52	0.015867		
spr1936- *tktC*	Catalytic activity	−1.51	0.042128		

#Statistical analysis was carried out y Student's *t*-test from three independent RNA samples. *P* value < 0.05 was classified as significant.

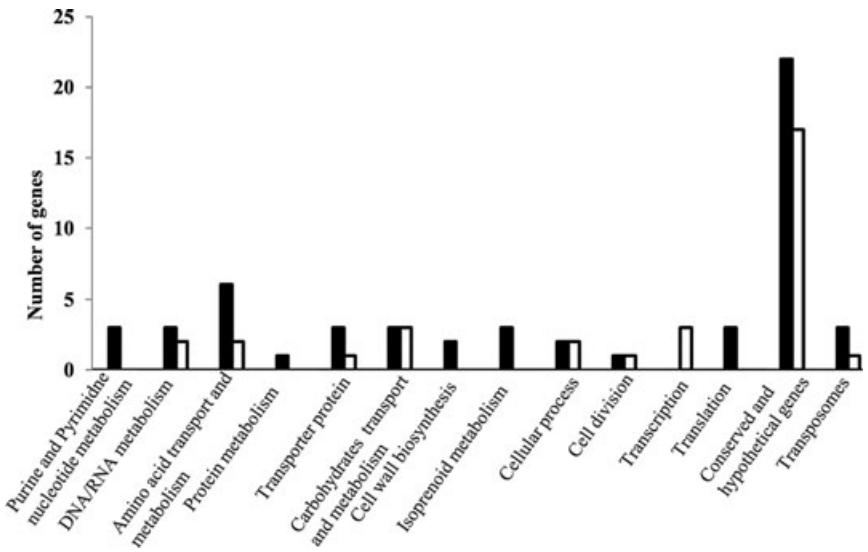

Fig. 1. Functional classification of genes expressed differentially in biofilm and planktonic cells.

Purine and pyrimidine nucleotide metabolism coding genes

Our microarray and real-time RT-PCR results showed up expression of a cluster of three genes associated with ribonucleotide biosynthesis in *S. pneumoniae* biofilms: *dut* codes for deoxyuridine 5′-triphosphate nucleotidohydrolase, which functions in dUTP metabolism; *pnpA* encodes for poly-nucleotide phosphorylase, having function in RNA processing and binding; whereas *purN* encodes a protein involved in *de novo* inosine monophosphate biosynthesis. The latter gene has been implicated in the *de novo* purine biosynthetic pathway responsible for IMP synthesis.

Isoprenoid metabolism

The two important isoprenoid metabolism pathway genes (*mvaS* and *mvd1*) were up regulated in *S. pneumoniae*

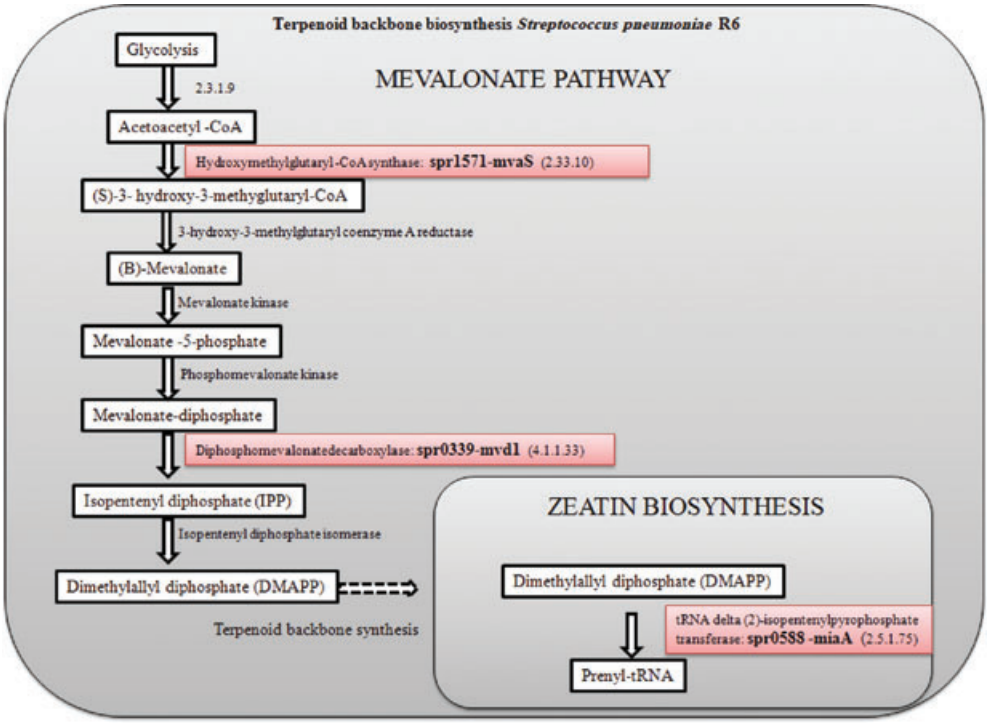

Fig. 2. Isoprenoid biosynthesis pathway in *Streptococcus pneumoniae*.

biofilms. The fold increase in gene expression was 2.9 ($P = 0.01$) and 3.0 ($P = 0.01$) respectively. *mvaS* and *mvd1* encode hydroxymethylglutaryl-CoA synthase and diphosphomevalonate decarboxylase, respectively, which function in isoprenoid biosynthesis through the mevalonate pathway (Fig. 2). Another gene up regulated by 2.8 fold ($P = 0.02$) in the biofilms was *miaA*, which encodes tRNA delta 2-isopentenylpyrophosphate transferase protein. The protein functionally connects the mevalonate pathway with the zeatin biosynthesis pathway in *Escherichia coli* by catalyzing the addition of a Δ^2-isopentenyl group from dimethylallyl diphosphate to the N^6-nitrogen of adenosine adjacent to the anticodon at position 37 in *E. coli* tRNA.

Translation related genes

The three genes (*rpsF*, *rplO* and *asnS*) involved in translation were up regulated in *S. pneumoniae* biofilms. The up-expression of these ribosomal genes ($P < 0.05$) indicates that *S. pneumoniae* in biofilm are actively involved in protein synthesis.

Transcription regulatory protein coding genes

The three regulatory proteins encoding genes (*ccpA*, *rr13* and *marR)*, were exclusively up regulated in planktonic cells. *ccpA* and *rr13* were up regulated by more twofold ($P < 0.05$) and encode negative repressor proteins, which reduce gene expression under normal growth conditions. *ccpA* encodes catabolite control protein A, which is a negative repressor protein with a regulatory role in carbohydrate metabolism. *rr13* codes for a response regulator protein of a two-component system, while the m*arR* gene encodes an iron-dependent transcriptional regulator protein.

Transporter proteins

The virulent related genes *cglD*, *amiF*, *amiE* and *glnP* were differentially regulated in biofilms and planktonic *S. pneumoniae*. *cglD* encodes a competence protein. *amiF* and *amiE* encode oligopeptide ABC transporter proteins that each have an ATP-binding protein domain and function in peptide transport. All three genes were up regulated in biofilms. *glnP* gene encodes for amino acid ABC transporter permease protein; this gene was up regulated in planktonic cells.

Real-time reverse transcriptase polymerase chain reaction assay

To validate the results of microarray, we performed real-time RT-PCR on 13 differentially expressed genes and one constitutively expressed gene from six different functional

groups. We calculated the fold-changes in gene expressions after normalization of each gene relative to the constitutively expressed gene *gyrB* using the comparative threshold method (22). The real-time RT-PCR results of 13 differentially regulated genes were completely in agreement with the microarray data (Tables 2 and 3).

DISCUSSION

The ability of *S. pneumoniae* to persist in the nasopharynx and cause chronic disease under appropriate conditions may be associated with its ability to form biofilms on mucosal epithelium (6, 23, 24). The sessile bacteria in a biofilm differ both physiologically and metabolically from their planktonic counterparts, as well as demonstrating tissue specific gene expression of a wide variety of virulence and pathogenic genes (25–28). Moreover, Oggioni *et al*. have demonstrated that the transcriptional profile of several known virulence-related genes in *S. pneumoniae* isolated from lungs and brains of infected mice is similar to that of those in *in vitro* biofilms and that the gene expression profile of pneumococci in blood is similar to planktonic bacteria in mid-log phase in TSB medium (13). In this study, we utilized a microarray approach to study pneumococcal gene expression changes in biofilm and planktonic cells.

The microarray analysis detected 89 genes that were differentially expressed in biofilm and planktonic cells. The real-time RT-PCR results of 13 differentially regulated genes were completely in agreement with the microarray data. The overall expression pattern demonstrated that more genes are up regulated in biofilm than in planktonic cells. The functional annotation demonstrated that three functional group genes are exclusively expressed in biofilms, and one cluster is exclusively up regulated in planktonic cells.

The up regulation of peptidoglycan biosynthesis genes and ribosomal genes demonstrates that, in *S. pneumoniae* biofilm, cells are growing and may be involved in protein synthesis for various processes, unlike in planktonic cells. The down regulation of ribosomal protein genes in planktonic cells indicates that free-floating *S. pneumoniae* has reduced transcription capacity (26).

Moscoso *et al*. reported that glucose supplementation of a semisynthetic medium best allows biofilm formation, while limiting the typical autolytic behavior of pneumococci, and that *S. pneumoniae* R6 strain is a strong biofilm producer (29). In this study, we used TSB medium supplied with 1% glucose for biofilm growth. Here, we detected up regulation of ribonucleotide biosynthesis genes (*dut*, *pnpA* and *purN*) in biofilm cells. The *pnpA* gene of *S. pneumoniae*, which encodes polynucleotide phosphorylase, has not been investigated in detail. However, in *Streptococcus mutans*, PnpA is reportedly up regulated under stress conditions such as acidic pH, and is significant for cell viability and mRNA turn over in *E. coli* (30, 31). Moreover, *pnpA* gene expression is unchanged in transcriptional analysis of *S. pneumoniae* R6 strain in response to acid tolerance (32). *purN* is up regulated when incubated in animal blood (26) or when treated with a sublethal concentration of antibiotic (33). Our results suggest that biofilm cells are possibly under stress conditions and that stress-related genes are up regulated to support biosynthesis of new nucleic acid.

In this study, one striking observation was up regulation of the two isoprenoid biosynthesis genes, *mvaS* and *mvd1*, which encode hydroxymethylglutaryl-CoA synthase and diphosphomevalonate decarboxylase, respectively. The polyisoprenoid compound undecaprenyl phosphate is required for biosynthesis of cell wall peptidoglycan in Gram-positive bacteria. *mvaS* encodes a protein that catalyzes synthesis of 3-hydroxy-3-methylglutaryl-coenzymeA by 3-hydroxy-3-methylglutaryl-coenzymeA synthase, and is the first and rate-limiting enzyme in isopentenyl diphosphate synthesis. *mvd1* catalyzes diphosphomevalonate decarboxylase, which functions in the fifth step of decarboxylation of diphosphomevalonate to IPP. Entry of IPP and DMAPP to the zeatin biosynthesis pathway is catalyzed by MiaA, which encodes tRNA delta 2-isopentenylpyrophosphate transferase catalysis (34). These mevalonate pathway enzymes are essential for the survival of *S. pneumoniae* in lungs and serum (35), and are essential in the other Gram-positive cocci, since they also lack the genes predicted to encode the enzymes of the GAP-pyruvate pathway. Disruption of the individual genes encoding HMG-CoA reductase and *mvaS*, which catalyzes the production of mevalonate, prevents growth of *S. pneumoniae* in the rat lung and results in a growth requirement for concentrations of mevalonate that far exceed those found in serum (35, 36). The recent discovery that diphosphomevalonate, an intermediate in the mevalonate pathway, potently and allosterically down regulates the activity of *S. pneumoniae* mevalonate kinase without inhibiting the human enzyme (37) provides an opportunity for developing a new class of antimicrobials that are capable of killing this bacterium without detriment to the host.

The finding of up regulation of three regulatory proteins encoding genes (*ccpA*, *rr13* and *marR)* in planktonic cells is similar to the finding of a previous report (26). *S. pneumoniae* adapts to changing growth conditions through catabolite repression mediated by CcpA. In a previous study, CcpA was reported to be required for colonization of the nasopharynx and survival and multiplication in the lung (38). MarR proteins regulate aromatic catabolism, expression of virulence factors and the

response to antibiotic, antimicrobial stress and oxidative stress (39, 40). In a previous study, *marR* mutants demonstrated a strain specific role in adherence to A549 cells (41). *rr13* encodes a response regulator protein of a two-component system. Two-component signal transduction systems enable bacteria to sense, respond and adapt to changes in their environment or in their intracellular state.

The transporter protein coding genes *cglD, amiF* and *amiE* are up regulated in biofilms. The cglD gene encoding proteins belongs to the Cgl family of proteins and is important in the production of competence in *S. pneumoniae* (42). In addition, *amiF* and *amiE* encode oligopeptide ABC transporters, whose ATP-binding protein domains function in competence. Similar up regulation of *amiF* gene in meningitis bacteria has been reported (26). The up regulation of these competence and peptide transporter genes indicates that cells in biofilms may be involved in natural competence.

Our study detected a few virulence and pathogenic genes whose expressions were up regulated in *in vitro* early biofilms. Similarly, previous studies have demonstrated that early biofilm formation (less than 18 hrs) on microtiter plates is unable to express many virulence and pathogenic gene, and may not correlate with invasive disease potential (18, 20). However, the gene expression patterns of most biofilm genes are close to previous gene expression reports *in vivo* (26) and *in vitro* (13). This study reports a few genes that are unique to early *in vitro* biofilms. Moreover, many studies have demonstrated strain-specific gene regulation in *S. pneumoniae* (41, 43), gene expressions differing with the richness of growth media, bacteria seeding, and strain capsulation, factors which affect biofilm formation on microtiter plates (9,13, 14, 29, 44).

In conclusion, the exclusive up regulation in biofilms of genes involved in the mevalonate pathway, cell wall biosynthesis, translation and purine and pyrimidine nucleotide metabolic pathway suggests that expression of these genes may be required for initial biofilm formation, and growth and survival of bacteria in biofilms. The cells in biofilms may be under stress conditions and actively involved in the protein synthesis required to adapt to the new environment. The significant up regulation of genes encoding mevalonate pathway enzymes indicates that these genes may be essential for *in vitro* early biofilm formation and should be the subject of further investigation.

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DISCLOSURE

The authors report that they have no financial relationships or interests to disclose.

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