Transcriptional analysis of the acid tolerance response in *Streptococcus pneumoniae*

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Streptococcus pneumoniae, one of the major causes of morbidity and mortality in humans, faces a range of potentially acidic conditions in the middle and late stages of growth *in vitro*, in diverse human fluids during the infection process, and in biofilms present in the nasopharynx of carriers. *S. pneumoniae* was shown to develop a weak acid tolerance response (ATR), where cells previously exposed to sublethal pHs (5.8–6.6) showed an increased survival rate of up to one order of magnitude after challenge at the lethal pH (4.4, survival rate of 10⁻⁴). Moreover, the survival after challenge of stationary phase cells at pH 4.4 was three orders of magnitude higher than that of cells taken from the exponential phase, due to the production of lactic acid during growth and increasing acidification of the growth medium until stationary phase. Global expression analysis after short-term (5, 15 and 30 min, the adaptation phase) and long-term (the maintenance phase) acidic shock (pH 6.0) was performed by microarray experiments, and the results were validated by real-time RT-PCR. Out of a total of 126 genes responding to acidification, 59 and 37 were specific to the adaptation phase and maintenance phase, respectively, and 30 were common to both periods. In the adaptation phase, both up- and down-regulation of gene transcripts was observed (38 and 21 genes, respectively), whereas in the maintenance phase most of the affected genes were down-regulated (34 out of 37). Genes involved in protein fate (including those involved in the protection of the protein native structure) and transport (including transporters of manganese and iron) were overrepresented among the genes affected by acidification, 8.7 and 24.6 % of the acid-responsive genes compared to 2.8 % and 9.6 % of the genome complement, respectively. Cross-regulation with the response to oxidative and osmotic stress was observed. Potential regulatory motifs involved in the ATR were identified in the promoter regions of some of the regulated genes.

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INTRODUCTION

Streptococcus pneumoniae is an important human pathogen and a major cause of pneumonia, meningitis, otitis media and sepsis. This broad pathogenic potential reflects its ability to adapt to different habitats within the human host.

In otitis media, *S. pneumoniae* is suspected to be present in biofilms on the mucosal surface of the middle ear, and possibly also during asymptomatic colonization of the nasopharynx (Donlan & Costerton, 2002). Physiological changes due to the biofilm mode of growth may include a decreased pH, as in dental plaque where bacterial crowding and production of lactic acid by the closely related *Streptococcus mutans* and *Streptococcus gordonii* occurs (Cotter & Hill, 2003). In other stages of infection, *S. pneumoniae* must also face a mild acidification of the invaded body fluids, i.e. blood, cerebrospinal fluid and inflammatory fluids, which decrease from pH 7.3–7.4 to 6.8–7.0 as a consequence of the inflammatory response (Andersen *et al.*, 1989; Light *et al.*, 1980).

Some bacterial species show an increased survival rate after challenge at lethal acid pH if they are first subjected to a period of mild sublethal acidic conditions, a process known

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Abbreviation: ATR, acid tolerance response.

The raw microarray data is available as supplementary material with the online version of this paper.

as the acid tolerance response (ATR), which has been described in *Escherichia coli* (Goodson & Rowbury, 1989), *Salmonella typhimurium* (Foster & Hall, 1990) and oral streptococci (Svensater *et al.*, 1997). The lethal pH and the range of pH that trigger the ATR are characteristic for each species, and are indicative of their acid resistance capacity (Svensater *et al.*, 1997). The bacterial responses to different types of physiological stresses often partially overlap, such that exposure to one stressful condition commonly gives some protection against several others (Svensater *et al.*, 2000). Whereas the ATR has been relatively well characterized in oral streptococci, it has not been investigated in *S. pneumoniae*.

One of the systems used by Gram-positive bacteria to respond to acidic pH is the system involving the F_0F_1 ATPase, an enzyme that hydrolyses ATP to generate a proton gradient for a variety of transport processes, and regulates the intracellular pH via proton extrusion (Cotter & Hill, 2003; Futai *et al.*, 1989). In the oral streptococci (Bender *et al.*, 1986), *Enterococcus hirae* (Kobayashi *et al.*, 1986), lactic acid bacteria (Koebmann *et al.*, 2000; Kullen & Klaenhammer, 1999) and *S. pneumoniae* (Martín-Galiano *et al.*, 2001), the activity of the F_0F_1 ATPase increases as the pH of the medium decreases. This increase in activity is regulated in *S. pneumoniae* at the level of transcription initiation (Martín-Galiano *et al.*, 2001). Global changes in gene expression in response to acidification have been analysed by microarray technology in bacteria in which acid resistance is crucial for their pathogenesis, such as *Helicobacter pylori* (Ang *et al.*, 2001) and *Mycobacterium tuberculosis* (Fisher *et al.*, 2002). Here we have characterized the ATR in *S. pneumoniae*, and analysed the adaptive and maintenance phases of the response by global expression analysis.

METHODS

Culture conditions, RNA extraction and microarray experiments. To characterize the ATR, *S. pneumoniae* R6 was grown at 37 °C in Todd-Hewitt medium (Difco) with 0.5% yeast extract (THYE medium), and survivors were counted after 16 h of growth by plating on THYE medium plus 1% agar in an atmosphere containing 5% CO₂. In the short-term ATR experiment, the R6 strain was grown in THYE medium at pH 7.8 until mid-exponential phase (OD₆₂₀ 0.25). At this moment, the pH of the medium was reduced to pH 6.0 (the more acidic pH that allows normal growth) with 1 M HCl, and RNA was extracted from 50 ml aliquots of culture collected at 0, 5, 15 and 30 min. In the long-term ATR experiment, R6 was grown in THYE medium at pH 7.8 until OD₆₂₀ 0.10, the culture was diluted 50-fold in fresh THYE medium buffered at starting pH values of 7.8 and 6.0, and 50 ml samples of culture were taken when the OD₆₂₀ reached 0.25, i.e. after approximately 200 min (referred as 200 min throughout this paper). Cells were then chilled on a water-ice bath, washed with cold 0.9% NaCl and stored at -80 °C. Pellets were thawed and cells lysed for 15 min at 37 °C in 10 mM Tris, 1 mM EDTA (pH 8.0), 0.1% sodium deoxycholate. RNA was extracted using the RNeasy midi kit (QIAGEN), including a DNase treatment according to the manufacturer's instructions, precipitated with ethanol, washed and resuspended in 40 µl RNase-free water. The concentration and purity of the RNA samples were measured using the 2100 Bioanalyser according to the manufacturer's

protocols (Agilent). Details of the construction of the microarrays used in this study have been described previously (Dagkessamanskaia *et al.*, 2004). The microarrays included probes for all strain TIGR4 annotated genes (2236) and probes for 117 R6-specific genes (i.e. those with less than 90% similarity to any of the TIGR4 genes, as deduced by BLAST analysis). The labelling of cDNA with Cy3- or Cy5-labelled CTP, and the microarray hybridization were performed as described previously (Martín-Galiano *et al.*, 2004). Microarrays were scanned with an Axon 4000A using GenePix Pro 3.0 software.

Microarray data normalization and analysis. All hybridization experiments were carried out in triplicate comparing two mRNA samples from three independent replicate cultures. Hybridization datasets were normalized and analysed as described by Holmes *et al.* (2005). In short, normalized data from triplicate hybridization datasets were unified in one single dataset and reanalysed. However, for the data analysis described here, the mean of the fluorescence values of single genes from each hybridization dataset were unified, as single genes were represented by at least four features on the array. The boundary between genes that were equally and differentially expressed was arbitrarily chosen as about twofold greater intensity in either the Cy3 or the Cy5 fluorescence values. The genes that potentially had increased or decreased expression levels were first identified as those with mean intensities outside the boundary, and were tested statistically by two *F*-tests. According to these tests, genes were classified as either differentially or equally expressed in both populations. With this boundary, the error in classification on the control dataset of the short-term ATR experiment and long-term ATR experiment was 0 and 1.45%, respectively.

The normalized and unified control experiments were also used to estimate the boundaries between genes that were equally and differentially expressed in the two samples (Holmes *et al.*, 2005). This boundary would allow the detection of changes equivalent to about 1.5-fold and 1.9-fold greater intensity in one of the fluorescence channels for the short-term ATR experiment and long-term ATR experiment, respectively. Differentially expressed genes identified from this analysis are shown in Table 2 if they are part of an operon, and at least one of the genes of the operon was found to have at least twofold changes in expression level.

Real-time RT-PCR experiments. Analysis of selected genes was carried out with 20 µl reverse transcription reactions containing 1 µg RNA, 0.5 mM each dNTP, 0.2 µM each gene-specific primer (Table 1), 40 U RNaseOUT ribonuclease inhibitor and 200 U SuperScript III RNase H⁻ reverse transcriptase (Invitrogen), in the buffer recommended by the manufacturer. The reactions were incubated at 55 °C for 60 min, terminated by 15 min incubation at 70 °C, and the template RNA was removed by 20 min incubation at 37 °C in the presence of 20 U RNase H (Amersham Biosciences). The cDNA obtained was stored at -20 °C. The LightCycler FastStart DNA Master SYBR Green I system (Roche) was used for real-time PCR (LightCycler 2.0 instrument) in a 20 µl reaction containing 2 µl diluted cDNA, 0.4 µM each primer, a variable amount of MgCl₂ (2–3 mM) and 2 µl SYBR Green mix. PCR amplifications were carried out as follows: 1 initial cycle of denaturation (10 min at 95 °C), and 42 cycles of amplification [95 °C, 0 s; test annealing temperature, 5 s; 72 °C, 8 s elongation and signal acquisition (single mode)]. To check the purity of the amplification product, 1 cycle of melting (95 °C, 0 s; test annealing temperature, 10 s; 95 °C, 0 s at the step acquisition mode) was performed. For relative quantification of the fluorescence values, a calibration curve of serial dilutions of cDNA samples of each amplicon was made. To normalize the three independent cDNA replicate samples, values were divided by

Table 1. Validation of microarray data by real-time RT-PCR

R6 locus/gene	PCR			Relative expression level analysis			
	Primers (5'→3')*	Product (bp)	Annealing/ melting temperature (°C)	Time (min)	Mean relative fold change†		RT-PCR/ microarray ratio
spr0444/ <i>glnA</i>	GGATCCAATGGCGAACCCCTTAC, + CCTTGCGCTCTTCTGCTGTCAT, -	134	64/81	5	-2.7	-3.9	0.7
				15	-5.5	-6.5	0.9
				30	-3.8	-7.0	0.5
				200	-1.3	-2.1	0.6
spr0455/ <i>dnaK</i>	CTTTCGGATGCAGGTTTGAG, + GGGTTTACTGATTGTGTTGTTCT, -	131	59/82	5	5.4	3.2	1.6
				15	4.7	1.9	2.4
				30	5.4	1.9	2.8
				200	-3.2	-1.6	2.0
spr1122/ <i>zwf</i>	CGGAGCGCTTGGGTGTAGAA, + CATGGCGAGGAGCGAAAGAA, -	107	64/85	5	-1.5	-2.8	0.5
				15	1.3	-3.3	0.4
				30	-0.9	-3.3	0.3
				200	-2.1	-0.7	1.2
spr1514/ <i>atpC</i>	GCTTATGTATTGCCTGTA, + TTGATAATAAATGAGAAGAC, -	177	48/80	5	1.5	1.1	1.5
				15	3.1	1.2	2.5
				30	3.8	1.1	3.4
				200	2.6	1.4	1.8
spr1538	TGTTTCCACGCTTCCATCCT, + TGCGTGCATAGACCTTTCCTT, -	113	61/82	5	1.8	-1.0	1.8
				15	1.2	-2.3	0.5
				30	-1.2	-3.0	0.4
				200	-3.1	-4.9	0.6
spr1625	AACTAAAGAACAGCATGAAGCAGA, + GAGAAACCAGATCCAAGACCAG, -	129	59/81	5	1.2	-1.3	0.9
				15	1.2	-2.2	0.5
				30	2.1	-2.2	0.9
				200	-1.8	-2.0	0.9
spr1680	TGCTAGCCCATCATATTCGTTTGT, + CAGTTGCGCAGCCAGTTTCA, -	138	62/82	15	2.8	2.1	1.3
				30	3.5	2.2	1.6
				200	1.7	2.2	0.8
spr1863/ <i>cglB</i>	ATGCACGTGAATGGGGGAATA, + TTGAGCCAGATCTTGACCGACTT, -	116	62/81	5	-	-1.5	-
				15	-	-1.6	-
				30	-	-1.9	-
				200	-	-1.0	-

* +, forward primer; -, reverse primer.

† RT-PCR fluorescence values were normalized with those obtained from the amplification of spr1863.

those obtained for the amplification of spr1863, a gene not showing variation in the microarrays. The mean of the relative values of the three replicates (coefficient of variation $\leq 25\%$) was used.

Searching for regulatory motifs in the upstream regions of transcriptionally altered genes. The 100 nt region located upstream of the start codon of genes was analysed. To assign genes to operons, the gene organization on The Institute for Genomic

Research (TIGR) web page (<http://www.tigr.org>) was analysed, based on the method published by Ermolaeva *et al.* (2001), and with STRING (von Mering *et al.*, 2005). Two contiguous genes were considered as part of the same operon if they had a confidence value over 75% according to the TIGR web page and/or a STRING score higher than 0.5. When genes were predicted to be part of the same operon, or located next to each other with similar transcriptional profiles, only the upstream region of the first gene of the operon was

Table 2. Genes with altered transcript levels in ATR, listed by role and subrole

Role or subrole	R6 locus	Gene/protein*	Mean relative fold change†				
			5 min	15 min	30 min	200 min	
Amino acid biosynthesis							
Glutamate family	spr0443‡§	<i>glnR</i> /transcription regulator	−2·8	−3·9	−4·2	−1·9	
	spr0444‡	<i>glnA</i> /Gln synthetase type I	−3·9	−6·5	−7·0	−2·1	
	spr1181	<i>gdhA</i> /NADP-specific glutamate DH	−2·2	−4·0	−4·3		
Pyruvate family	spr1935	<i>ilvD</i> /dihydroxyacid dehydratase			−2·1		
Biosynthesis of cofactors, prosthetic groups and carriers							
Lipoate	spr1047	Lipoate protein ligase A			−2·0		
Riboflavin, FMN and FAD	spr0161‡	<i>ribH</i> /riboflavin synthase, β chain		1·5			
	spr0163‡	<i>ribE</i> /riboflavin synthase, α subunit	2·0	2·3	2·4		
	spr0164‡§	<i>ribD</i> /riboflavin biosynthesis, a deaminase			1·6		
Cell envelope							
B/D surface liposaccharides/ polysaccharides	spr0096	LysM domain protein		−2·5	−3·1	−4·9	
	spr1875	LysM domain protein		−2·9	−3·3	−4·0	
Cell processes							
Adaptation to atypical conditions	spr1625	General stress protein 24		−2·2	−2·2	−2·0	
Cell division	spr2046	SpspJ protein				−2·4	
Detoxification	spr0692	<i>gor</i> /glutathione reductase			2·0		
Toxin production and resistance	spr0040	<i>blpU</i> /bacteriocin BlpU				−2·4	
	spr0472	<i>blpY</i> /immunity protein		−2·1	−2·0		
Disrupted reading frame							
	spr1274	<i>pspC</i> /PspC choline-binding protein			2·1	2·8	
Energy metabolism							
Amino acids and amines	spr1955	<i>arcA</i> /arginine deiminase		−2·7		−3·3	
B/D polysaccharides	spr0059	<i>bgal</i> β -galactosidase		−2·4	−2·0	−3·7	
	spr0276	<i>bglA</i> /6-phosphate- β -glucosidase				−4·0	
	spr1073‡	<i>lacD</i> /tagatose-1-6-diphosphate aldolase		1·7	2·2		
	spr1074‡	<i>lacC</i> /tagatose-6-phosphate kinase			1·6		
	spr1239	α -Amylase precursor			−2·3		
	spr1538	Xylan esterase 1		−2·3	−3·0	−4·9	
	spr1666‡	Conserved hypothetical protein		1·7	2·1		
	spr1667‡	Galactose-1-phosphate uridylyltransferase	1·7		1·9		
	spr1698	Dextran glucosidase DexS			−2·4	−2·3	
	Glycolysis/gluconeogenesis	spr0779‡§	1-phosphofructokinase	3·6	2·7	4·1	
		spr0780‡	PTS, fructose-specific IIABC components	2·2	2·1	2·6	
	Pentose phosphate pathway	spr1122	<i>zwf</i> /glucose-6-phosphate 1-DH	−2·8	−3·3	−3·3	
		spr1936‡	<i>tktC</i> /transketolase, C-terminal subunit			1·8	
		spr1937‡§	<i>tktN</i> /transketolase, N-terminal subunit		1·7	2·0	
Sugars	spr1972‡	<i>fucA</i> /L-fucose phosphate aldolase				−3·8	
	spr1973‡§	<i>fcsK</i> /L-fucose kinase		−2·1		−3·2	
Hypothetical proteins							
	spr0470	Hypothetical protein	−2·7	−3·3	−3·7		
	spr0086‡	Hypothetical protein	−2·8	−6·7	−7·0	−2·9	
	spr0087‡	Hypothetical protein	−2·6	−5·5	−5·7	−3·0	
	spr0088‡	Hypothetical protein	−2·5	−4·2	−4·3	−2·2	
	spr0089‡§	Hypothetical protein	−2·6	−4·0	−3·8	−2·9	
	spr0110	Hypothetical protein				−2·5	
	spr0127	Hypothetical protein				−3·0	
	spr0138	Hypothetical protein				2·4	
	spr0159	Hypothetical protein				−2·8	
	spr0173	Hypothetical protein				−2·4	
	spr0174	Hypothetical protein				−2·0	

Table 2. cont.

Role or subrole	R6 locus	Gene/protein*	Mean relative fold change†			
			5 min	15 min	30 min	200 min
	spr0600	Hypothetical protein				−3·8
	spr0616	Hypothetical protein				−2·2
	spr0617	Hypothetical protein		2·0		
	spr0652	Hypothetical protein				−2·0
	spr0730	Conserved hypothetical proteins			2·0	
	spr0931	Hypothetical protein		−2·1	−2·3	
	spr1080‡	Hypothetical protein				−2·2
	spr1081‡§	Hypothetical protein		−2·0		−2·8
	spr1205	Hypothetical protein		2·1	2·8	
	spr1283	Hypothetical protein		−2·1	−2·1	
	spr1551‡	Hypothetical protein				−2·8
	spr1552‡§	Hypothetical protein				−2·4
	spr1612	Hypothetical protein				−2·3
	spr1623‡§	Hypothetical protein		−2·0	−2·2	
	spr1624‡	Hypothetical protein			−1·7	−2·2
	spr1626‡	Hypothetical protein				−2·4
	spr1666	Hypothetical protein			2·1	
	spr1731	Hypothetical protein				−2·5
	spr1858	Hypothetical protein			−2·3	−2·4
	spr1915	Hypothetical protein				−3·0
	spr1938	PTS, IIC component			2·2	2·1
Protein fate						
Degradation of polypeptides	spr0307	<i>clpL</i> /ClpL protease, ATP-binding subunit	2·3			
	spr0561	<i>prtA</i> /serine protease precursor, subtilase family	2·0	3·1	6·1	4·5
	spr1204	<i>ptrB</i> /prolyl oligopeptidase family		2·2	2·6	2·7
	spr1282	<i>amy</i> /peptidase, U32 family		−2·1	−2·3	
	spr2045	Serine protease				−2·5
Protein folding and stabilization	spr0453‡§	<i>hrcA</i> /heat-inducible repressor	2·4			
	spr0454‡	<i>grpE</i> /heat-shock protein	3·1	1·8		
	spr0455‡	<i>dnaK</i> /heat-shock protein	3·2	1·9	1·9	
	spr0456‡	<i>dnaJ</i> /heat-shock protein	2·0		1·5	
	spr1993	<i>hslO</i> /chaperonin 33 kDa				2·2
	spr2011	<i>yfiA</i> /ribosomal subunit interface protein		−3·2	−2·5	−2·9
Protein synthesis						
Ribosomal proteins: synthesis and modification	spr0555	<i>rplK</i> /ribosomal protein L11				−2·2
	spr2020‡	<i>rpsB</i> /20S ribosomal protein S2			−1·5	−2·1
Translation factors	spr2019‡§	<i>tsf</i> /translation elongation factor Ts				−2·1
Purines, pyrimidines nucleosides and nucleotides						
Pyrimidine ribonucleotide biosynthesis	spr0865‡§	<i>pyrK</i> /dihydroorotate DH electron transfer subunit		2·0		
	spr0866‡	<i>pyrDb</i> /dihydroorotate DH		2·1	1·9	
	spr1153‡	<i>carB</i> /carbamoyl phosphate synthase (ammonia), heavy subunit		2·5	2·2	
	spr1154‡	<i>carA</i> /carbamoyl phosphate synthase (glutamine hydrolysing), light subunit	1·7	2·3	2·1	
	spr1155‡§	<i>pyrB</i> /aspartate carbamoyltransferase	1·8	2·8	2·2	
Regulatory functions						
DNA interactions	spr1072	<i>lacT</i> /transcription antiterminator				−2·5
	spr0279	Transcription antiterminator BglG family				−7·8

Table 2. cont.

Role or subrole	R6 locus	Gene/protein*	Mean relative fold change†				
			5 min	15 min	30 min	200 min	
Signal transduction							
Phophotransferase system	spr0060‡§	PTS, sugar-specific IIB component				−4.9	
	spr0061‡	PTS, sugar-specific IIC component				−4.0	
	spr0230	PTS, sugar-specific IIB component				−2.7	
	spr0278	PTS, IIB component				−3.5	
	spr0280	PTS, IIA component				−5.5	
	spr1969	PTS, sugar-specific IIB component				−3.3	
Transcription							
Other	spr1440	Probable ATP-dependent RNA helicase			−2.2		
Transport							
Amino acids, peptides and amines	spr0551	<i>brnQ</i> /branched-chain amino acid transport system carrier protein		2.1	2.5		
	spr1120‡§	ABCT, membrane-spanning permease – Gln transport	−2.8	−4.3	−4.5		
	spr1121‡	ABCT, ATP-binding protein – Gln transport	−2.8	−4.0	−4.5		
Anions	spr1382	<i>aliB</i> /ABCT oligopeptide-binding protein		2.0	2.6		
	spr1677‡	<i>proWX</i> /ABCT membrane-spanning permease – choline transporter	1.7	1.9	2.0		
	spr1678‡	<i>proV</i> /ABCT ATP-binding protein – choline transporter	1.9	2.0	2.3	2.6	
	spr1679‡	Hypothetical protein	1.8	2.1	2.3		
	spr1680‡§	Transcription regulator, MarR family	1.9	2.1	2.2	2.2	
	spr1561‡	<i>natB</i> /ABCT, membrane-spanning permease, Na export			1.9		
	spr1562‡§	ABCT, ATP-binding protein, Na export			2.0		
	spr1897‡	<i>pstA</i> /phosphate-ABCT, membrane-spanning permease	−1.7	−1.6	−1.9		
	spr1898‡	<i>pstB</i> /phosphate-ABCT, ATP-binding protein				−3.3	
	spr1899‡	<i>phoU</i> /phosphate-transport system regulatory protein	−1.5	−1.6	−1.8		
	Carbohydrates, organic alcohols and acids	spr1699	<i>treP</i> /PTS, trehalose-specific IIABC components			−3.2	
		spr1700	<i>treR</i> /trehalose operon transcriptional repressor				−3.8
spr1410		P-type ATPase-Ca transporter		−2.4	−2.1		
spr1430		Non-haem Fe-containing ferritin			−2.1	−2.5	
spr1492‡§		<i>psaB</i> /Mn ABCT, ATP-binding protein		2.3	3.7	2.9	
spr1493‡		<i>psaC</i> /Mn ABCT, membrane-spanning permease	1.7	2.5	3.8	2.7	
spr1494‡		<i>psaA</i> /Mn ABCT, Mn-binding protein	1.7	2.6	4.0	2.9	
Cations	spr1672	Cation efflux system protein				−2.6	
	spr1684‡§	<i>fatD</i> /ABCT, membrane-spanning permease – ferric iron		2.5	3.8		
	spr1685‡	<i>fatC</i> /ABCT, membrane-spanning permease – membrane-spanning		2.8	3.8		
	spr1686‡	<i>fecE</i> /ABCT, ATP-binding protein – membrane spanning		2.6	3.8		
	spr1687‡	<i>fatB</i> /ABCT, substrate-binding protein – membrane spanning			2.2		
	Other	spr1344	Glycerol uptake facilitator protein			−2.4	

Table 2. cont.

Role or subrole	R6 locus	Gene/protein*	Mean relative fold change†			
			5 min	15 min	30 min	200 min
Unknown substrate	spr0474	<i>pncP</i> /ABCT ATP-binding protein – unknown substrate				–3.3
	spr1202‡	ABCT, ATP-binding protein		1.8	1.9	
	spr1203‡§	ABCT, ATP-binding/membrane-spanning permease		2.2	2.8	2.5
	spr1559	ABCT, ATP-binding protein	2.2			
	spr1817	ABCT, ATP-binding protein		–2.2		
Unknown function	spr0246	Oxidoreductase, putative			–2.3	
	spr0677	VanZF-related protein				2.0

*ABCT, ATP-binding cassette transporter; B/D, biosynthesis and degradation; DH, dehydrogenase; PTS, phosphotransferase system.

†Only genes whose transcript level showed significant fold variations ≥ 2 -fold are shown. Additionally, genes with variations in the short term experiment ≥ 1.5 -fold and in the long-term experiment ≥ 1.9 -fold are listed if present within operons having at least one other gene with a ≥ 2 -fold change in expression.

‡Genes could be part of an operon.

§, indicates the first gene of the putative operon. In three cases, the first gene of the operon is not in the table: spr1076 (spr1076–spr1073 operon); spr1668 (spr1668–spr1666 operon); and spr1895 (spr1895–spr1899 operon).

considered. The *P* value was calculated by comparing the potential motif with its random appearance considering the mean GC content of *S. pneumoniae* (40 mol% G+C). All sequences presented in at least two promoter regions of similar controlled genes with $P \leq 2 \times 10^{-3}$ were analysed as potential acid-dependent regulatory motifs.

RESULTS

Acid tolerance response of *S. pneumoniae*

The lethal pH, defined by a survival rate of 10^{-4} after an exposure of 2 h, was estimated in order to undertake the subsequent ATR experiments, and corresponded to pH 4.4 (Fig. 1a). Exposure of bacteria to mild sublethal acid conditions (pH 5.8–6.6) for 2 h prior to lethal pH challenge, increased the cellular survival rate threefold to tenfold (Fig. 1b). Moreover, the survival rate was notably increased, by up to three orders of magnitude, when cells at stationary phase were compared to cells at exponential phase, without any additional period at sublethal pH (Fig. 1c) due to the gradual acidification of the medium caused by the production of lactic acid. The pH values at which the ATR is triggered in the optical density experiment are concordant with those in the mild acid shock assay (compare Figs 1b, c).

Global transcription analysis

The short-term (5, 15 and 30 min) and long-term (200 min) transcriptome response to low pH was analysed in exponentially growing cells by labelling cDNA from the control and test samples with different fluorescent dyes (Cy3 and Cy5), followed by subsequent co-hybridization on a single microarray slide. For these experiments *S. pneumoniae* R6

cultures were grown in triplicate as described in Methods. Cells diluted at pH 6.0 suffered a transition stage of around 30 min as deduced from the serious delaying effect on the growth rate. After that period, the cells recovered displaying equivalent duplication times (34 min) to the parallel control culture at pH 7.8 (38 min). Although the stationary-phase ATR was the predominant form of acid adaptation, the exponential phase of growth was chosen to compare the transcriptional status of cells. This decision was made on the basis of discerning among acid adaptation and other characteristics dependent of the phase of growth, such as a lack of primary sources of carbon and nitrogen, accumulation of metabolic by-products, alterations in the duplication rate or potential activation of quorum-sensing processes. Triplicate hybridization datasets were normalized and analysed as described in Methods. Most genes remained unaltered, showing comparable expression levels between the samples at pH 7.8 and pH 6.0, whereas 6.0% of genes (126 out of 2084 R6 strain genes) had a significantly different signal intensity. A similar number of genes were affected in experiments comparing *S. pneumoniae* gene expression in different body fluids (Orihuela *et al.*, 2004). We have detected variations of up to 6.1-fold for genes with increased expression levels, and up to 7.8-fold for genes with decreased expression levels. The magnitude of these changes were small compared to the 10-fold to 100-fold changes detected in the levels of certain proteins during the ATR of *S. mutans* (Len *et al.*, 2004a, b), *Streptococcus sobrinus* (Nascimento *et al.*, 2004) and *Streptococcus oralis* (Wilkins *et al.*, 2001). Of the 126 genes with altered transcript levels, 59, 30 and 37 were associated with short-term, both short- and long-term, or long-term ATRs, respectively (Table 2). The patterns of expression were very consistent, with only two exceptions.

Fig. 1. Characterization of the ATR of *S. pneumoniae* R6: lethal pH determination (a), ATR (b) and optical density dependence of the ATR (c). (a) Cells grown to OD₆₂₀ 0.1 at pH 7.8 were diluted 1/50 and incubated for 2 h in media with increasingly acidic pHs. (b) Cells grown to OD₆₂₀ 0.1 were diluted 1/50 in medium with the indicated pHs. Then, a volume corresponding to 10⁶ cells (as measured by OD₆₂₀ determination) was inoculated into 5 ml medium at pH 4.4 and incubated for 2 h. (c) Fractions of a culture corresponding to 10⁶ cells grown in medium at pH 7.8 were incubated for 2 h at pH 4.4. Survival rates (grey bars) are the mean $\pm$ SEM of three independent experiments. $\blacktriangle$, pH.

Genes *spr1667* and *spr0456* (*dnaJ*) showed increased expression at 5 and 30 min, but not at 15 min. In these two cases, the increases at 15 min were of 1.4-fold, just below the threshold limit. Whilst the number of genes showing up- or down-regulation was nearly equivalent in the short-term acid response (i.e. 64.4% were up-regulated), the majority of genes (91.9%) specifically altered in the long-term acid response were down-regulated. An intermediate situation was observed for genes with altered expression in both periods (66.7% were down-regulated). The number of genes with an altered transcription, and their function and general type of regulation (the balance of up-/down-regulated genes) strongly support the inference of a different type of regulation in short-term adaptation compared to long-term maintenance responses to acidification.

Genes encoding products predicted to have a role in protein fate were overrepresented among those affected by acidification, i.e. they made up 8.7% of the acid responsive genes compared to 2.8% in the genome complement. Similarly, 24.6% of the acid responsive genes were predicted to have a role in transport, whereas only 9.6% of the genome set have this putative function (Table 2).

Around half of the genes (59 out of 126) with an altered expression could be grouped in 23 putative operons comprising 2–4 genes (Table 2), as deduced by parallel expression patterns and contiguous localization in the chromosome of their genes. Almost all of these operons (87%) were also computationally predicted by the algorithm utilized by TIGR (<http://www.tigr.org>) and/or the STRING database procedure.

Verification of transcriptome data by real-time RT-PCR

The reverse transcription products of seven genes (i.e. six acid responsive genes and one non-varying gene, *spr1863*, as an internal standard) were measured by real-time RT-PCR at 5, 15, 30 and 200 min after acid shock. Representative acid-responsive genes were selected on the basis of their different temporal patterns of expression as revealed by transcriptome analysis. One of these genes (*dnaK*) showed an elevated transcription level at 5 min. Gene *spr1680*, encoding a transcription regulator of the MarR family, was selected as this was up-regulated at 5, 15, 30 and 200 min. Four genes showing decreased transcript levels were analysed. Two of them were down-regulated at 5, 15 and 30 min (*glnA* and *zwf*). The other two were down-regulated at 15, 30 and 200 min (*spr1538*, coding xylan esterase I and *spr1625*, coding a general stress protein). Additionally, relative expression levels of *spr1514* (*atpC*), the first gene of an operon that was increased in response to low pH (Martín-Galiano *et al.*, 2001), encoding the *c* subunit of the F₀F₁ ATPase, was also included. Ratios of microarray and real-time RT-PCR expression measurements showed a good correlation, with a slope of 1.08 (Fig. 2).

Fig. 2. Correlation of the measured change in RNA transcript levels (at pH 7.8 versus pH 6.0) using data from microarray hybridizations and real-time quantitative RT-PCR, $r^2 = 0.74$.

Gene clustering and promoter analysis

The 100 nt regions located upstream of the predicted start codon for the genes with altered expression were analysed for the presence of potential regulatory motifs, as described in Methods. For genes belonging to operons, only the region upstream of the first gene of the operon was analysed. In this way, 29 regions of 51 up-regulated genes, and 59 regions of 75 down-regulated genes were analysed. Almost one-third (35 out of 110, 31.8 %) of the genes had typical promoters consisting of two conserved hexameric boxes (matching the TTGACA-17 ± 1 nt-TATAAT consensus). Four of the nineteen genes up-regulated at 5 min had specific regulatory sequences: a CIRCE element was found in the promoter region of the *hrcA* gene, the first gene of the *hrcA-grpE-dnaK* operon (Zuber & Schumann, 1994), and a CtsR-repressor-binding sequence is located upstream of *clpL*, as described by Kwon *et al.* (2003) (Fig. 3). Sequence comparisons revealed a number of putative regulatory sequences. The GTTTRTWTTWTWTCARAAATT sequence (where R is A or G, and W is A or T), was found 3 nt upstream of the -35 box of the putative promoters of the manganese (*psaB-psaC-psaA*) and iron (*fatD-fatC-fecE-fatB*) ABC transporter operons, which are up-regulated at 15 and 30 min, with very similar fold changes. Two up-regulated ABC transporters (*spr1203* and *spr1382/aliB*) share a GAGGTTTT sequence located 10–22 bp upstream of the putative -35 region. The putative promoters of the first genes of two down-regulated operons, the one encoding Gln synthetase (*glnR*) and the five-gene operon encoding a phosphate ABC transporter (*pstS*), have the sequence TTAGACTATAA overlapping their potential -10 box.

DISCUSSION

S. pneumoniae has been shown to be an acid-sensitive lactic acid bacterium according to the optimum pH (7.5) of its F₀F₁ ATPase (Martín-Galiano *et al.*, 2001) and, as shown

here, also by the characteristics of its ATR (high lethal pH, high pH values triggering the response). All the pH values are noticeably higher than those estimated for *S. mutans*, an aciduric oral streptococcus (Hamilton & Svensater, 1998). This weak ATR of *S. pneumoniae* was further enhanced by the growth of bacteria to stationary phase, where the pH steadily decreases due to the production of lactic acid as an end product of fermentation, as also observed in *S. mutans* (Li *et al.*, 2001). Then, the predominant ATR in this organism seems to occur at stationary phase. Additionally, it is worth mentioning that the ATR assays were performed on the R6 laboratory strain, and it cannot be completely ruled out that other strains would display slightly different behaviours in response to acid.

Our analysis of the global transcription response of *S. pneumoniae* to low pH is, as far as we know, the first reported study on global expression of the ATR for any member of the genus *Streptococcus*. A total of 126 genes were shown to be involved in ATR. Based on their temporal pattern of expression, we classified acid tolerance responsive genes as those involved in an adaptation response (with altered expression at 5, 15 and 30 min after the acid shock) and those involved in the maintenance of the response (altered in the long term). Six of the nineteen genes with elevated expression levels at 5 min are involved in protein fate. Four are part of the *hrcA-grpE-dnaK-dnaJ* operon, encoding GrpE, DnaK and DnaJ chaperones, the HrcA repressor and two proteases (*clpL* and *prtA*). Early activation of *clpL* might play a role in the degradation of misfolded proteins due to acidification, and also as a chaperone to assist the refolding of proteins (Kwon *et al.*, 2003). DnaK and ClpL proteins has been shown to increase in *S. mutans* at acidic pH (Len *et al.*, 2004b), and an increase in the transcription of *clpL* has been observed in response to heat and cold shock in *Lactococcus lactis* (Skinner & Trempey, 2001). Proteins that cannot be folded by molecular

Locus	Gene		P	Cases
spr0307	<i>clpL</i>	TACATAGAGTAGCGATTAGCTAGTCTAAATTTTTTAAAGAAAAGGTCAAAGATAGTCAA-37 nt-ATG CtsR repressor binding sequence	1.6×10 ⁻⁷	0 (1, 1)
spr0453	<i>hrcA</i>	TTGACA TTCTCTTCTAAGGGTGT TATAAT AGAATCATGAGTTAGCACTTGGATGCATTGAGTGCTAA-26 nt-ATG CIRCE Element	6.3×10 ⁻⁹	0 (1, 2)
spr1492	<i>psaB</i>	GTTATTATTTTAAATTTTTCAAAAATTAACT TTGACT TAATTTTTTTTTTAATG TATATT -24 nt-ATG	1.4×10 ⁻⁸	0 (2, 2)
spr1684	<i>fatD</i>	GTGTGATTATTAAATTTGTCAGAAATTTT TTGACA GATTAGAAAAATAATGT TACATT -34 nt-ATG		
spr1203		TGGAGGTTTTTACTCCATCAGATGTTCTCTTAATGATTGATACTTT TAAATT -31 nt-ATG	2.0×10 ⁻³	4 (14, 16)
spr1382	<i>aliB</i>	GAGGTTTTTTGATTTTTTGGAAAAACA TTGACT AAACAAACCAAGAGTTG TATAAT -32 nt-ATG		
spr1895	<i>pstS</i>	TTTACA AAGACTTG TTAGAC TATAAA -48 nt-ATG	7.0×10 ⁻⁵	0 (2, 2)
spr0443	<i>glnR</i>	TTGACG TTTGCTTTTT TTAGAC TATAAT -23 nt-ATG		

Fig. 3. Common sequences found in the 5' upstream regions of genes with altered expression. The -35 and -10 boxes are underlined twice, those bases matching the consensus sequence are shown in bold. Sequences with P values of 2.0×10^{-3} or lower are shown, this value was calculated as the random possibility of finding the sequence considering a genome with a content of 40 mol% G + C. Cases indicates the number of sequences expected in the genome of R6. The real number found in R6 and TIGR4, respectively, are shown in parentheses.

chaperones may be degraded, in order to recycle amino acids for *de novo* protein synthesis. This might explain the early up-regulation of the *prtA* and *ptrB* protease genes, with *prtA* having the highest increase in expression of all up-regulated genes at 30 min. The elevated proteolytic activity would generate assimilated nitrogen sources, such as ammonia, thus possibly explaining why expression of the two enzymes involved in the synthesis of glutamine from 2-oxoglutarate and ammonium, NADP-glutamate dehydrogenase (*gdhA*) and glutamine synthetase (*glnA*), showed a reduction in expression. In accordance with this idea, *gdhA* is down-regulated in *S. mutans* at low pH (Wilkins *et al.*, 2002), and in *Bacillus subtilis* the *glnRA* operon is autoregulated such that expression is lowered when the cellular nitrogen content is high (Wray *et al.*, 1996). On the other hand, the decreased expression of the proteases encoded by *spr1282* and *spr2045*, and the up- and down-regulation of various amino acid transporters, suggests that low pH might induce changes in the amino acid metabolism as an adaptation to the new demands for protein synthesis.

A change in nucleotide metabolism was also observed after 5 min, involving elevated expression of the *pyrB-carA-carB* and *pyrK-pyrDb* operons involved in the first four steps of the *de novo* biosynthesis of pyrimidine ribonucleotides. In addition, reduced activity of the oxidative branch of the pentose phosphate pathway was predicted, due to the down-regulation of the *zwf* gene, which encodes glucose-6-phosphate dehydrogenase, the first and key enzyme of the pathway. Conversely, most genes of the non-oxidative route of the pentose phosphate pathway were up-regulated, suggesting that the pathway was redirected to the production of pentose phosphate instead of NADPH, possibly to support the biosynthesis of new nucleic acids (Kamada *et al.*, 2003).

Maintenance of the new cellular state should involve energy-demanding mechanisms, which would require a cellular energetic shift. Glycolysis appeared to be enhanced since the gene encoding its rate-limiting enzyme, 1-phosphofructokinase, was transcribed at higher levels. Additionally, the incorporation of galactose intermediates into glycolysis through the tagatose pathway (involving the *lacC* and *lacD* genes) was also enhanced. For efficient utilization of galactose in *S. mutans*, a combined function of both tagatose and Leloir pathways is required (Abranches *et al.*, 2004). In this respect, the gene encoding the galactose-1-phosphate uridyl transferase (*spr1667*) of the Leloir pathway was up-regulated in the pneumococcal ATR. However, transcript levels of several genes encoding enzymes that degrade carbohydrate polymers like maltodextrines, xylan, dextran and glycogen were decreased: α -amylase; acetyl xylan esterase, dextran glucosidase and β -galactosidase. Interestingly, dextran glucosidase was also down-regulated in the ATR of *S. mutans* (Wilkins *et al.*, 2002).

A link between ATR and other kinds of stress responses, such as oxidative and osmotic stress responses, was found. Acid stress induced increased transcript levels of the *gor* gene

encoding glutathione reductase, an enzyme that regenerates glutathione to avoid oxidation of many cellular molecules, including proteins and fatty acids. Accordingly, several genes (*ribD*, *ribE*, *ribH*) of the operon that is involved in riboflavin biosynthesis appeared to be up-regulated. As *S. pneumoniae* has an anaerobic metabolism and lacks a respiratory chain, we speculate that proteins containing riboflavin as a cofactor (flavoproteins) might be involved in redox reactions that are relevant to the oxidative branch of the ATR. Additionally, the genes encoding the three subunits of the transporter for manganese, a cofactor required for superoxide dismutase function, were up-regulated. This enzyme plays a role in anti-oxidative defences and is important for virulence (Tseng *et al.*, 2002). The 33 kDa chaperone encoded by the gene *hslO* was up-regulated in the maintenance phase of the ATR. It is well documented that this chaperone is subject to regulation under different environmental conditions (e.g. oxidative stress) compared to other chaperones, such as DnaK (Winter *et al.*, 2005), which were up-regulated within a few minutes of acidification in our experiments.

A possible link between the ATR and osmotic stress was also indicated by the increased expression of the operon encoding a choline transporter (genes *proV* and *proWX*). These genes are homologues of the permease and ATPase subunits of the *B. subtilis* *opuC* ABC transporter, which transports glycine betaine (Kappes *et al.*, 1996). This kind of transporter is able to concentrate osmoprotectants like proline and glycine betaine, which can retain water under adverse osmotic circumstances (Baliarda *et al.*, 2003; Kappes *et al.*, 1999; Lucht & Bremer, 1994; Proctor *et al.*, 1997). In accordance with this, the *S. mutans* response to acid partially protects against osmotic stress and also oxidative stress, and 25–40% of the proteins expressed in response to these stresses are common between them (Svensater *et al.*, 2000).

Especially interesting are the manganese and iron transporters. The acquisition of both elements is crucial for bacterial pathogens since they are in limiting amounts inside the host body. Manganese metabolism is important during biofilm formation in *S. gordonii* (Loo *et al.*, 2003) and essential in the pneumococcal colonization of the nasopharynx (Tseng *et al.*, 2002). Iron is an essential cofactor for the function of several enzymes, for example alcohol dehydrogenases, that take part in alternative energetic pathways, and possibly also play a role in the anti-oxidative response (Echave *et al.*, 2003).

In other bacteria, the ATR involves alternative sigma factors. The alternative sigma B factor of *Listeria monocytogenes* is involved in acid response and osmotic stress (Wemekamp-Kamphuis *et al.*, 2004). RpoS, the alternative *Escherichia coli* sigma factor, is regulated under acid shock at the translational level (Audia & Foster, 2003). In *S. pneumoniae*, different patterns of expression in response to low pH, and the absence of common sequences in the promoters, would indicate multiple regulatory mechanisms. A similar expression profile could occur *in vivo* during the infection or in

different stages of the carrier and transmission process of *S. pneumoniae*.

Although expression of the operon encoding the F_0F_1 ATPase did not show a significant (≥ 2 -fold) increase in microarray experiments, the 1.5-fold increase detected for the first gene of the operon (*atpC*) was in agreement with other quantitative measurements using primer extension (1.6-fold), Northern blot (1.4-fold) and dot blot (1.5-fold) (Martín-Galiano *et al.*, 2001). This indicates that the inherent variability of microarray data may lead to some biologically important changes in gene expression being statistically excluded from the analysis.

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