Biofilm formation and induction of stress response genes is a common response of several serotypes of the pneumococcus to cigarette smoke condensate

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Running title: Pneumococcal stress responses to smoking

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Summary

Objectives: Exposure to cigarette smoke impacts on the virulence of *Streptococcus pneumoniae* (pneumococcus) by mechanisms including induction of biofilm formation. Most studies, however, have focused on individual strains of the pneumococcus. Accordingly, the current study has investigated the commonality of the pneumococcal stress response to cigarette smoke condensate (CSC), using five different strains of the pathogen.

Methods: Following exposure to CSC at final concentrations of 80 and 160 $\mu g.mL^{-1}$ during a 16 hour incubation period, biofilm formation was measured using a crystal violet-based spectrophotometric procedure. Expression of stress genes seemingly linked to biofilm formation *viz. hk11* and *rr11* [histidine kinase and response regulator of the two-component regulatory system 11 (TCS11) respectively], *cat eff* (cation efflux system protein), *abc* (ATP-binding component of an ATP-binding cassette transporter) and *2005-hyp* (hypothetical gene) was measured by sequential extraction of RNA, cDNA synthesis and real-time qPCR.

Results: Exposure of all five strains of the pneumococcus to CSC, resulted in significant biofilm formation, as well as induction of all five test stress genes.

Conclusions: Augmentation of induction of selective stress genes and biofilm formation are common, possibly linked, responses of various serotypes of the pneumococcus to CSC, favouring both persistence of the pathogen and decreased efficacy of antibiotics.

Keywords: ATP-binding cassette transporter; biofilm; cation efflux; cigarette smoke condensate; pneumococcus; serotype; *Streptococcus pneumoniae*; stress genes; two component regulatory system 11.

Introduction

Cigarette smoking is well recognized as being a prominent, independent risk factor for the development of invasive pneumococcal disease (IPD).[1] In addition, smokers who develop pneumococcal pneumonia have a 5-fold increased risk of mortality, irrespective of age, comorbidities and early implementation of guideline-compliant antimicrobial chemotherapy.[2] Smoke-mediated impairment of pulmonary host defences is considered to be a major cause of increased susceptibility for development of severe pneumococcal infection.[3] However, we have previously reported that direct exposure of two strains of the pneumococcus (both serotype 23F) to cigarette smoke condensate (CSC) *in vitro* also impacts significantly on the virulence of this dangerous pathogen.[4,5] In this setting, we have observed that brief exposure of the pneumococcus to CSC results in the induction of a limited number of stress-related genes, which precedes formation of biofilm.[5] Biofilm is a self-generated, extensively-hydrated, extracellular, polymeric matrix comprised mostly of nucleic acids, polysaccharides and proteins. Concealed in biofilm in a quiescent state, the pneumococcus can persist in the respiratory tract, withstanding immune- and antibiotic-mediated assault, re-emerging opportunistically to cause invasive disease when the host immune system is weakened.

In our earlier study, the limited set of genes upregulated following exposure of the pneumococcus to CSC included those encoding the two-component regulatory system (TCS) 11, comprising the sensor kinase and response regulator genes (*hk11,SP2001*, and *rr11,SP2000*, respectively), as well as those encoding the *cat eff* (*SP1857*; cation efflux system protein), *abc* (*SP2003*; ATP-binding component of an ATP-binding cassette transporter) and *2005-hyp* (hypothetical) genes.[5] These findings were recently confirmed by Manna *et al.* who noted upregulation of

expression of the genes comprising TCS11 following exposure of two strains of the pneumococcus, belonging to serotypes 16F and 19F, to cigarette smoke *in vitro.*[6] In this context, it is noteworthy that TCS11 has been linked, directly or indirectly, to biofilm formation by *S. mutans*[7] and possibly the pneumococcus.[8] To date, however, only the studies reported by Mutepe *et al*.[4] and Cockeran *et al*.[5] have focused on the sequential effects of exposure of the pneumococcus to CSC on gene expression and biofilm formation, albeit using only two strains of the pathogen (strains 172 and 3328, both serotype 23F).

This limitation of our earlier studies has been addressed in the current study, in which stress gene expression and biofilm formation were measured following exposure to CSC of five different strains of the pneumococcus, encompassing three of the most common disease-causing serotypes, 6A, 19F and 23F.[9,10]

Materials and methods

Bacterial strains

Five different strains of the pneumococcus, all clinical isolates, were provided by the National Institute of Communicable Diseases, Johannesburg, South Africa. These strains with serotypes and multi-locus sequence types (MLSTs) shown in parenthesis were 172, 521 and 2507 (all serotype 23F; respective MLSTs: ST81, ST85, ST81), 4916 (serotype 6A; MLST: ST2289) and 49619 (serotype 19F; MLST not known), two of which were intrinsically macrolide resistant mediated via *mefA* gene (strain 521)- or *ermB* gene (strain 2507)-dependent mechanisms, while strain 49619 manifested moderate penicillin resistance. These strains were grown overnight in tryptone soy broth (TSB, Merck, Darmstadt, Germany) and adjusted

thereafter to concentrations of 6 x 10^6 or 2 x 10^8 colony-forming units (cfu).mL^{-1} for experiments designed to investigate the effects of exposure to CSC on biofilm formation and gene expression respectively.[5]

Cigarette smoke condensate (CSC)

CSC (Murty Pharmaceuticals, Lexington, KY, USA) was provided as a concentrate in dimethyl-sulphoxide (DMSO) at a concentration of 40 mg.mL^{-1} and used at final concentrations of 80 and 160 µg.mL^{-1} (biofilm production) or 160 µg.mL^{-1} only (gene expression). Solvent controls were included in all experiments, which equated to a final concentration of 0.4% for systems in which the CSC was used at a final concentration of 160 µg.mL^{-1}. The total amount of condensate generated during the combustion of one cigarette is 26.3 milligrams.[11]

Gene expression analysis

This procedure involved sequential isolation of bacterial RNA, conversion to and amplification of cDNA, and detection with real-time reverse transcription polymerase chain reaction (RT-qPCR).

Bacteria

Following exposure of all five strains of the pneumococcus to DMSO (control system) or CSC (160 µg.mL^{-1} only) for 15 or 60 min in TSB, the bacterial cells were concentrated by centrifugation and the pellets snap frozen in liquid nitrogen and stored at -80°C until extraction. Three separate experiments were performed on different days for each treatment.

RNA extraction

RNA extraction was performed in a two-step process, firstly by disintegrating the bacterial cells using lysozyme (Roche, Basel, Switzerland), glass beads (Sigma-Aldrich) and a TissueLyser (Qiagen, Hilden, Germany) followed by isolation of the bacterial RNA using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Any contaminating DNA was removed by treatment of the isolated RNA with the RNase-free DNase 1 (New England Biolabs, Ipswich, MA) followed by an RNA clean-up using the RNeasy mini kit (Qiagen). The RNA concentration was measured spectrophotometrically using a Nanodrop 2000™ (ThermoScientific Inc., Waltham, MA, USA) and stored at -80°C. The quality of the extracts was assessed by resolving 1 µL RNA aliquot on a 3% agarose gel.

cDNA synthesis

The high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) was used according to the manufacturer's instructions for the generation of the cDNA. Briefly, 10 µL of reverse transcription reaction mixture was prepared by adding 2.1 µL Best Quality Water SABAX (Adcock, Johannesburg, SA), 1 µL RT buffer, 0.4 µL 25x deoxynucleotide (dNTP) mix (100 mM), 1 µL 10x random primers, 0.5 µL reverse transcriptase and 5 µL of total RNA (100 ng.μL^{-1}). For non-reverse transcribed (NRT) negative controls, nuclease-free water was added as a substitute for the reverse transcriptase. Samples were incubated at 25°C for 10 min followed by incubation at 37°C for 120 min, enzyme inactivation at 85°C for 5 sec and a cooling step at 4°C using an MJ Mini Personal Thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Table 1: Primers for real-time PCR

Primer Name	Primer Sequence	Strains
SP_1857-Cation Efflux F	5’ – CATTGACTGGTTGCGGATGC – 3’	All
SP_1857-Cation Efflux R	5’ – CACCTTGGGCTATAAGCGGT – 3’	
SP_2000-RR11-F	5’ – GGGCAAGAAGCAATCCAAC – 3’	All
SP_2000-RR11-R	5’ – TCCACTCCAAGACTTCAAGAC – 3’	
SP_2001-HK11-F1	5’ – TTCCCCATCCTGTCTGTAG – 3’	172,2507
SP_2001-HK11-R1	5’ – AGGCAGTATTCCCAACTACAT – 3’	
SP_2001-HK11-F2	5’ – CTTTGACATCCGAACTAGAGAC – 3’	4916
SP_2001-HK11-R2	5’ – AATCATAGAAGCCATTGACTCC – 3’	
SP_2001-HK11-F3	5’ – TGGAAAATCTCAAATCACGGAC – 3’	521,49619
SP_2001-HK11-R3	5’ – TCAACCTCAACCTCAATCCC – 3’	
SP_2003-ABC-F1	5’ – ATCGTGACTCCTTGCGCTTT – 3’	172,2507,4916
SP_2003-ABC-R1	5’ – TCTTGACCTTGATTGGGCGA – 3’	
SP_2003-ABC-F3	5’ – CGCCCAATCAAGGTCAAAAC – 3’	521,49619
SP_2003-ABC-R3	5’ – CCAGCAACAAAAAGCCCAG – 3’	
SP_2005-Hyp-F	5’ – GCTCCTTTTTCACATCTCCGAC – 3’	All
SP_2005-Hyp-R	5’ – TGTTTTCGGGATTGGATGAGAGT – 3’	
SP_0806-gyrB-F	5’ – TCAGCCAAATCTGGTCGTAACCGT – 3’	All
SP_0806-gyrB-R	5’ – AATTCTGCGCCAAATCCTGTTCCC – 3’	
SP_1219-gyrA-F	5’ – AATCTTGCTCATACGTGCCTCGGT – 3’	All
SP_1219-gyrA-R	5’ – ATGGTGGAGCTACCGTTACATGCT – 3’	

Strain shows which primer pair was used for the indicated strain. “All” indicates that the same primer pair was used for all five strains.

PCR primers

Pneumococcal stress response gene expression was detected using real-time reverse transcription polymerase chain reaction (RT-qPCR). The forward and reverse primers for the test stress genes are shown in Table 1. The genes are: *hk11* (*SP2001*, TCS 11 sensor histidine kinase) and *rr11* (*SP2000*, TCS 11 transcriptional response regulator); ii), *cat eff* (*SP1857*, cation efflux system protein); iii) *abc* (*SP2003*, ATP-binding component of an ATP-binding cassette transporter); and iv) *2005-hyp* (*SP2005*, encodes a hypothetical protein). Gene expression was normalised against two reference genes *viz. DNA gyrase subunit A* (*SP1219*) and *DNA gyrase subunit B* (*SP0806*) designed using "Primer Quest DNA Technologies".[12]

Real time PCR

RT-qPCR was performed in duplicate using PowerUP ™ SYBR ™ Green master mix (Applied Biosysytems) on a CFX96 thermocycler (BioRad, Hercules, CA). Each reaction consisted of 10 ng cDNA; 1 X SYBR Green master mix and 200nM of each primer. Thermocycling parameters included UDG activation at 50°C for 2 mins; initial denaturation at 95°C for 2 mins; and 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Comparison of gene expression between the treated and untreated samples was performed using relative quantification of genes normalised to multiple reference genes on the qbase software (Biogazelle, Zwijnwaarde, Belgium).[13] In brief, change in quantification cycle (ΔCq) values for each sample were calculated for both the genes of interest and the reference genes by subtracting the Cq value of the treated sample (CSC-treated or DMSO control) from the Cq value of the untreated

pneumococcal cells). The relative quantities ($2^{\Delta Cq}$) of the genes of interest and the geometric mean of the reference genes were calculated.[13] The normalised relative quantity of each gene of interest was determined by dividing the relative quantity of the gene of interest by the geometric mean. These normalised relative quantities of the test genes were log-transformed prior to statistical analysis.

Biofilm formation

The bacteria in TSB growth medium were exposed to CSC (80 or 160 µg.mL^{-1}) or the solvent control for 16 hours at 37°C, 5% CO_2, in a six-well tissue culture plate to facilitate adherence and biofilm formation. Following incubation, the non-adherent bacteria and medium were removed, and the wells washed three times with phosphate-buffered saline (PBS, 0.15 M; Becton Dickinson and Co., Sparks, MD, USA). After removal of the unbound bacteria, the biofilm was stained with 0.1% crystal violet, the excess dye removed and the wells washed five times with PBS. The crystal violet was released from the adherent bacteria by the addition of 96% ethanol and the amount of biofilm formed determined spectrophotometrically at a wavelength of 570 nm using the PowerWaveX (Bio-Tec Instruments Inc., Winooski, VT, USA) plate reader.

Expression and statistical analysis of results

With respect to biofilm formation studies, a total of 3-6 experiments with 2-3 replicates for each system in each experiment was performed. The results are expressed as the mean values ± standard deviations (SDs) and the data analysed using the Mann-Whitney *U* test. For the gene expression data, descriptive and inferential statistical techniques were used in the analyses. The results are also

expressed as mean values ± SDs of three different experiments with 2 replicates for each system. Analysis of variance was measured using one-way ANOVA with Scheffe *post-hoc* analysis. Statistical significance was set at a p-value <0.05. GraphPad InStat 3 (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analysis.

Results

Gene expression

As shown in Figure 1, exposure of the pneumococcus to CSC for 60 min resulted in statistically significant ($p<0.05$) upregulation of expression the *hk11*, *rr11*, *abc, cat eff* and *2005-hyp* genes by all five test strains of the pneumococcus when compared to the DMSO-treated controls. Gene expression in CSC-treated and DMSO-treated controls was normalised to that of untreated cells and log transformed to approximate normal distribution. The untreated cells are thus represented with baseline gene expression levels of 0.The CSC-and DMSO-treated cells are represented with positive (up-regulated) and negative (down-regulated) values compared to the baseline (untreated cells). Down-regulation of gene expression noted in the DMSO-treated systems of strains 172, 2507 and 49619 of the pneumococcus may indicate modest inhibitory effects on gene expression and possible under-estimation of the effects of CSC on up-regulation of the test genes.

Expression levels of these genes were comparable between strains. Fifteen min exposure to CSC had similar, but less striking effects on gene expression, compared with 60 min exposure (data not shown).

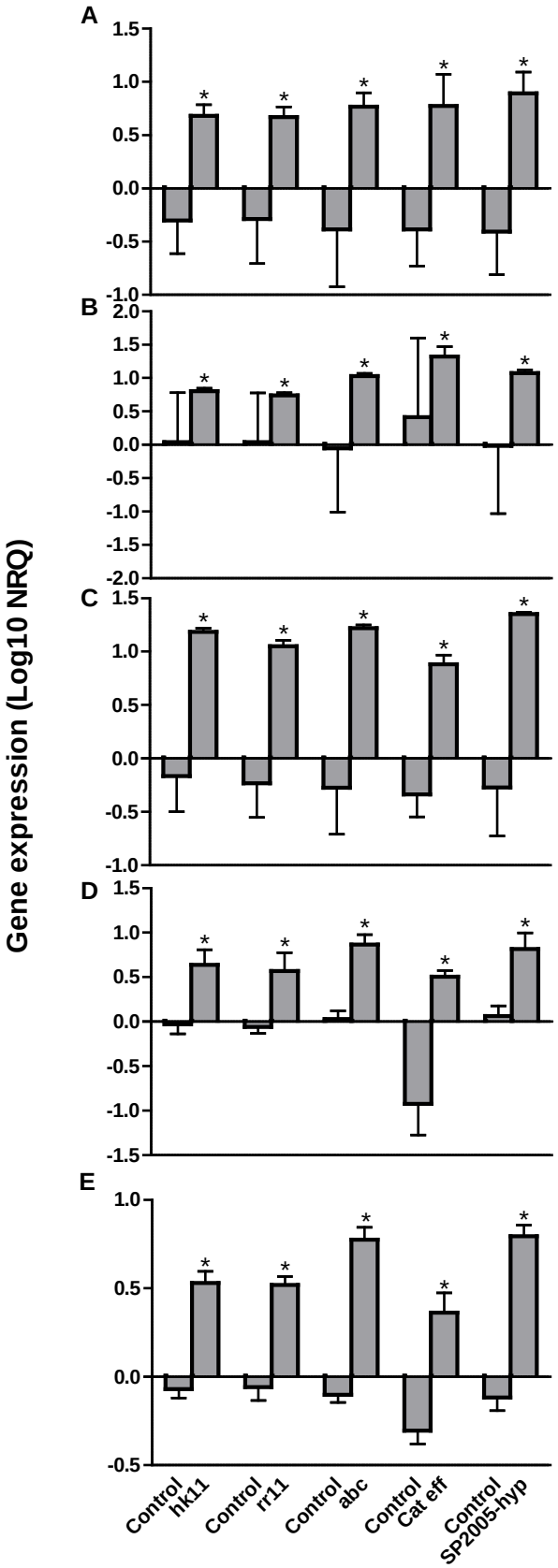
Gene expression (Log10 NRQ)
A
B
C
D
E
Control
hk11
Control
rr11
Control
abc
Control
Cat eff
Control
SP2005-hyp

Figure 1: The effects of exposure of strains 172 (A), 521 (B), 2507 (C), 4916 (D) and 49619 (E) of the pneumococcus to CSC (160 µg.mL^{-1}) for 60 min on the relative expression of the *hk11, rr11, cat eff, abc* and *SP*2005 genes. The results of 3 different experiments with 2 replicates for each system are expressed as normalised relative quantities (NRQ, mean values ± SDs). Gene expression levels, either up- or down-regulated, of the DMSO control and CSC-treated systems, are presented relative to those of untreated bacteria (DMSO- and CSC-free) represented by the baseline for each of the five strains of the pneumococcus. Significance, when compared to the control system, is indicated where $p<0.05$.

Biofilm formation

The results for biofilm formation by the various clinical strains of the pneumococcus, *viz.* strains 172, 521, 2507, 4916 and 49619 exposed to CSC (80 and 160 µg.mL^{-1}) overnight are shown in Figure 2. Treatment of all five strains with both concentrations of CSC showed a concentration-dependent, increase in biofilm formation, which was statistically significant ($p<0.05$) at both concentrations of CSC for all strains of the pneumococcus, with the exception of strain 172 for which statistical significance was noted only at the 160 µg.mL^{-1} concentration of the condensate. Differences in the magnitudes of CSC-mediated biofilm formation were noted between strains, the most and least prolific producers being strains 49619 and 172 respectively. The mean percentages augmentation of biofilm formation following exposure of strains 172, 521, 2507, 4916 and 49619 of the pneumococcus were 78, 39, 50, 73 and 621%, respectively.

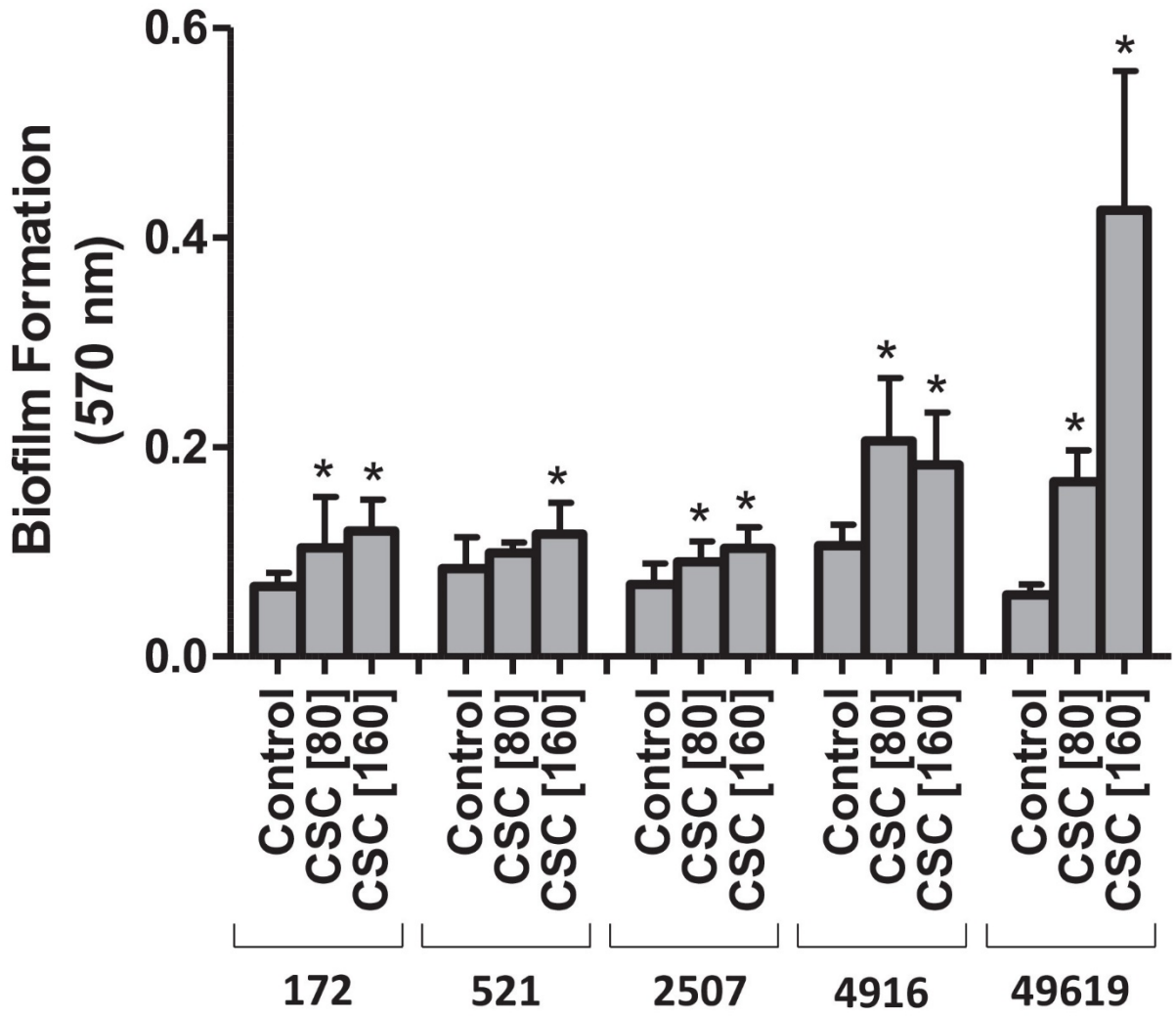

Figure 2: The effects of exposure of strains 172, 521, 2507, 4916 and 49619 of the pneumococcus to CSC (80 and 160 μg.mL^{-1}) on biofilm formation following 16 hours of incubation. The results of 3-6 different experiments with 2 to 3 replicates in each system are expressed as the mean values ± SDs. Significance, when compared to the control system, is indicated where $p<0.05$.

Discussion

Our findings demonstrate that induction of biofilm formation appears to be a common response of various strains of the pneumococcus following exposure to CS. This contention is based on the observation that all five strains of the pathogen

investigated in the current study exhibited significant augmentation of biofilm formation following exposure to CSC *in vitro*. The five strains of the pneumococcus used in this study belong to three serotypes, 6A, 19F and 23 F, these being predominant disease-causing serotypes of the pneumococcus, all of which are represented in the currently recommended pneumococcal 13-valent conjugate vaccine, PCV13. Although statistically significant for all strains tested, the magnitude of CSC-mediated formation of biofilm differed considerably between strains. This may reflect differences in the cellular machinery involved in biofilm formation or, alternatively, differences in sensitivity to the condensate.

In a previous study focused on a single strain (172) of the pneumococcus, we reported that CSC-mediated augmentation of biofilm formation, detected using similar experimental conditions to those used in the current study, resulted in significantly altered expression of only a small number of genes, six of which were increased and one decreased.[5] Global gene expression was measured using the TIGR4 DNA microarray chip with the results confirmed by RT-qPCR.[5] With two exceptions *viz. SP2004* (hypothetical gene – up-regulated) and *ply* (pneumolysin – down-regulated), the genes analysed in the current study correspond to those which were previously found to undergo significantly increased expression following exposure of strain 172 of the pneumococcus to CSC.[5] Upregulation of these genes, specifically *hk11, rr11, cat eff, abc* and *SP2005*, was also found to coincide with increased biofilm formation by all five test strains of the pneumococcus in the current study, possibly indicative of a mechanistic link between these events. In this context, *hk11* and *rr11*, which comprise the TCS11 transcriptional regulatory pathway, seem most likely to play a role in biofilm formation,[8] while upregulation of the other stress-

related genes appears to result in activation of mechanisms which promote efflux of CSC-derived toxicants.

The proposed involvement of TCS11 of the pneumococcus in CSC-mediated enhancement of biofilm formation, although speculative, is based on several lines of evidence. Firstly, on an earlier report by Li *et al.,* which was the first to describe the existence of TCS11 in *S. mutans*, as well as its apparent involvement in biofilm formation.[7] This contention was based on observations that inactivation of the genes encoding TCS11 resulted in decreased biofilm mass and structural integrity, as well as acid resistance.[7] In this context, it is noteworthy that HK11 shares homology with SPY1622, also known as YvqE, a histidine kinase two-component system sensor of *S. pyogenes*, which is also involved in biofilm formation and acid production.[14] Secondly, exposure of the pneumococcus to the glycopeptide antibiotic, vancomycin, also a stress-related inducer of biofilm formation,[15] is accompanied by induction of an array of stress-related genes (n=175), including those encoding TCS11.[16]

Although gene knockout studies may contribute additional insights into the possible involvement of TCS11 in stress-related induction of pneumococcal biofilm, it is, however, noteworthy that the pneumococcus, like many other bacterial pathogens, appears to possess several mechanisms which trigger biofilm formation.[17,18] Apart from underscoring the key role of biofilm in promoting bacterial persistence and survival, this also raises interpretational issues related to possible over-expression of compensatory mechanisms of biofilm formation in TCS11 gene knockouts. In addition, it seems likely that the 97 different serotypes of the pneumococcus may vary with respect to utilisation of different mechanisms of biofilm formation, possibly underpinning the differences in the magnitudes of biofilm formation observed in the current study following exposure of the various strains of

the pathogen to CSC. With respect to the current study, we concede that the evidence linking up-regulation of the various test genes to biofilm formation is circumstantial. Nevertheless, given the limited number of genes up-regulated following exposure of the pneumococcus to CSC, the association with biofilm formation is unlikely to be coincidental.

Of the remaining genes, *abc* and *cat efflux*, as mentioned above, are most likely involved in the expulsion of smoke-derived toxicants, while the role of the SP2005 protein remains to be elucidated. In the case of CSC exposure-mediated induction of the *abc* and *cat efflux* genes, it is noteworthy that cigarette smoke (CS) contains an array of potentially damaging pro-oxidative and other types of low molecular weight, chemical toxicants. These include organic and inorganic stable and unstable, highly-reactive free radicals, as well as heavy metals. Collectively, these promote the induction of various stress response genes, which counteract damage to key cellular proteins and organelles, as well as nucleic acid. In this setting, the cation efflux pump mediates the removal of toxic heavy metal ions, while the ABC transporter, albeit of unknown substrate specificity, is likely to promote the removal of smoke-derived, chemical toxicants. This latter contention is strengthened by the finding that the ABC transporter encoded by the *SP2003* gene was the only one of the more than 60 ABC transporters of the pneumococcus[17] found in our earlier study[5] to undergo significant induction following exposure of the pneumococcus to CSC. Interestingly, the *SP2003 abc* gene, like those comprising TCS11, has also been reported to be induced following exposure of the pneumococcus to vancomycin, consistent with a role in mediating multidrug resistance.[19,20]

To our knowledge, the only other study focused on transcriptomic profiling following exposure of the pneumococcus (strains pmp1287, serotype 16F, and EF3030, serotype 19F) to CS *in vitro* is that which was recently reported by Manna *et al.*[6] These authors also noted significant up-regulation of the genes encoding TCS11 by both strains of the pathogen following exposure to smoke-related stress. There, is however, a notable distinction between the earlier study reported by Cockeran *et al.*[5] and that of Manna *et al.*,[6] specifically in relation to the substantial difference in the numbers of genes which demonstrated significantly altered expression levels following exposure of the test pneumococcal strains to CS/CSC. In comparison with the small group of genes detected in the study by Cockeran *et al.*,[5] which also represented the focus of the current study, Manna *et al*. detected up-regulation and down-regulation of 59 and 63 genes respectively, following exposure of strain EF3030 to CS.[6] We believe that differences in the profiles of gene expression between the two studies are related to differences in the types and intensities of smoke exposure to which the bacteria were subjected. In their study, Manna *et al.* used CS extracts prepared by bubbling smoke generated from the combustion of four 3R4F research grade cigarettes through 10 mL of bacteriological culture medium, which retained the aqueous component of CS. This was considered to represent the maximum exposure, which induced the “strongest transcriptional response” without affecting bacterial viability.[6] The relatively moderate conditions of exposure used in our current and earlier studies may therefore be more discerning in respect of identifying those genes which undergo preferential activation following exposure of the pneumococcus to CS.

In conclusion, the findings of the current study strengthen the apparent association between CSC exposure-mediated augmentation of biofilm formation and

early induction of genes encoding the components of TCS11, as well those involved in expulsion of smoke-derived toxicants, a response to CSC, which is seemingly common to several major disease-causing serotypes of the pneumococcus.

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SP_1857-Cation Efflux F	5’ – CATTGACTGGTTGCGGATGC – 3’	All
SP_1857-Cation Efflux R	5’ – CACCTTGGGCTATAAGCGGT – 3’	
SP_2000-RR11-F	5’ – GGGCAAGAAGCAATCCAAC – 3’	All
SP_2000-RR11-R	5’ – TCCACTCCAAGACTTCAAGAC – 3’	
SP_2001-HK11-F1	5’ – TTCCCCATCCTGTCTGTAG – 3’	172,2507
SP_2001-HK11-R1	5’ – AGGCAGTATTCCCAACTACAT – 3’	
SP_2001-HK11-F2	5’ – CTTTGACATCCGAACTAGAGAC – 3’	4916
SP_2001-HK11-R2	5’ – AATCATAGAAGCCATTGACTCC – 3’	
SP_2001-HK11-F3	5’ – TGGAAAATCTCAAATCACGGAC – 3’	521,49619
SP_2001-HK11-R3	5’ – TCAACCTCAACCTCAATCCC – 3’	
SP_2003-ABC-F1	5’ – ATCGTGACTCCTTGCGCTTT – 3’	172,2507,4916
SP_2003-ABC-R1	5’ – TCTTGACCTTGATTGGGCGA – 3’	
SP_2003-ABC-F3	5’ – CGCCCAATCAAGGTCAAAAC – 3’	521,49619
SP_2003-ABC-R3	5’ – CCAGCAACAAAAAGCCCAG – 3’	
SP_2005-Hyp-F	5’ – GCTCCTTTTTCACATCTCCGAC – 3’	All
SP_2005-Hyp-R	5’ – TGTTTTCGGGATTGGATGAGAGT – 3’	
SP_0806-gyrB-F	5’ – TCAGCCAAATCTGGTCGTAACCGT – 3’	All
SP_0806-gyrB-R	5’ – AATTCTGCGCCAAATCCTGTTCCC – 3’	
SP_1219-gyrA-F	5’ – AATCTTGCTCATACGTGCCTCGGT – 3’	All
SP_1219-gyrA-R	5’ – ATGGTGGAGCTACCGTTACATGCT – 3’	

Strain shows which primer pair was used for the indicated strain. “All” indicates that the same primer pair was used for all five strains.

FIGURE LEGENDS

Figure 1: The effects of exposure of strains 172 (A), 521 (B), 2507 (C), 4916 (D) and 49619 (E) of the pneumococcus to CSC (160 µg.mL^{-1}) for 60 min on the relative expression of the *hk11, rr11, cat eff, abc* and *SP*2005 genes. The results of 3 different experiments with 2 replicates for each system are expressed as normalised relative quantities (NRQ, mean values ± SDs). Gene expression levels, either up- or down-regulated, of the DMSO control and CSC-treated systems, are presented relative to those of untreated bacteria (DMSO- and CSC-free) represented by the baseline for each of the five strains of the pneumococcus. Significance, when compared to the control system, is indicated where $p<0.05$.

Figure 2: The effects of exposure of strains 172, 521, 2507, 4916 and 49619 of the pneumococcus to CSC (80 and 160 µg.mL^{-1}) on biofilm formation following 16 hours of incubation. The results of 3-6 different experiments with 2 to 3 replicates in each system are expressed as the mean values ± SDs. Significance, when compared to the control system, is indicated where $p<0.05$.

FIGURE 1

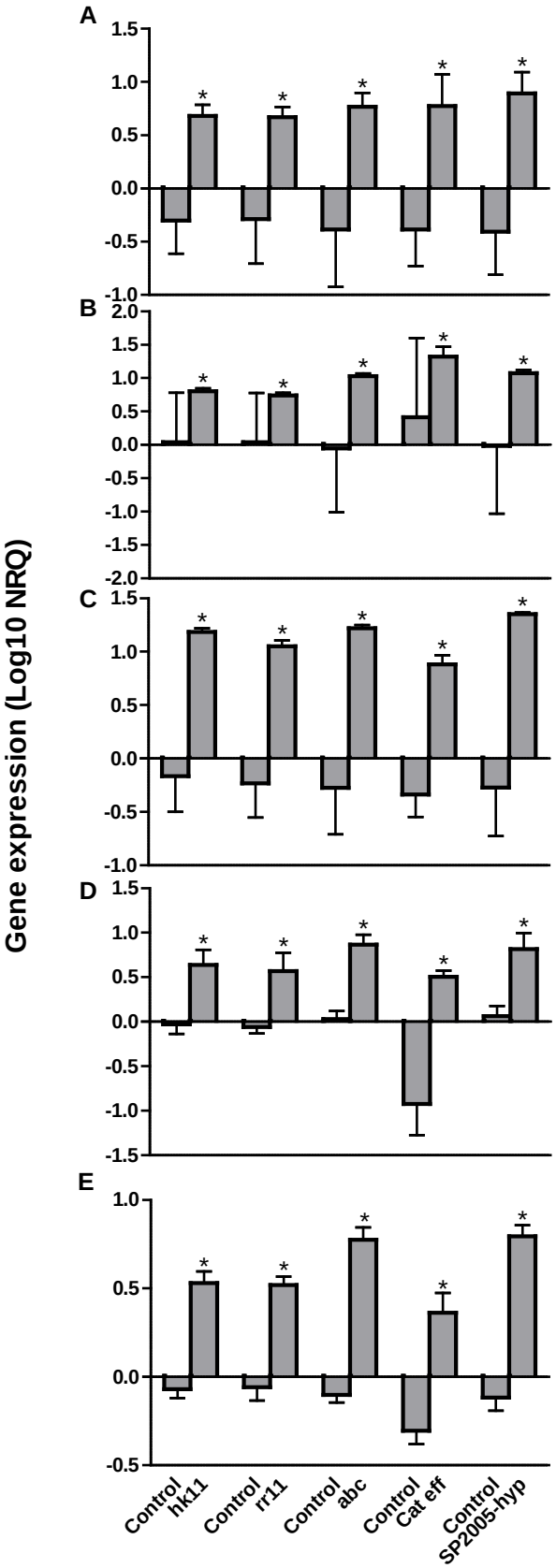
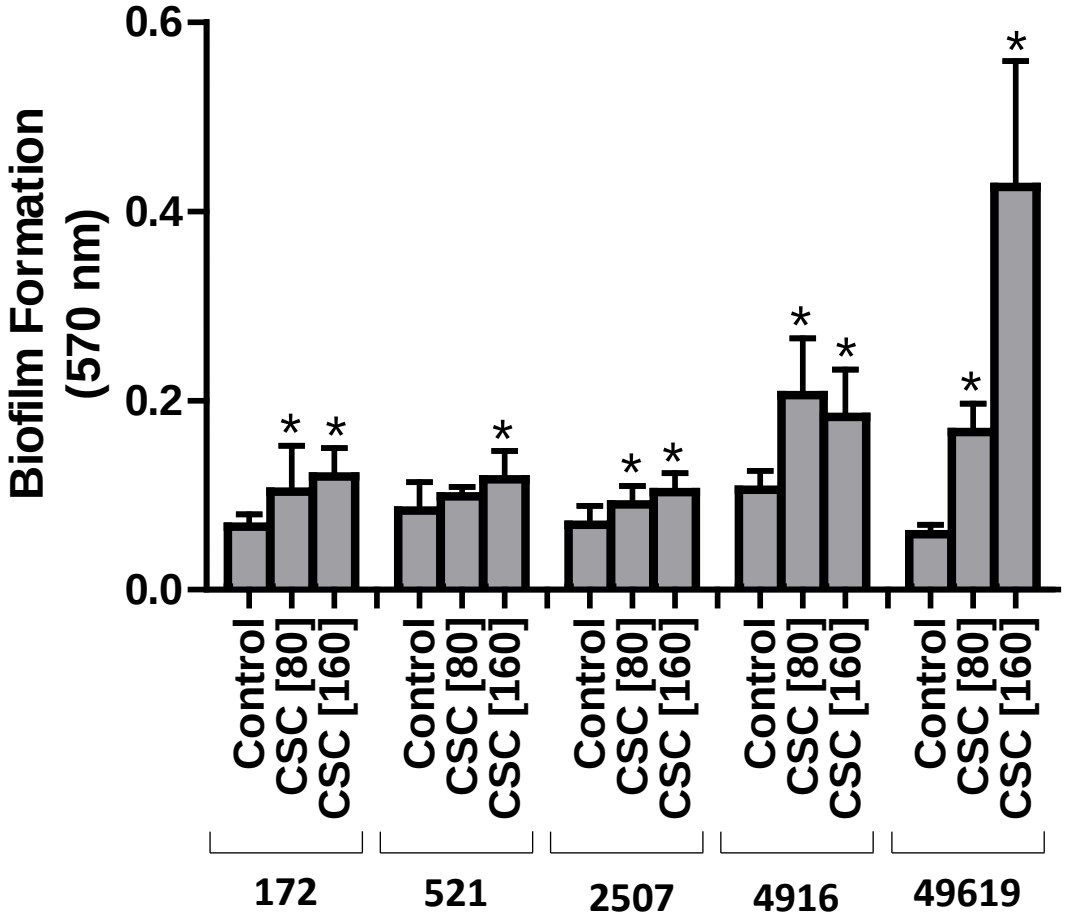

A
B
C
D
E
Gene expression (Log10 NRQ)
Control
hk11
Control
rr11
Control
abc
Control
Cat eff
Control
SP2005-hyp

FIGURE 2


Biofilm Formation
(570 nm)
0.6
0.4
0.2
0.0
*
Control
CSC [80]
CSC [160]
172
521
2507
4916
49619