CodY-affected transcriptional gene expression of *Streptococcus pyogenes* during growth in human blood

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In an attempt to expand the available knowledge of pathogen–host interactions during *ex vivo* growth of *Streptococcus pyogenes* (GAS) in nonimmune whole human blood, the extents to which the expression of 51 genes including regulators with known targets, established virulence factors, physiologically important transporters and metabolic enzyme genes was differentially affected in the presence or absence of a functional *codY* gene were determined. The results obtained by quantitative real-time PCR using the M49 strain NZ131 showed that CodY influenced GAS gene activity in a dynamic fashion, with differential responses detected for 26 genes and occasionally characterized by discordance in the blood environment compared to laboratory medium. Degenerate derivatives of the recently discovered CodY box potentially serving as a *cis*-regulatory element for CodY action were identified in the upstream regions of 15 genes of the NZ131 genome, and these genes featured sequence motifs identical to the NZ131 CodY box in all completely sequenced *S. pyogenes* genomes. As none of these genes represented a genuine virulence factor, it seems likely, therefore, that the observed differential transcription of the majority of virulence genes was caused by indirect actions of CodY as part of a regulatory network.

Received 4 October 2006
Accepted 27 January 2007

INTRODUCTION

Streptococcus pyogenes (group A streptococcus, GAS) is a human-specific pathogen that causes high morbidity characterized by many types of skin and upper respiratory tract infections. The organism has also attained notoriety by causing occasionally life-threatening invasive disease characterized by deep tissue or bloodstream infections. Although GAS has long been known to be capable of multiplying in non-opsonizing blood and several virulence factors contributing to immune evasion have been identified (Cunningham, 2000), the full spectrum of bacterially encoded regulatory proteins mediating pathogen–host interactions remains elusive. Recently, *hasABC*-encoded hyaluronic acid capsule synthesis has been shown to be rapidly induced in the bloodstream of GAS-infected mice but the nature of the stimulus remained unknown (Gryllos *et al.*, 2001). During the initial period of growth in human blood, transcription of the two-component regulatory system CovRS, which negatively affects *has* expression

(Federle *et al.*, 1999), increases but transcript levels return to normal 30 min after adaptation to blood (Graham *et al.*, 2005). Moreover, the latter comprehensive study of GAS transcriptome dynamics during growth in human blood provided ample evidence for an important role of the CovRS system in the remodelling of GAS metabolism and virulence-associated functions in this environment (Graham *et al.*, 2005).

In previous studies, the CodY protein, highly conserved in the low-G + C Gram-positive bacteria, has been identified as a pleiotropic transcriptional regulator that is activated by branched-chain amino acids and controls many genes which become operative when the cells face nutrient limitation (den Hengst *et al.*, 2005; Guédon *et al.*, 2005; Levnikov *et al.*, 2006; Malke *et al.*, 2006; Sonenshein, 2005). In *S. pyogenes* grown in Todd–Hewitt broth or chemically defined medium, CodY affects, directly or indirectly, the expression of certain regulatory genes, virulence factors, transporters and metabolic enzyme genes, often in a medium- and nutritional status-dependent fashion (Malke *et al.*, 2006). To expand these observations to include an environment with clinical relevance, we examined the same functional categories of genes during cultivation of *S. pyogenes* in whole human blood and show here that CodY influences not only *covRS* expression in a dynamic

Abbreviation: GAS, group A streptococcus.

Tables showing the targets and primers used for real-time reverse transcription PCR and the mean transcript values of the genes and strains are available as supplementary material with the online version of this paper.

fashion but similar to CovR has a much broader role in structuring GAS gene activity in an environment mimicking streptococcal septicaemia.

METHODS

Bacterial strains and culture conditions. *S. pyogenes* strain NZ131 (M type 49) and its *codY1* mutant were used throughout this study. The *codY1* mutation, constructed by nonpolar insertion mutagenesis and confirmed as described previously (Malke *et al.*, 2006), disrupted *codY* at codon 197, implying that the sequence segment encoding the helix–turn–helix DNA-binding motif (Levdikov *et al.*, 2006) was removed from the 3′-portion of the truncated *codY* gene. Cells were grown statically at 37 °C in Todd–Hewitt broth containing 0.2 % yeast extract (THY broth) or fresh heparinized nonimmune whole human blood drawn on different occasions from a healthy 68-year-old male individual according to a protocol approved by the Institutional Review Board for Human Subjects. The blood was used without previous heat treatment to preserve complement components. Seventeen millilitres of mid-exponential phase THY cultures grown to OD₆₀₀ 0.6 were centrifuged and cells were resuspended at zero time in the same volume of blood at a multiplicity of ~10 c.f.u. per leukocyte. At 0, 1, 2 and 3 h of incubation, samples were taken for colony counts on blood agar plates, and 5 ml aliquots were withdrawn and added to 13 ml RNeasy lysis buffer (Qiagen) for RNA stabilization.

RNA isolation, cDNA synthesis and quantitative real-time PCR.

To lyse erythrocytes, 25 ml EL buffer (Qiagen) was added and the mixtures were incubated on ice for 20 min. After centrifugation at 6000 g and washing with EL buffer, bacterial cells were subjected to total RNA isolation with subsequent rDNase I treatment by the RiboPure-Bacteria Protocol (Ambion) as previously described in detail (Malke *et al.*, 2006). RNA integrity was assessed electrophoretically (Malke *et al.*, 2006). cDNA synthesis from total RNA with random primers, dNTPs and Superscript II reverse transcriptase (Invitrogen) followed by RNase A and RNase H treatment has also been detailed previously (Malke *et al.*, 2006). The amounts of gene-specific cDNAs were quantified by the comparative C_T ($\Delta\Delta C_T$) method using the Bio-Rad MyiQ Real-Time PCR Detection system with fluorescein-spiked SYBR Green as fluorophore (Malke *et al.*, 2006). PCR primers specified in Supplementary Table S1 in the online version of this paper were designed as described before (Malke *et al.*, 2006). Primer specificity was verified by running amplification plus melt-curve protocols for every target gene and experimental condition. Expression fold changes of the genes of interest were normalized for both strains to the constitutively expressed *gyrA* gene, which served as endogenous reference (see Supplementary Table S2 in the online version of this paper). Quantitative real-time PCR was performed in duplicate on RNA purified from two to four independently grown cultures. The significance of fold changes of gene expression in the mutant relative to the wild-type strain was assessed by determining statistical probabilities based on Student's two-tailed *t*-test, with *P* ≤ 0.0500 being considered significant.

RESULTS AND DISCUSSION

Gene expression analysis

c.f.u. counts show that both wild-type and the *codY1* mutant strains were capable of multiplication during exposure to blood, with any differences between them not attaining significance. This observation is relevant because it rules out the possibility that differential gene

expression was attributable to differences in growth rates between the two strains. Growth rates were highest during the first hour of growth and then declined such that c.f.u. numbers increased by a factor of ~3.2 within the 3 h incubation period (Fig. 1).

A total of 51 genes were compared between the wild-type and the *codY* mutant for *ex vivo* expression in blood using quantitative real-time PCR (Table 1). These genes included transcription factors with known targets, prominent virulence-associated genes and transporters as well as metabolic enzyme genes physiologically important to this polyauxotrophic pathogen. Compared to the trendier but inherently more variable microarray technique, this approach limited the number of genes assayed but was considered more efficient and reliable in detecting low-abundance transcripts (Graham *et al.*, 2002), and in yielding significant transcription differences with expression fold changes smaller than two (Table 1). Of the 51 genes examined, the transcription of 26 genes representing 23 transcription units was differentially affected in the two strains. Among the transcription factors, *codY* transcript levels, measured by PCR primers targeting the expressed 5′ segment of the truncated *codY1* allele (Malke *et al.*, 2006), were most strongly affected, with derepression in the mutant being highest during the first hour of blood culturing. Derepression of *codY* activity prevailed at a lower level during the next 2 h of incubation, an observation accountable for by the exhaustion of free branched-chain amino acids required for full CodY activity (Guédon *et al.*, 2001; Shivers & Sonenshein, 2004). Similar to *codY* mutant cells grown in THY broth, those cultured in blood expressed the two-component regulatory systems CovRS and SptRS at higher levels than the wild-type. In the case of *covRS*, upregulation decreased with increasing time of incubation in blood, again suggestive of decreasing CodY

Fig. 1. Growth of NZ131 wild-type and NZ131 *codY1* in human blood. Error bars indicate standard deviations calculated from four independent experiments using blood drawn on four different occasions.

Table 1. CodY-dependent gene expression in *S. pyogenes* strain NZ131 grown in human blood

Growth (h)*	Gene†	Mean FC‡	SEM§	t(df)	P¶	Functional assignment (SPy number)#
Regulatory genes						
0	<i>codY</i>	13.5	0.7	49.4(6)	<0.0001	Transcriptional pleiotropic regulator (1777)
1	<i>codY</i>	13.9	1.9	7.2(10)	<0.0001	
2	<i>codY</i>	8.8	1.1	20.8(14)	<0.0001	
3	<i>codY</i>	8.4	0.4	30.4(14)	<0.0001	
0	<i>covR</i>	1.9	0.2	6.7(4)	0.0026	Two-component response regulator (0336)
1	<i>covR</i>	2.1	0.3	6.0(14)	<0.0001	
2	<i>covR</i>	1.5	0.1	2.7(14)	0.0187	
3	<i>covR</i>	1.5	0.0	5.1(14)	0.0002	
0	<i>covS</i>	2.4	0.2	6.2(6)	0.0008	Two-component sensor histidine kinase (0337)
1	<i>covS</i>	2.3	0.5	3.7(14)	0.0024	
2	<i>covS</i>	1.6	0.1	2.6(14)	0.0233	
3	<i>covS</i>	1.2	0.1	1.4(14)	0.1842	
0	<i>fasX</i>	1.0	0.1	0.0(4)	0.9768	Regulatory RNA (–) associated with the two-component <i>fasBCA</i> system (0242–0245) (Kreikemeyer <i>et al.</i> , 2001)
1	<i>fasX</i>	0.5	0.0	3.3(10)	0.0087	
2	<i>fasX</i>	0.4	0.1	2.2(10)	0.0515	
3	<i>fasX</i>	0.4	0.0	2.5(10)	0.0293	
0	<i>mga</i>	0.3	0.0	7.0(6)	0.0196	Multiple gene transcriptional activator (2019)
1	<i>mga</i>	0.5	0.0	3.5(10)	0.0059	
2	<i>mga</i>	0.4	0.2	2.9(10)	0.0156	
3	<i>mga</i>	0.4	0.1	6.4(10)	<0.0001	
0	<i>sptR</i>	2.9	0.6	4.3(6)	0.0500	Two-component response regulator contributing to saliva persistence (0874) (Shelburne <i>et al.</i> , 2005)
1	<i>sptR</i>	1.7	0.2	3.0(10)	0.0140	
2	<i>sptR</i>	2.0	0.2	4.6(14)	0.0004	
3	<i>sptR</i>	2.0	0.4	3.4(14)	0.0043	
0	<i>sptS</i>	2.9	0.0	23.6(6)	0.0018	Two-component sensor histidine kinase contributing to saliva persistence (0875) (Shelburne <i>et al.</i> , 2005)
1	<i>sptS</i>	2.0	0.5	2.3(14)	0.0375	
2	<i>sptS</i>	2.0	0.1	4.2(10)	0.0020	
3	<i>sptS</i>	1.6	0.1	2.3(10)	0.0443	
Virulence genes						
0	<i>cfa</i>	0.7	0.0	6.2(6)	0.0035	CAMP factor haemolysin (1273)
1	<i>cfa</i>	0.6	0.2	2.3(14)	0.0349	
2	<i>cfa</i>	0.4	0.1	4.0(14)	0.0014	
3	<i>cfa</i>	0.1	0.1	5.2(14)	0.0001	
0	<i>emm49</i>	0.8	0.0	5.5(6)	0.0313	Type 49 M protein (–)
1	<i>emm49</i>	0.3	0.1	3.3(10)	0.0075	
2	<i>emm49</i>	0.2	0.0	7.6(14)	<0.0001	
3	<i>emm49</i>	0.3	0.1	4.2(10)	0.0018	
0	<i>graB</i>	49.3	2.5	71.8(6)	<0.0001	Protein G-related α_2 macroglobulin binding protein (1357)
1	<i>graB</i>	5.6	1.7	3.2(10)	0.0089	
2	<i>graB</i>	3.2	0.4	3.0(14)	0.0091	
3	<i>graB</i>	2.9	0.5	3.3(14)	0.0050	
0	<i>hasA</i>	0.4	0.0	46.2(6)	<0.0001	Capsular hyaluronate synthase A (2200)
1	<i>hasA</i>	7.7	2.1	4.9(10)	0.0006	
2	<i>hasA</i>	5.8	0.9	2.9(10)	0.0163	
3	<i>hasA</i>	6.6	0.5	4.8(10)	0.0008	
0	<i>ideS</i>	0.2	0.0	30.2(6)	<0.0001	IgG-degrading endopeptidase (0861)
1	<i>ideS</i>	0.5	0.0	5.3(6)	0.0018	
2	<i>ideS</i>	0.4	0.0	7.0(6)	0.0004	

Table 1. cont.

Growth (h)*	Gene†	Mean FC‡	SEM§	t(df)	P¶	Functional assignment (SPy number)#
3	<i>ideS</i>	0.7	0.0	6.7(6)	0.0006	NAD glycohydrolase (0165)
0	<i>nga</i>	0.5	0.1	6.3(6)	0.0008	
1	<i>nga</i>	0.5	0.1	4.6(14)	0.0004	
2	<i>nga</i>	0.4	0.1	7.5(14)	<0.0001	
3	<i>nga</i>	0.3	0.1	5.0(10)	0.0005	Cell surface proteinase (0416)
0	<i>prtS</i>	0.04	0.0	23.3(6)	<0.0001	
1	<i>prtS</i>	0.5	0.1	5.1(10)	0.0004	
2	<i>prtS</i>	0.5	0.1	9.4(10)	<0.0001	
3	<i>prtS</i>	1.2	0.2	0.4(10)	0.6978	Collagen-like surface protein (1054)
0	<i>scl</i>	0.03	0.0	16.5(6)	<0.0001	
1	<i>scl</i>	0.1	0.0	4.3(10)	0.0016	
2	<i>scl</i>	0.2	0.0	12.9(10)	<0.0001	
3	<i>scl</i>	0.3	0.1	2.4(10)	0.0404	Complement C5a peptidase (2010)
0	<i>scpA</i>	0.1	0.0	28.5(6)	<0.0001	
1	<i>scpA</i>	0.2	0.0	11.7(10)	<0.0001	
2	<i>scpA</i>	0.2	0.0	10.7(10)	<0.0001	
3	<i>scpA</i>	0.7	0.0	7.8(6)	0.0002	Streptokinase (1979)
0	<i>ska</i>	3.9	0.6	6.3(6)	0.0007	
1	<i>ska</i>	0.4	0.1	3.1(10)	0.0120	
2	<i>ska</i>	0.8	0.1	2.3(10)	0.0436	
3	<i>ska</i>	1.5	0.2	1.3(10)	0.2401	Streptolysin O (0167)
0	<i>slo</i>	0.6	0.1	3.7(6)	0.0104	
1	<i>slo</i>	0.3	0.0	3.5(14)	0.0034	
2	<i>slo</i>	0.3	0.1	7.8(14)	<0.0001	
3	<i>slo</i>	0.4	0.1	3.7(10)	0.0039	Serum opacity factor (–)
0	<i>sof</i>	0.6	0.0	23.4(6)	<0.0001	
1	<i>sof</i>	0.3	0.1	5.9(10)	0.0002	
2	<i>sof</i>	0.3	0.0	2.6(10)	0.0264	
3	<i>sof</i>	1.0	0.0	0.0(6)	0.9673	Pyrogenic exotoxin H (1008)
0	<i>speH</i>	0.2	0.0	8.9(6)	0.0009	
1	<i>speH</i>	0.3	0.0	11.7(6)	<0.0001	
2	<i>speH</i>	0.3	0.0	69.2(6)	<0.0001	
3	<i>speH</i>	0.6	0.0	6.2(6)	0.0008	
Transporters						
0	<i>braB</i>	40.6	1.2	63.2(6)	<0.0001	Branched-chain amino acid transport protein (0323)
1	<i>braB</i>	5.1	1.5	3.9(10)	0.0031	
2	<i>braB</i>	4.6	0.7	3.8(10)	0.0038	
3	<i>braB</i>	4.9	1.3	3.9(10)	0.0028	
0	<i>dppA</i>	0.3	0.0	12.2(6)	0.0003	Dipeptide ABC transporter substrate binding protein (2000)
1	<i>dppA</i>	0.7	0.1	1.3(14)	0.2029	
2	<i>dppA</i>	0.7	0.0	2.5(14)	0.0250	
3	<i>dppA</i>	0.9	0.0	2.1(14)	0.0509	
0	<i>ptsG</i>	0.6	0.2	1.8(6)	0.1452	Enzyme II of glucose-specific phosphotransferase system (1986)
1	<i>ptsG</i>	3.1	0.6	2.8(14)	0.0147	
2	<i>ptsG</i>	3.4	0.5	4.1(14)	0.0011	
3	<i>ptsG</i>	2.9	0.6	3.5(14)	0.0038	
0	<i>pyrP</i>	0.8	0.1	2.9(6)	0.0463	Uracil permease (0831)
1	<i>pyrP</i>	0.4	0.1	6.0(10)	0.0001	
2	<i>pyrP</i>	1.3	0.2	0.6(14)	0.5642	
3	<i>pyrP</i>	1.1	0.1	0.4(14)	0.7215	
Metabolic enzymes						
0	<i>arcA</i>	0.6	0.1	4.6(6)	0.0101	Arginine deiminase (1547)
1	<i>arcA</i>	6.5	1.8	6.8(6)	0.0005	

Table 1. cont.

Growth (h)*	Gene†	Mean FC‡	SEM§	t(df)	P¶	Functional assignment (SPy number)#
2	<i>arcA</i>	7.0	0.8	17.4(6)	<0.0001	Asparagine synthetase (1539)
3	<i>arcA</i>	1.6	0.3	2.9(6)	0.0281	
0	<i>asnA</i>	4.7	0.7	10.7(6)	0.0004	
1	<i>asnA</i>	1.7	0.1	5.1(6)	0.0022	
2	<i>asnA</i>	1.8	0.1	25.2(6)	<0.0001	
3	<i>asnA</i>	1.2	0.1	1.6(6)	0.1690	

*Zero hour growth point corresponding to mid-exponential phase cells (OD₆₀₀ 0.6) precultivated in THY broth.

†The following genes (SPy numbers) not included in Table 1 showed insignificant differential expression at any time point during blood culture of the two strains. Regulatory genes: *ccpA* (0514), *fasA* (0245), *msmR* (–), *phoU* (1240), *pyrR* (0830), *relA* (1981), *ropB* (2042); virulence genes: *sagA* (0738), *sagB* (0739), *sda* (2043), *speB* (2039); transporters: *malX* (1294), *oppA* (0293), *oppB* (0294), *pbuX* (1137), *potA* (1102), *pstB* (1241), *scrA* (1815); metabolic enzyme genes: *dtd* (1980), *glnA* (1877), *nadE* (1652), *pepB* (1393), *pepF* (0606), *pncA* (1776) and *xpt* (1136).

‡FC, Expression fold change in NZ131*codY1* relative to wild-type NZ131.

§SEM, Standard error of the mean expression fold change.

||t(df), Student's *t* value (including the degree of freedom).

¶P, *P* value based on Student's *t*-test, with figures in bold indicating insignificant fold changes.

#SPy numbers based on annotation of the SF370 (M type 1) genome (Ferretti *et al.*, 2001). A dash indicates that no SPy number has been assigned because the gene is absent from SF370 or assignment is pendent.

activity during the course of incubation. Contrary to the aforementioned regulatory genes, the multigene activators *mga* and *fasX* were downregulated in NZ131*codY1*, an effect not observed for *fasX* in cells grown in THY broth. The failure to find *fasA* to be downregulated as well may be explained by the observation that *fasX* shows strong independent transcription from the *fasBCA* members of the *fas* operon (Kreikemeyer *et al.*, 2001; Steiner & Malke, 2001, 2002). Thus CodY-mediated positive control of *fasX* transcription appears to depend on the blood environment and to be directed toward the independent mode of transcription of the terminal member of the *fas* operon.

This finding is important in view of the fact that FasX was found to stimulate streptococcal aggressiveness towards host cells by promoting cell adherence and internalization, cytokine expression and release, as well as host cell apoptosis (Klenk *et al.*, 2005).

Consistent with underexpression of *mga* in the *codY1* mutant strain, virulence genes known to be activated by Mga (*emm49*, *scl*, *scpA*, *sof*) (Hynes, 2004) were strongly downregulated in blood as well, with underexpression tending to become less severe, or even absent in the case of *sof*, towards the end of the exposure time. Additional

Table 2. NZ131 genes displaying a CodY motif in their upstream region

SPy number*	Gene	Motif†	Position/ATG‡	(Putative) Functional assignment
0293	<i>oppA</i>	AATaTTCAGAtAATT	–110	Oligopeptide periplasmic binding protein
0323	<i>braB</i>	AATTTTCAGtcAATT	–151	Branched-chain amino acid transport protein
0647	<i>budC</i>	tAgTTTCAGAAAATa	–152	Acetoin reductase
0870	<i>fms</i>	AATaTTCAGAAAATT	–385	Formylmethionine deformylase
1414	–	AATTTTCTGAAAAG	–78	Potassium uptake protein
1654	<i>aapA</i>	AATTTTCTGAAAaAa	–38	Amino acid permease
1676	<i>tkl</i>	gATTTTCTGAAAAG	–73	Transketolase
1738	<i>manL</i>	AtTTTTCAGAAAATg	–320	Mannose-specific phosphotransferase component IIAB
1758	<i>phaB</i>	AATTaTCAGAAAAGa	–8	Enoyl-CoA hydratase
1777	<i>codY</i>	AATTTTCTGAtAATT	–66	Transcriptional pleiotropic regulator
1825	<i>uvrA</i>	gATTTTCTGAAAaT	–85	UvrABC system protein A
1827	–	AtTTTTCAGAAAATc	–71	Divalent cation (Mg ⁺⁺) transport protein
1881	<i>pgk</i>	tATTTTCTGAAAATa	–117	Phosphoglycerate kinase
2150	<i>argR</i>	AtTTTTCAGAAAATg	–259	Arginine repressor
2151	<i>argS</i>	cATTTTTCAGAAAaT	–118	Arginyl-tRNA synthetase

*Based on annotation of the SF370 (M type 1) genome (Ferretti *et al.*, 2001).

†Lower-case letters indicate mismatches from the consensus sequence AATTTTCWGAATAATT (den Hengst *et al.*, 2005; Guédon *et al.*, 2005).

‡Distance (number of bases) of the CodY motif from the translational start codon.

prominent virulence factors showing decreased transcript levels in the mutant included *cfa*, *ideS*, *nga*, *slo*, *ska* and *speH*, genes often influenced in an incubation time-dependent fashion. Of the latter, *ska* and *ideS* are under CovR control (Federle *et al.*, 1999; Hynes 2004) and apparently were negatively affected by the increased CovR levels in the mutant. Additionally, decreased levels of FasX in the *codY1* mutant may contribute to the downregulation of *ska*, which is under positive FasX control (Kreikemeyer *et al.*, 2001). Of relevance, CodY-mediated stimulation of *ska*, *slo* and *ideS* expression implicates this regulator in the positive control of genes encoding nutrient-releasing proteins, thus tying in with a role in not only sensing nutrient availability but also contributing to nutrient release. In contrast to the majority of the virulence genes with lower activity, two important virulence factors, *graB* and *hasA*, showed highly increased transcript levels in the *codY1* mutant. Interestingly, for *hasA*, this behaviour was discordant with transcription in THY broth but similar derepression of the *has* operon has been observed in a *covR* mutant during growth in blood (Graham *et al.*, 2005), consistent with negative transcriptional control of the *has* genes by CovR.

Transporters strongly upregulated during *codY1* mutant growth in blood included *braB* and *ptsG*. Of special relevance is *braB*, which is also under strong negative stringent control during isoleucine plus valine starvation (Malke *et al.*, 2006). This shows that the wild-type, in repressing *braB* during growth in complex media, exploits a nutritional status-specific, cost-effective means for the abrogation of futile transport processes. Repression in the wild-type of glucose transport (*ptsG*) as well as temporal repression of amino acid synthesis or amino acid catabolic enzymes (*asnA* and *arcA*, respectively) may be regarded as examples of the involvement of CodY in the control of basic metabolic processes during growth in blood. Concerning arginine deiminase encoded by *arcA*, this enzyme, by releasing ammonia from arginine, might also support the survival of *S. pyogenes* under acidic conditions such as those present in phagolysosomes (Degnan *et al.*, 2000).

Identification of the CodY box in NZ131

A 15-nucleotide palindromic sequence (AATTTTCWGA-AAATT) serving as a high-affinity binding site for CodY has recently been identified in *Lactococcus lactis* (den Hengst *et al.*, 2005; Guédon *et al.*, 2005). Derivatives of the CodY box with high similarity scores were also detected by *in silico* analysis in the upstream regions of genes from other Gram-positive bacteria (den Hengst *et al.*, 2005; Guédon *et al.*, 2005), including five genes from *S. pyogenes* (Guédon *et al.*, 2005). Using BLAST searches (www.ncbi.nlm.nih.gov) against the genomic sequence of NZ131 and a threshold value of 80 % sequence identity (three mismatches accepted), we detected the CodY box in the upstream regions of 15 NZ131 genes. These included *codY*, *braB* and *oppA* (Table 2), genes also found by Guédon *et al.* (2005) in strains of M types other than M49. While *codY* and *braB* were strongly

responsive to CodY repression in the present experiments (Table 1), *oppAB* repression was evident only in laboratory media (Table 1, footnote†; Malke *et al.*, 2006). This provides another example of environmentally dependent gene responses to the action of CodY. As *codY* is transcribed both monocistronically and dicistronically from the co-oriented upstream *aat* gene encoding aspartate aminotransferase (Malke *et al.*, 2006), it is interesting to point out that the CodY box precedes only the coding region of *codY* but not *aat*. This suggests that negative transcriptional autoregulation of *codY* applies only to monocistronic transcription of this gene. The dicistronic mode adds a strong autonomous component to *codY* expression, a phenomenon not observed in the nonpathogenic *Bacillus subtilis* and *L. lactis* species (den Hengst *et al.*, 2005; Guédon *et al.*, 2005; Sonenshein, 2005). Confirming this notion, the results of previous experiments indicated that *aat* is not under the control of CodY (Malke *et al.*, 2006), and the same situation applies to the organization and expression pattern of the *aat* and *codY* genes from the M1 strain SF370 (Ferretti *et al.*, 2001; Malke *et al.*, 2006). This situation may have developed to facilitate growth of this fastidious organism under various environmental conditions.

Table 2 shows that the majority of genes carrying the CodY box encoded regulators, transporters or proteins involved in nitrogen and carbon metabolism. The genes with CodY motifs detected in NZ131 were also present in all GAS genomes accessible at www.ncbi.nlm.nih.gov. As an interesting detail, the CodY box precedes the coding sequence of a Mg⁺⁺ transport protein (Spy1827). Since the CovS sensor histidine kinase is specifically activated by environmental magnesium (Gryllos *et al.*, 2003), these observations may provide a clue as to how the CodY and CovRS systems interact during GAS growth in human body fluids, which contain low magnesium concentrations (Gryllos *et al.*, 2003): CodY-mediated repression of magnesium uptake would place the CovRS system in an inactive state, resulting in the derepression of virulence factors controlled by CovR. There were also two instances where the CodY box was located in the intergenic region between adjacent genes with opposite orientations. Most interestingly, these included *argR* and *argS*, strongly suggesting that the arginine repressor and arginyl-tRNA synthetase are coregulated by CodY. Notably, all virulence factors responsive to CodY action in this and a previous study (Table 1; Malke *et al.*, 2006) lacked the CodY box. In line with this observation, a substantial proportion of the known CodY target genes of *B. subtilis* and *L. lactis* do not display the CodY box (den Hengst *et al.*, 2005; Guédon *et al.*, 2005). In the same vein, the most differentially expressed *S. pyogenes* genes responsive to CovR action do not reveal a conserved sequence motif in their regulatory region (Federle & Scott, 2002; Graham *et al.*, 2002). Thus higher levels of degeneracy of the CodY box, higher order DNA structures enabling CodY binding, or, most likely, indirect effects caused by the amplification of CodY action through its interaction with other regulators may contribute to the broad action of this

regulator (den Hengst *et al.*, 2005; Guédon *et al.*, 2005; Malke *et al.*, 2006; Serror & Sonenshein, 1996).

Conclusions

The present data provide strong evidence that the pleiotropic action of CodY observed in complex or defined laboratory media (Malke *et al.*, 2006) extends to the human blood environment which, despite the experimental curtailment involved in *ex vivo* cultivation (Graham *et al.*, 2005), is of greater clinical relevance than the artificial media. This is highlighted by our observation that transcriptional responses directed by CodY are not always concordant during growth in laboratory media versus growth in blood, suggesting the action of biological cues which are missing in THY broth or chemically defined medium. From the viewpoint of a more comprehensive understanding of virulence regulation, it is apparent that CodY contributes to the regulation of expression of key virulence genes. The observations that many virulence genes are upregulated in the wild-type following the initial phases of blood culture (Graham *et al.*, 2005) but remain down-regulated in the *codY1* mutant (Table 1) strongly suggest that CodY contributes to the dynamics of gene expression during GAS growth in blood. As in the case of other pleiotropic regulators acting in GAS (Chaussee *et al.*, 2004; Graham *et al.*, 2002, 2005), more information is necessary to distinguish between direct and indirect CodY effects on gene expression. Genes showing the highest differential expression levels in the strain pair used here would appear to qualify as prime candidates for being direct CodY targets (*codY*, *braB*, *graB*, *scl* and *scpA*). However, this assumption seems to be coherent with the presence of the CodY box only for *codY* itself and *braB*. Concerning the majority of the virulence genes that show transcription differences between the wild-type and the *codY* mutant, it seems likely that indirect actions of CodY as component of a regulatory network are involved. In any case, any indirect CodY effects do not diminish the physiological role of CodY and its clinical relevance. A particularly interesting situation with the potential of shedding additional light on the interaction of CodY and CovR has recently been discovered by Gusa *et al.* (2007). They found that CovR stimulates *dppA* transcription *in vivo* but not *in vitro* using purified RNA polymerase and major sigma factor from GAS. As not only CovR but also CodY is required for activation of *dppA* transcription *in vivo* (Malke *et al.*, 2006 and Table 1; Gusa *et al.*, 2007), CodY might act as co-activator of CovR in promoting *dppA* expression (Gusa *et al.*, 2007). Clearly, a *codY-covR* double mutant may help unravel the complex mechanism of *dppA* expression and possibly that of other multiply controlled GAS genes.

ACKNOWLEDGEMENTS

We thank C. Bennet for phlebotomy and M. McShan for providing the genomic sequence of strain NZ131 before publication. This work was supported by NIH/NIAID grant AI054473 to H. M.

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