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Identification of differentially expressed genes in a *Giardia lamblia* WB C6 clone resistant to nitazoxanide and metronidazole

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***Objectives*: The characterization of differential gene expression in *Giardia lamblia* WB C6 strain C4 resistant to metronidazole and nitazoxanide using microarray technology and quantitative real-time PCR.**

***Methods*: In a previous study, we created and characterized the *G. lamblia* WB C6 clone C4 resistant to nitazoxanide and metronidazole. In this study, using a microarray-based approach, we have identified open-reading frames (ORFs) that were differentially expressed in C4 when compared with its wild-type WB C6. Using quantitative real-time PCR, we have validated the expression patterns of some of those ORFs, focusing on chaperones such as heat-shock proteins in wild-type and C4 trophozoites. In order to induce an antigenic shift, trophozoites of both strains were subjected to a cycle of en- and excystation. Expression of selected genes and resistance to nitazoxanide and metronidazole were investigated after this cycle.**

***Results*: Forty of a total of 9115 ORFs were found to be up-regulated and 46 to be down-regulated in C4 when compared with wild-type. After a cycle of en- and excystation, resistance of C4 to nitazoxanide and metronidazole was lost. Resistance formation and en-/excystation were correlated with changes in expression of ORFs encoding for major surface antigens such as the variant surface protein TSA417 or AS7 ('antigenic shift'). Moreover, expression patterns of the cytosolic heat-shock protein HSP70 B2, HSP40, and of the previously identified nitazoxanide-binding proteins nitroreductase and protein disulphide isomerase PDI4 were correlated with resistance and loss of resistance after en-/excystation. C4 trophozoites had a higher thermotolerance level than wild-type trophozoites. After en-/excystation, this tolerance was lost.**

***Conclusions*: These results suggest that resistance formation in *Giardia* to nitazoxanide and metronidazole is correlated with altered expression of genes involved in stress response such as heat-shock proteins.**

Keywords: drug resistance, microarray analysis, differential gene expression, antigenic variation, encystation, excystation

Introduction

Giardia lamblia (syn. *Giardia duodenalis*; *Giardia intestinalis*), a flagellated protozoan, is the most common causative agent of persistent diarrhoea worldwide.[1] The life cycle of *G. lamblia* includes two major stages: the proliferative trophozoite and the non-proliferative, infectious cyst. Infection occurs upon peroral ingestion of cysts. Following excystation in the upper part of the small intestine, cysts release an excyzoite, a transient stage of the life cycle, immediately dividing into four trophozoites.[2] As demonstrated *in vitro* in the case of *G. lamblia* isolate WB, clone C6, the process of excystation coincides with a change in the expression of the major surface antigens, namely variant surface proteins (VSPs).[3]

Since metronidazole (commercially known as Flagyl®) and other nitroimidazoles efficiently kill *Giardia* trophozoites *in vitro*, these drugs have been used as therapy of choice against giardiasis.[4,5] Metronidazole efficiently enters giardial trophozoites and is supposed to be reduced into a toxic nitro radical by the metabolic anaerobic enzyme pyruvate:ferredoxin

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oxidoreductase (POR).[6] Resistance to metronidazole and other nitroimidazoles has been induced *in vitro*, and various genotypically distinct isolates with reduced drug susceptibility have also been found in human patients.[4,7] Formation of giardial resistance to metronidazole is associated with down-regulation of the POR activity.[5] This is consistent with the involvement of POR in metronidazole activation.[8]

In the mid-1990s, a new antiparasitic agent, the thiazolide nitazoxanide (commercially known as Alinia®), was introduced to the market.[9,10] Nitazoxanide, a nitrothiazolyl-salicylamide, is the first agent with proven efficacy against cryptosporidiosis and has been approved in the USA for the treatment of cryptosporidiosis and giardiasis in children and adults.[9] Upon oral uptake, nitazoxanide is rapidly deacetylated to tizoxanide and further metabolized to tizoxanide–glucoronide.[9] Tizoxanide has been reported to display antimicrobial activity similar to nitazoxanide, whereas tizoxanide–glucuronide is largely inactive against a number of pathogens.[11] Tizoxanide and tizoxanide–glucuronide are further metabolized, or excreted, in urine and/or bile and faeces.

Thiazolides without the thiazole-associated nitro group have been shown to exhibit decreased efficacy against *Giardia*.[12,13] Therefore, an involvement of the nitro group in the mechanism of action, with the participation of POR similar to metronidazole, has been postulated.[14] The nitro group is also required for the *in vitro* activity against anaerobic bacteria.[15] In *G. lamblia*, we have identified and characterized a nitroreductase (NR) as a nitazoxanide-binding protein.[16] Inhibition of the anaerobic energy metabolism through nitazoxanide by interfering in functional activity of POR has recently been reported for other protozoan parasites,[17] but this process is thought to be only one of several pathways by which the drug exhibits antiprotozoal activity.[9] In fact, more recent studies have shown that nitazoxanide derivatives lacking the nitro group exhibit profound *in vitro* activity against intracellular pathogens such as *Neospora caninum*, *Cryptosporidium parvum* and *Besnoitia besnoiti*,[11,18] suggesting that the nitro group is not required for the activity against intracellular parasites.

In order to elucidate the biochemical nature of resistance formation to nitazoxanide and metronidazole, we have generated metronidazole- and nitazoxanide-resistant *G. lamblia* clones and compared them to the wild-type WB C6 with respect to their growth behaviour and to the expression pattern of genes that are potentially involved in resistance formation.[19] In order to identify novel genes that could be involved in resistance formation, we have chosen a microarray-based screen in order to identify genes that are differentially expressed in the previously characterized, double-resistant strain C4 by a factor of 4 or more when compared with wild-type trophozoites. Some of the positive hits were then further investigated by RT–PCR in independent experiments. Respective gene expression patterns were also determined for trophozoite populations that, after a cycle of en- and excystation, had completely lost resistance to both nitazoxanide and metronidazole.

Materials and methods

Tissue culture media, biochemicals and drugs

Unless otherwise stated, all biochemical reagents were from Sigma (St Louis, MO, USA). Nitazoxanide was synthesized at the Department of Chemistry and Biochemistry, University of Berne (C. Leumann). The structural formulas are described elsewhere.[13] Nitazoxanide and metronidazole were kept as 100 mM stock solutions in DMSO at 4°C.

Axenic culture of Giardia trophozoites and en-/excystation of the parasite

Trophozoites from *G. lamblia* WB clone C6 were grown under anaerobic conditions in 10 mL culture tubes (Nunc, Roskilde, Denmark) containing modified TYI-S-33 medium as described previously.[13] In order to initiate subcultures, cultures with confluent trophozoite lawns were incubated on ice for 15 min. Suspended living (adherent) trophozoites were counted (Neubauer chamber, 200×). Subcultures were initiated by adding 10^4 trophozoites to a new culture tube. The double-resistant clone C4 was cultivated in the presence of 40 μM nitazoxanide.

In vitro induction of encystation of the parasite was performed as described previously.[20] For *in vitro* excystation, 2-day encysting cells were water-treated at 4°C to destroy trophozoites and any incompletely formed cysts. Approximately, 1×10^5 of pre-treated cysts were excysted by using a two-step procedure that included: (i) a 20 min incubation at 37°C in a low pH excystation solution (pH 4.0) containing reduced glutathione and L-cysteine in Hanks buffer (stage I) and (ii) a 1 h of incubation at 37°C in excystation stage II solution (pH 8.0) containing 1 mg/mL trypsin (Type 2, from porcine pancreas, Sigma) in Tyrode's salt solution.[21] Excysted trophozoites were grown in drug-free, modified TYI-S-33 medium until near confluency was reached and then subcultivated in order to test both drug resistance and thermotolerance as described below.

In order to determine the IC_{50} values of different *G. lamblia* lines, 10 mL trophozoite cultures were grown at different drug concentrations as described previously.[13] For thermotolerance experiments, trophozoites were inoculated in 24-well plates (10^4 in 1 mL medium per well) and incubated for 2 days in anaerobic growth chambers at temperatures ranging from 37 to 43°C.

Processing of RNA samples and cDNA synthesis

To quantify gene expression by real-time RT–PCR, trophozoites of wild-type WB C6 and resistant clone C4 were grown until near confluency was reached. Cells were harvested as described, and RNA was extracted using the Qiagen RNeasy™ kit, including a DNase I digestion (to remove residual genomic DNA) according to the instructions provided by the manufacturer. RNA was eluted with 50 μL of RNase-free water and stored at −80°C.

Synthesis of cDNA for quantitative RT–PCR was performed using the Qiagen Omniscript™ kit as described.

For synthesis of aminoallyl-cDNA for microarray analysis, the reaction mixture contained 5 μL of Omniscript buffer 10×, 2.5 μL of random primer (Promega), 0.6 μL of RNAsin (40 U/μL; Promega), 2.5 μL of Omniscript RTase, 0.6 μL of dNTP (25 mM; aminoallyl-dUTP 2:1), 5 μL of DTT (0.1 M), 1 μg of RNA and water of highest purity in a total volume of 50 μL. The reaction was incubated overnight at 37°C. Then the reaction was stopped by adding 10 μL of EDTA (0.5 M) and 10 μL of NaOH (1 M). The reaction was incubated for 10 min at 65°C in order to hydrolyse RNA and was neutralized by adding 30 μL of Tris Cl− (1 M, pH 7). Aminoallyl-cDNA was purified using a Roche PCR purification kit (Roche, Basel, Switzerland). Then, 500 μL of binding buffer from the kit was added to the sample, and the sample was applied to the column and centrifuged. In order to avoid the carry-over of amino groups to the subsequent labelling reaction, the column was

washed first with 500 μL and then with 200 μL of phosphate wash buffer (5 mM K-PO_4, pH 8.5, 80% EtOH). Subsequently, cDNA was eluted with 2× 50 μL of phosphate elution buffer (4 mM K-PO_4, pH 8.5), lyophilized and stored at −20°C.

Dye labelling of aminoallyl-cDNA

For dye labelling,[22] lyophilized aminoallyl-cDNA was suspended in 5 μL of 100 mM $NaCO_3$, pH 9.3. The appropriate dye, namely Cy3 or Cy5 mono-reactive (GE Healthcare, Little Chalfont, UK), was dissolved in 75 μL of DMSO, and 5 μL was added to the cDNA. The remaining dye was stored at −20°C. The reaction was incubated for 2 h at room temperature in the dark and stopped by adding 35 μL of 100 mM Na-Acetate, pH 5.2. Dye-labelled cDNA was purified by Roche PCR purification kit according to the protocol. Eluted cDNA was lyophilized and stored in the dark until microarray hybridization.

Microarray hybridization

The microarrays were kindly provided by the Pathogen Functional Genomics Research Center (J. Craig Venter Institute, Rockville, MD, USA). The arrays (*Giardia lamblia* microarrays version 1) contained 19 230 elements consisting of duplicates of 70-mer oligomers derived from 9115 predicted open-reading frames (ORFs) including the clearly identified 6470 ORFs of the genome of *G. lamblia* WB C6[23] as described in the *Giardia* database[24] and of 500 *Arabidopsis thaliana* control oligomers. To pre-hybridize, slides were incubated in Falcon tubes containing 50 mL of 5× SSC, 0.1% SDS and 12.5 mg of herring sperm DNA at 55°C for 1 h. Slides were then washed in deionized water for 2 min at continuous shaking. Washes were repeated five to six times. Slides were then dipped into isopropanol for 2 min, dried under an air-stream and immediately hybridized.

For hybridization, labelled aminoallyl-cDNAs were dissolved in 30 μL of hybridization buffer, i.e. sterile-filtered pre-hybridization buffer containing 0.1 mM DTT and denatured for 10 min at 95°C followed by cold shock on ice. For each hybridization, one Cy3- and one Cy5-labelled cDNA were combined yielding ∼60 μL of probe mix. Then, 40 μL of probe mix was added to a pre-hybridized slide and covered with a 24 × 60 mm clean, isopropanol washed, dry cover slip.

Slides were then placed into a 50 mL Falcon tube on top of a paper strip wetted with hybridization buffer, placed horizontally into a 55°C oven and incubated for 3 h.

After hybridization, slides were dipped into low stringency buffer (2× SSC, 0.1% SDS) until the cover slip loosened and washed at low stringency for another 20 min, followed by a wash at medium stringency (1× SSC) for another 20 min and a wash at high stringency (0.1× SSC, 2% EtOH) for another 5 min. All washes were performed at room temperature in the dark. Slides were then dried in an air-stream and scanned with a GenePix Personal 4000B scanner (Molecular Devices, Sunnyvale, CA, USA). Annotation and evaluation of the hybridized slides were performed using the GenePix Pro software package. Two independent hybridizations (two screens) were performed. Prior to analysis, signals were normalized by the GenePix Pro software. Signal strengths were defined as median feature pixel intensity minus the median feature background intensity at 535 nm (Cy3-labelled probes) or 632 nm (Cy5-labelled probes). For the screens presented here, only signal strengths higher than 800 were retained. The corresponding ORFs were regarded as differentially expressed when the ratio between signals at 535 and 632 nm was higher than 4.

Quantitative RT–PCR

First-strand cDNA was synthesized using the Qiagen Omniscript™ RT kit as described by the manufacturer with a polyT-ANC primer[25] for subsequent real-time PCR (for primer sequences, see Table 1). Quantitative PCR was performed with 4 μL of 1:100 diluted cDNA using the Quanti Tect™ SYBR Green PCR Kit (Qiagen) in a 10 μL standard reaction containing a 0.5 μM concentration of forward and reverse primers (MWG Biotech, Ebersberg, Germany). Furthermore, a control PCR included RNA equivalents from samples that had not been reverse transcribed into cDNA (data not shown) to confirm that no DNA was amplified from any residual genomic DNA that might have resisted DNase I digestion (see above). PCR was started by initiating the 'Hot-Start' *Taq* DNA-polymerase reaction at 95°C (15 min). Subsequent DNA amplification was performed in 40 cycles including denaturation (94°C for 15 s), annealing (60°C for 30 s) and extension (72°C for 30s); temperature transition rates in all cycle steps were 20°C/s. Fluorescence was measured at 79°C (TSA417 and VSPtot-PCR) or 82°C (all other PCRs listed in Table 1) during the temperature shift after each annealing phase. For statistical analysis, five independent experiments were performed. Expression levels of the genes summarized in Table 1 were given as values in arbitrary units relative to the amount of constitutively expressed 'house keeping' gene ACT.

Statistics

IC_{50} values were calculated after to the logit–log transformation of the relative growth (RG; control = 1) according to the formula $\ln[(RG/(1 - RG)] = a \times \ln(\text{drug concentration}) + b$ and subsequent regression analysis. Regression analysis, analysis of variance and subsequent pairwise *t*-tests were performed using the corresponding software tool contained in the Excel software package (Microsoft, Seattle, WA, USA).

Results

Microarray screen for differentially expressed ORFs

In order to identify differentially expressed ORFs in the nitazoxanide-resistant clone C4 versus wild-type cells, as a first step, two microarray-based screens were performed [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. We considered ORFs as up- or down-regulated, when in at least one screen, a >4-fold difference between wild-type and C4 cDNA hybridization signals ($\log_2$ ratio of wild-type versus C4 signals >2 or <−2) was found. The mRNA corresponding to 40 ORFs was found to be up-regulated in C4 versus wild-type cells, and the mRNA corresponding to 46 ORFs was found to be down-regulated in C4 versus wild-type cells (Table 2).

Besides variable surface antigens, ORFs encoding for proteins potentially involved in cell signalling such as protein kinases and phosphatases were differentially regulated in wild-type versus C4 trophozoites. More striking were the differences in ankyrin-domain proteins (protein 21.1) with eight ORFs being up-regulated and one down-regulated, and chaperonins with five up-regulated ORFs. Among the chaperones ORFs up-regulated in C4 versus wild-type, three were heat-shock proteins (one HSP70 and two HSP90).

Table 1. Overview of primers used in this study

Gene, accession number or ORF	Region of CDS	Primer (5′→3′)
21.1 protein (21.1) ORF:12 139	CDS 1773–2046 plus 16 of 3′ utr	12139F GAACGACCGCACTCATGGA
		12139R GGTCCACACGCCGTGTTC
Actin (ACT) EAA39190	CDS 715–933	ACTquantF ACATATGAGCTGCCAGATGG
		ACTquantR TCGGGGAGGCCTGCAAAC
Chaperonin cpn60 (cpn60) ORF:7031	CDS 2744–2988 plus 14 of 3′ utr	7031F CTAACACGGCATCGGTACCA
		7031R GTTTCAATCCTAAATCACGGT
Co-chaperone p23 (cpnp23) ORF:10429	CDS 350–558 plus 13 of 3′ utr	10429F CCAACCCCAACGACATGGA
		10429R AATTGCCAGGCATTTACTCCT
Cysteine rich surface antigen ORF:10659	CDS 2003–2176 plus 34 of 3′ utr	10659F GGGGTGACTACAAGCTGGA
		10659R CATTGGTGTGGCCGCTTAC
Cyst wall protein 1 (CWP1) XM 766142.1	CDS 367–713	CWP1quantF GGCGATATTCCCGAGTGCATGTG
		CWP1quantR GTGAGGCAGTACTCTCCGCAGT
Glutamate dehydrogenase (GDH) XM_773614	CDS 761–893	GDHquantF AGGTCCTCACCTTCTCAGACT
		GDHquantR GGATACTTGTCCTTGAACTCGG
Heat-shock protein 40 (hsp40) ORF:17483	CDS 817–990 plus 28 of 3′ utr	17483F GTATCAGAGGTCATCATCCC
		17483R CGAAAAGCAGGAGTAGGGG
Heat-shock protein 70 B2, cytosolic form (hsp70B2) ORF:88765	CDS 1818–1995 plus 16 of 3′ utr	88765F GAGGCGATCGTCCATCCC
		88765R CCTTACACCACAGTTAGTCC
Heat-shock protein 70, ER form (erhsp70) ORF:17121	CDS 2204–2434 plus 45 of 3′ utr	17121F AACATCGTTGACAAGATCTCC
		17121R CATTGGTTAGTTTACAAGACCT
Heat-shock protein 70, mitochondrial form (mthsp70) ORF:14581	CDS 1733–1923 plus 55 of 3′ utr	14581F TCTTGTCCAAAGATATCGTGG
		14581R GGATGGGCGGCAGATACC
Heat-shock protein 90 (hsp90_1) ORF:13864	CDS 752–975 plus 27 of 3′ utr	13864F ACGACGACGCAGAGAACCT
		13864R CAAAGTAACACAACAAGTTCAC
Heat-shock protein 90 (hsp90_2) ORF:98054	CDS 846–1051 plus 39 of 3′ utr	98054F CCGAGGACGAGTACAAGGA
		98054R GGGGTGTACTTGTTGCACC
Nitroreductase (NR) EAA43030.1	CDS 526–794 plus 8 of 3′ utr	NRquantF CCTGCTGACAAGGCCGCA
		NRquantR AACACCAATTACTTAAATGTAATG
Protein disulphide isomerase 2 (PDI2) EAA42483.1	CDS 1055–1350 plus 13 of 3′ utr	PDI2quantF GGCCCAGGGCGAGGAGT
		PDI2quantR AGACAAGAACCGTTTACTTCTT
Protein disulphide isomerase 4 (PDI4) AF295634.1	CDS 775–1065 plus 1 of 3′ utr	PDI4quantF CCGAAGGACGAGACTTCCT
		PDI4quantR CTCAGAGCTCCTTGTCCCC
Pyruvate oxidoreductase 1 (POR1) ORF:17063	CDS 3341–3600 plus 9 of 3′ utr	POR1quantF ATCCAACGCGACCCAGAAG
		POR1quantR GTTCACTGCTTACTCCGCC
Pyruvate oxidoreductase 2 (POR2) ORF:114609	CDS 3325–3547	POR2quantF CTCGCACATGGTCCAGGG
		POR2quantR AGAGCCGCAGCCATCTCC
Variant surface protein (total)	see Nash and Mowatt[26]	VSP_{tot}quantF (MM16) GGCTTCCTCTGCTGGTGGTTC
		VSP_{tot}quantR (ANC) GACCACGCGTATCGATGTCGA
Variant surface protein TSA417 U89152.1	CDS 127–335	TSA417quantF TGTGGAACGTGTGCCAATAG
		TSA417quantR AGACACGTAGTACAGTCGG
Variant surface protein VSPAS7 ORF:137740	CDS 1171–1356 plus 25 of 3′ utr	137740F TGAGCACAGACGTCGATGG
		137740R ACTATCTAAGTACACAGCTAAG
Variant surface protein VSPAS7A ORF:98058	CDS 90–264 plus 52 of 3′ utr	98058F CCATCTAATAATCAAGGCTCC
		98058R AGTGGGCAGGTGCAATAGG

CDS, coding sequence; ORF, referring to *Giardia* database[24]; utr, untranslated.

Expression of chaperone genes

Given the custom-made nature of the microarrays, we validated the results obtained in the two screens by quantitative real-time RT–PCR assays using reverse transcripts of actin (ACT) as reference for the amount of total cDNA. The gene expression level of actin was not affected in the clone C4 when compared with the wild-type (see Müller *et al.*[13]). In a first study, quantitative real-time PCR was performed on chaperone ORFs that were found positive in the screens. As a non-chaperonin, we included one ankyrin ORF (protein 21.1.). The cytosolic HSP70 isoform B2 transcript had the highest expression level of all chaperones, and was nearly 10 times higher in C4 than in wild-type cells. HSP90 was expressed at levels three orders of magnitude lower. Expression was nearly three times higher in C4 (Figure 1a). Co-chaperone p23, chaperonin 60 and protein 21.1 had expression levels one order of magnitude lower than HSP70

Table 2. Overview of ORFs expressed in nitazoxanide/metronidazole double-resistant C4 trophozoites with a >4-fold difference to wild-type trophozoites in at least one of two independent microarray-based screens

(Hypothetical) function	Protein	ORF number in *Giardia* database	Log_2 ratio (C4/wild-type) screen 1	Log_2 ratio (C4/wild-type) screen 2
Up-regulated in C4 versus wild-type				
Chaperonin	co-chaperone p23 homologous	10429	2.31	3.18
	heat-shock protein 70 B2	88765	2.62	3.49
	heat-shock protein 90	13864	1.98	2.85
		98054	1.88	2.75
	spindle pole associated chaperonin 60	7031	1.96	2.83
Cytoskeleton, adhesion and organelle transport	ankyrin-repeat proteins (protein 21.1)	8174	1.26	2.13
		12139	2.26	3.13
		14859	1.66	2.67
		16532	1.80	2.66
		17046	1.89	2.76
		17551	1.91	2.78
		27925	2.02	2.89
	giardin	4812	1.75	2.62
Intermediary metabolism	carbamate kinase	16453	1.77	2.64
	cysteine protease	114773	1.22	2.80
Protein (de) phosphorylation	CDC14 phosphatase	9270	1.22	2.19
	NAF-domain containing protein kinase	16235	1.37	2.24
	NimA related protein kinase	8445	1.33	2.20
	protein kinase family protein	17558	1.38	2.25
	thymidine kinase	8364	1.46	2.33
Surface antigen	variant specific surface protein AS7	137740	1.24	2.10
	variant specific surface protein VSP9B10	115066	1.28	2.15
	VSP; cysteine rich protein	38901	1.01	2.46
	hypothetical protein	(17 ORFs)		
Down-regulated in C4 versus wild-type				
Cell division and gene expression	bacterial-like Sir2 family protein	16569	−4.08	−4.50
	cell division control protein 16	15248	−2.57	−4.41
	spliceosome-associated protein	13464	−2.58	−3.52
	valyl-tRNA synthetase	35428	−1.47	−2.75
Cytoskeleton, adhesion and organelle transport	ankyrin-repeat proteins (protein 21.1)	14872	−3.86	−8.35
	dynein regulatory complex	16540	−1.71	−3.24
	kinesin-9	6404	−3.86	−8.21
Intermediary metabolism	*N*-acetyl-glucosaminyl-phosphatidylinositol synthesis protein	113610	−4.81	−7.91
	cathepsin-L-like protease	9548	−3.37	−3.04
Protein (de) phosphorylation	serine/threonine protein kinase SKS1	14578	−2.86	−2.66
Surface antigen	trophozoite cysteine rich surface antigen 170	24391	−2.52	−1.65
	trophozoite cysteine-rich surface antigen 72	111874	−2.11	−1.23
		113954	−2.26	−1.39
	variant specific surface protein 417	97820	−3.21	−2.34
		113797	−3.72	−2.85
	variant specific surface protein AS12	114672	−2.20	−1.33
	variant-specific surface protein	113450	−3.13	−2.26
	variant-specific surface protein AS7A	98058	−2.48	−1.61
	hypothetical protein	(28 ORFs)		

Log_2 ratio (C4/wild-type) corresponds to the mean value of two technical replicates of the signal strengths of C4 cDNA versus wild-type cDNA in two independent microarray-based screens.

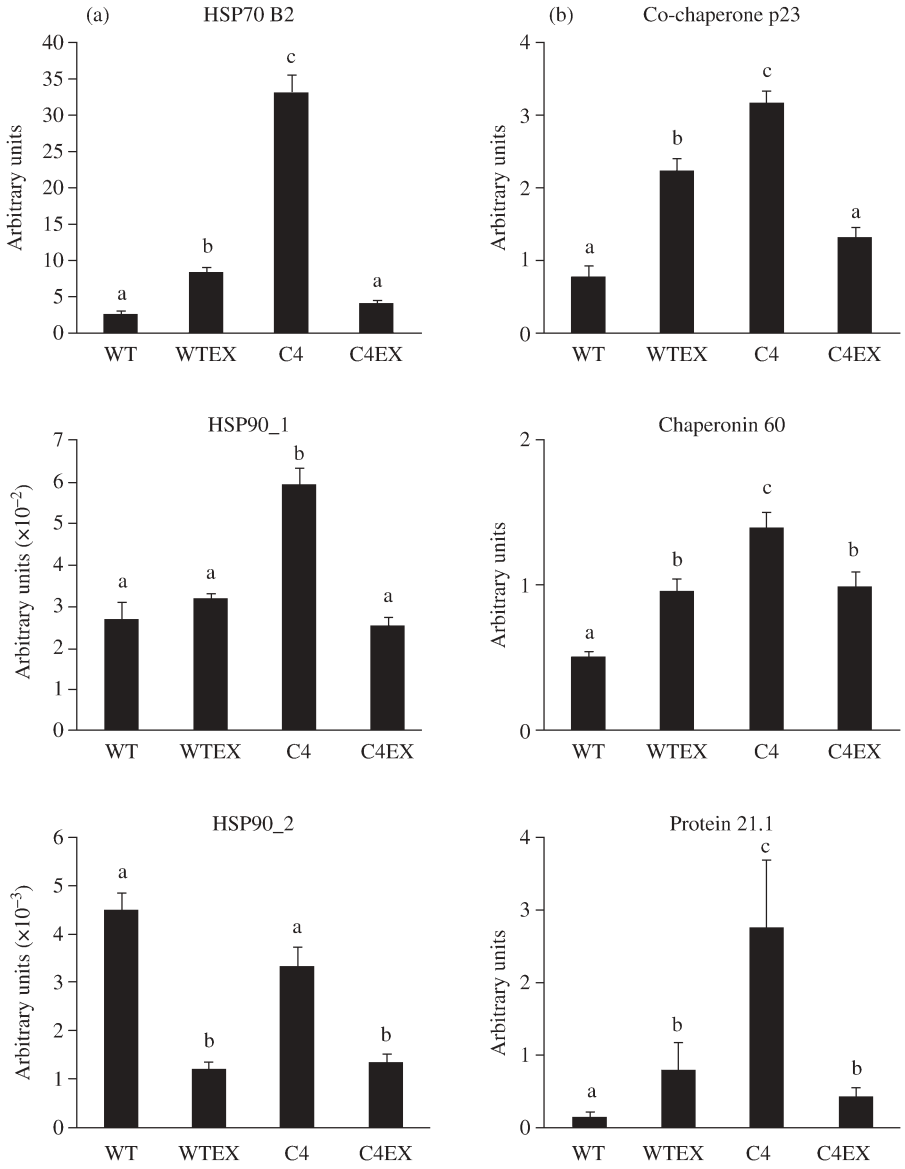

Figure 1. Quantification of gene expression in wild-type (WT) and nitazoxanide/metronidazole-resistant (C4) trophozoites and in the same lines (WTEX and C4EX) after en-/excystation by RT–PCR. Trophozoites were grown in normal culture medium until near confluency was reached. RNA was extracted and reverse transcribed to cDNA. Transcripts of the heat-shock protein genes encoding for the cytosolic HSP70 B2 (ORF:88765), HSP90_1 (ORF:13864) and HSP90_2 (ORF:98054) (a), and the chaperonins co-chaperonin p23 (ORF:10429) and chaperonin 60 (ORF:7031) and an ankyrin protein 21.1 (ORF:12139) (b) were quantified in relation to actin mRNA. The relation is expressed in arbitrary units. Mean values ($\pm$SE) of five independent experiments are given. Different letters indicate statistically significant differences (ANOVA followed by pairwise *t*-tests; $P < 0.01$). Note the differences in scales of ordinates.

with a more than four times higher expression in C4 than in wild-type, thus fully confirming the microarray results (Figure 1b).

In order to check whether heat-shock protein ORFs that were not retained in the screens were also overexpressed in C4 when compared with wild-type trophozoites, the expression of the endoplasmic reticulum (ER) isoform of HSP70,[27] the putative mitochondrial HSP70[28] and HSP40[24] was analysed by RT–PCR. The HSP70 ER isoform was up-regulated by a factor 2 in C4 versus wild-type trophozoites. Conversely, the mitochondrial HSP70 isoform and HSP40 isoform had similar expression levels in wild-type and C4 (Figure 2).

Loss of resistance and antigenic shift after en-/excystation

During long-term culture over 30 generations in the absence of a drug, C4 maintained a significant degree of resistance to nitazoxanide and metronidazole. During this time, the expression profile of some selected genes such as HSP70 B2, NR and others, as well as the expression of the major surface trophozoite antigen 417 (TSA417), did not change markedly [Figures S2, S3, S4 and S5, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Therefore, in order to induce a stage conversion and to test the stability of nitazoxanide/metronidazole double resistance in *G. lamblia* clone C4, C4 as well as

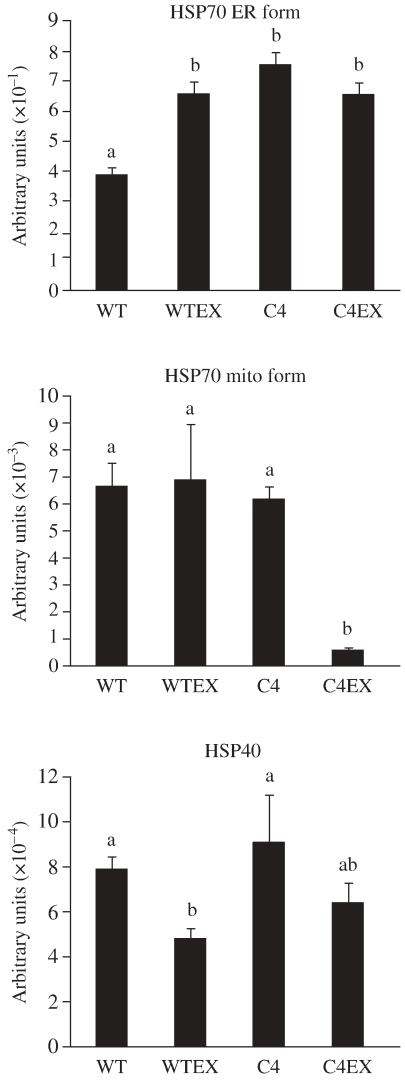

Figure 2. Quantification of heat-shock protein gene expression by RT–PCR in wild-type (WT), nitazoxanide/metronidazole-resistant (C4) trophozoites and in the same lines (WTEX and C4EX) after en-/excystation. Trophozoites were grown in normal culture medium until near confluency was reached. RNA was extracted and reverse transcribed to cDNA. Transcripts of the genes encoding for the ER and mitochondrial HSP70s (ORF:17121 and ORF:14581) and HSP40 (ORF:17483) were quantified in relation to actin mRNA. The relation is expressed in arbitrary units. Mean values ($\pm$SE) of five independent experiments are given. Different letters indicate statistically significant differences (ANOVA followed by pairwise *t*-tests; $P < 0.01$). Note the differences in scales of ordinates.

wild-type WB C6 trophozoites were subjected to one cycle of en- and excystation and were subsequently tested for growth in the presence of different drug concentrations (see the Materials and methods section). Both en- and excystation of clone C4 were conducted in the absence of the two drugs, since in the presence of the compounds, trophozoites of this strain did not encyst.[19] In this experiment, en-/excysted C4 trophozoites exhibited IC_{50} values (5.8 $\pm$ 1.1 μM nitazoxanide and 3.1 $\pm$ 1.7 μM metronidazole) similar to those of wild-type clone WB C6 (4.2 $\pm$ 1.2 μM nitazoxanide and 5.8 $\pm$ 1.4 μM metronidazole) and of en-/excysted WB C6 (2.5 $\pm$ 1.0 μM nitazoxanide and 2.3 $\pm$ 1.4 μM metronidazole). However, these values were much lower than those determined for the original clone C4 (33.8 $\pm$ 1.1 μM nitazoxanide and 38 $\pm$ 2.5 μM metronidazole). This observation indicated that upon completion of the life cycle, *G. lamblia* clone C4 had completely lost both nitazoxanide and metronidazole resistance.

As expected, en-/excystation was followed by an antigenic shift, the major surface antigen of WB C6, VSP417 being almost completely down-regulated (Figure 3) as in the resistant clone C4 before en-/excystation. After en-/excystation, C4 did not shift back to VSP417. The same was observed for a cysteine-rich surface antigen strongly down-regulated in C4 trophozoites and wild-type trophozoites after excystation. Instead, other surface antigens such as VSPAS7 and VSPAS7A were expressed (Figure 3).

Interestingly, after en-/excystation of C4 trophozoites, differences in expression of HSP and chaperonin-like genes reached levels similar to wild-type or en-/excysted wild-type trophozoites (Figures 1 and 2). In some cases (co-chaperone p23, chaperonin 60 and protein 21.1), en-/excysted wild-type trophozoites had significantly higher values than wild-type trophozoites, whereas values in C4 trophozoites were similar or lower after than before en-/excystation (Figure 1b). In one case, namely HSP90_2, expression levels in en-/excysted trophozoites were significantly lower than before en-/excystation (Figure 1a).

Moreover, the expression of genes encoding nitazoxanide-binding proteins such as NR, pyruvate oxidoreductases (POR1 and POR2) and protein disulphide isomerases (PDI2 and PDI4) was investigated in all four strains. As described previously,[13] the expression level of NR was significantly lower, but the expression levels of PDI2 and PDI4 were higher in C4 than in wild-type trophozoites. Upon en-/excystation, differences in NR and PDI4 expression were lost (Figure 4). Expression of the two POR genes showed only slight differences between all strains. In en-/excysted C4 trophozoites, POR2 expression was significantly lower than before en-/excystation. Conversely, expression of the gene encoding for the cyst wall protein CWP1 was significantly decreased in C4 when compared with wild-type trophozoites. After en-/excystation, expression levels were significantly decreased when compared with levels before en-/excystation (Figure 4).

Thermotolerance

The constitutive expression of heat-shock proteins, most notably of the highly expressed cytosolic isoform of HSP70 (B2), may cause thermotolerance. Thus, we inoculated 24-well plates with 10^4 wild-type and C4 trophozoites and cultured them at temperatures ranging from 37 to 43°C. After 2 days, the trophozoites grown at 37°C were confluent with a density of ca. 1.7×10^6 per well (Figure 5). After 2 days of culture at 39°C, C4 trophozoites had grown up to 80% of their 37°C-cell density, whereas the proliferation of wild-type trophozoites was severely impaired, reaching only 45% of the 37°C levels. After culture at 41°C, C4 trophozoites exhibited proliferation to 7% of their 37°C-cell density, whereas the cultures of wild-type trophozoites as well as en-/excysted trophozoites of both lines had died off and trophozoites were not detected anymore. At 43°C, no growth was observed in either cell line (Figure 5). Thus, the nitazoxanide/metronidazole double-resistant clone C4 was considered to be relatively thermotolerant when compared with the

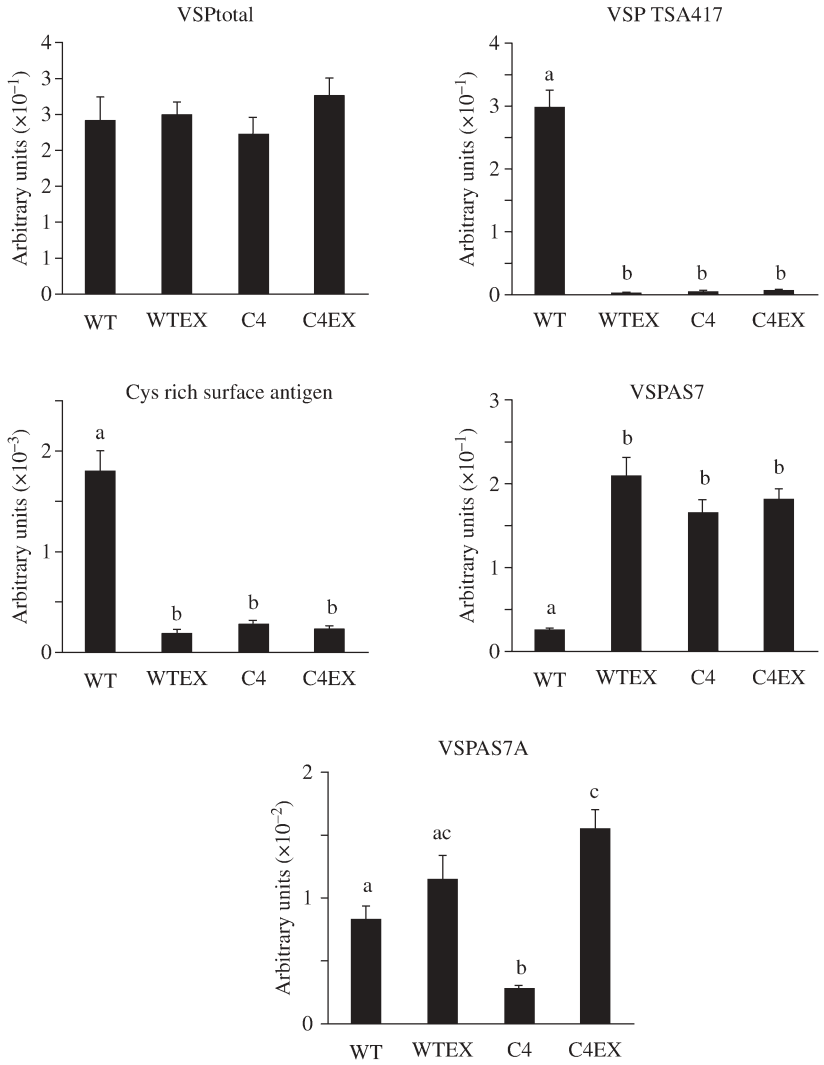

Figure 3. Antigenic variation in wild-type (WT) and nitazoxanide/metronidazole-resistant (C4) trophozoites and in the same lines (WTEX and C4EX) after en-/excystation. Trophozoites were grown in normal culture medium until near confluency was reached. RNA was extracted and reverse transcribed to cDNA. Transcripts of the genes encoding for VSPtotal, VSP TSA417, a cysteine-rich surface antigen (ORF:10659), VSPAS7 (ORF:137740) and VSPAS7A (ORF:98058) were quantified in relation to actin mRNA. The relation is expressed in arbitrary units. Mean values (±SE) of five independent experiments are given. Different letters indicate statistically significant differences (ANOVA followed by pairwise *t*-tests; $P < 0.01$). Note the differences in scales of ordinates.

respective wild-type, en-/excysted lines of C4 and wild-type trophozoites.

Discussion

In a previous study, we characterized the nitazoxanide/metronidazole double-resistant *G. lamblia* trophozoite clone C4 and the metronidazole-resistant clone C5.[19] In the present study, we performed two initial microarray screens in order to identify genes that were up- or down-regulated by a factor of 4 or more in C4 versus wild-type trophozoites. The differences in surface antigen expression observed in the microarray screen, especially the down-regulation of the trophozoite major surface-labelled trophozoite antigen 417 precursor (TSA417), confirm our previous results. A completely different pattern of up- and down-regulated mRNAs was observed in other lines such as the formononetin-resistant line C3 or the metronidazole-resistant strain C5. The only annotated ORFs down-regulated in all screens encoded the major surface trophozoite antigen 417 (TSA417), thus confirming previous results (data not shown). Clone C5 exhibits a slower growth capacity compared with C4, with a more pronounced lag phase. In addition, clone C5 does not excyst *in vitro*. Therefore, clone C5 cannot be directly compared with C4 and was not included in the present work.[19] Moreover, we have identified *vsp* genes that were up-regulated following the antigenic switch in clone C4. In addition, genes potentially involved in protein phosphorylation and network formation, especially ankyrins,[29–31] and chaperonins were up-regulated in clone C4.

In this study, we have investigated the expression of chaperones in more detail using RT–PCR. The chaperone with the highest expression level in clone C4 is the cytosolic form of HSP70.[27,28] Other HSPs such as HSP40, HSP90 and the ER isoform of HSP70 were also up-regulated in C4.

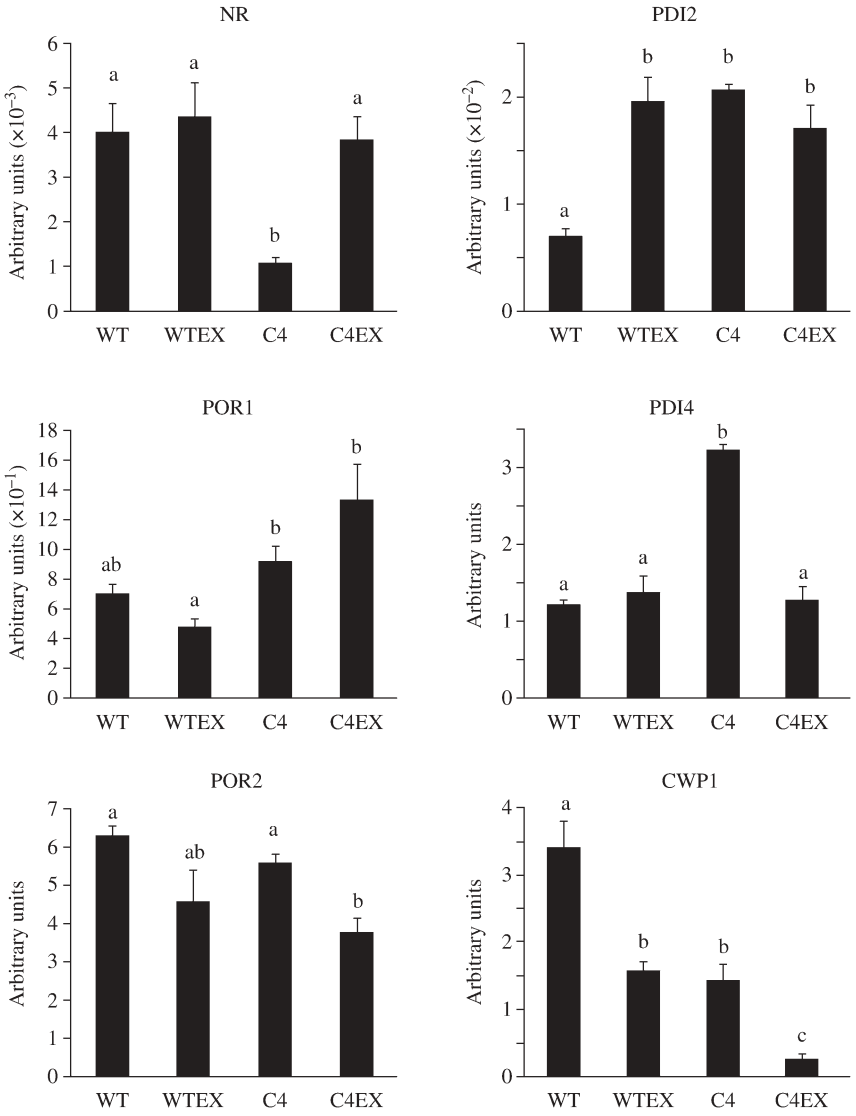

Figure 4. Expression of various genes in wild-type (WT) and nitazoxanide/metronidazole-resistant (C4) trophozoites and in the same lines (WTEX and C4EX) after en-/excystation. Trophozoites were grown in normal culture medium until near confluency was reached. RNA was extracted and reverse transcribed to cDNA. Transcripts of the genes encoding for nitroreductase (NR, EAA43030.1), pyruvate oxidoreductases (POR1, ORF:17063; POR2, ORF: 114609), protein disulphide isomerases (PDI2, EAA42483.1; PDI4, AF295634.1) and cyst wall protein 1 (CWP1, XM 766142.1) were quantified in relation to actin mRNA. The relation is expressed in arbitrary units. Mean values ($\pm$SE) of five independent experiments are given. Different letters indicate statistically significant differences (ANOVA followed by pairwise *t*-tests; $P < 0.01$). Note the differences in scales of ordinates.

The roles of these HSPs in *Giardia* are not well understood. In human cells, molecular chaperones such as HSP72 and HSP27 prevent cells from apoptosis induced by heat shock.[32] If similar mechanisms occur in *Giardia*, a general response to stress via induction of chaperones could be responsible for the increased thermotolerance as well as for the resistance to nitazoxanide/metronidazole treatment as observed in clone C4 *in vitro*.

In previous studies, we have shown that nitazoxanide inhibits the enzymatic activities of protein disulphide isomerases from *G. lamblia*[19] and *N. caninum*,[33] and that two protein disulphide isomerase genes were up-regulated in C4 when compared with the wild-type.[19] Inhibition of protein disulphide isomerases *in vivo* could lead to the formation of badly folded proteins and thus to an impairment of central metabolic and regulatory processes.[34,35] Cells that overexpress chaperones and proteins involved in the stabilization of subcellular structures such as ankyrins could overcome the deleterious effects due to protein disulphide isomerase inhibition. It is therefore possible that the resistance phenotype is caused by amino acid substitutions in target proteins rather than by changes in the expression level of the genes mentioned above. These changes may be compensatory, reducing the fitness cost of the true resistance mutation(s) but not having a causal relationship to that phenotype.

Resistance formation correlates with the massive down-regulation of the expression of the TSA417 gene locus, encoding the major surface antigen (VSP C6) of WB C6. Antigenic variation in *G. lamblia* allows the parasite to escape the host immune response. Moreover, *G. lamblia* can alter its gene expression profile in response to environmental pressure by inducing its

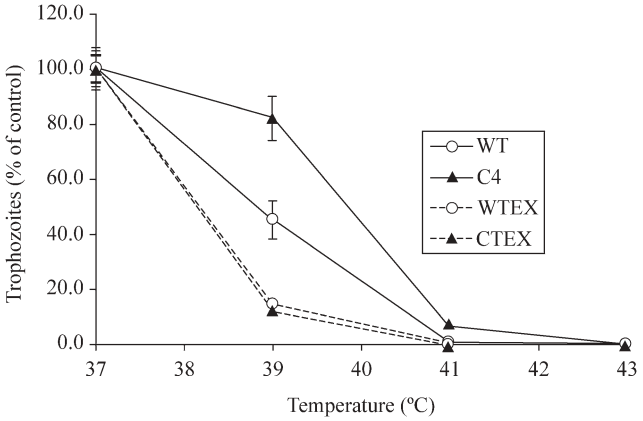

Figure 5. Growth of *G. lamblia* WB C6 wild-type (WT) and nitazoxanide/metronidazole-resistant (C4) trophozoites and of the same lines (WTEX and C4EX) after en-/excystation; 10^4 trophozoites were inoculated in normal culture medium. Sessile, motile trophozoites were counted after 2 days. Trophozoite numbers per well are given as mean values ($\pm$SE) from four experiments.

encystation and subsequent excystation, and this can be carried out *in vitro* using *G. lamblia* clone WB C6.[3] Thus, we investigated whether one en-/excystation cycle would render *G. lamblia* clone C4 non-resistant. As expected, the *vsp* expression profile was altered in all trophozoite lines upon en-/excystation. Moreover, excysted C4 trophozoites were now sensitive to metronidazole and nitazoxanide. Thus, the loss of resistance to nitazoxanide or metronidazole following en-/excystation indicates that resistances acquired through prolonged treatments cannot be passed from one generation to the other via the cysts.

It is possible that the resistance mutation(s) rendered the cell unable to encyst. The encystation procedure could have killed the resistant population, permitting only the susceptible revertants to survive the process. Moreover, en-/excystation of C4 trophozoites may have put a selective pressure for rapid growth on the trophozoites that is not compatible with high expression levels of those genes. Concerning the genes that we have investigated, only the expression patterns of HSP70 B2, NR, PDI4 and TSA417 correlate with resistance or susceptibility to nitazoxanide and metronidazole. By (unknown) adaptive mechanisms, these genes are then down-regulated to wild-type levels and resistance is lost. In a recent study, it has been found that the introduction of neomycin resistance into *G. lamblia* trophozoites via a transgene has led to drastic changes in gene expression patterns including two chaperones that we have also identified in our study.[36]

These results suggest that *Giardia* has very flexible mechanisms in order to respond to changes in the environment such as drug pressure, presence of antibodies, or even to changes in medium composition and changes to the intracellular physiology by introduction of transgenes. Complex gene expression patterns could be fine-tuned in order to find an optimal compromise between speed of growth and survival. This assumption is consistent with our finding that genes involved in cell division are down-regulated in resistant C4 when compared with wild-type trophozoites (Table 2).

A recent report suggested that epigenetic mechanisms, probably including histone acetylation, could be responsible for antigenic variation.[37] Very recently, evolution of antibiotic resistance initiated by increasing sublethal doses of various antibiotics has been attributed to epigenetic inheritance.[38] Similar adaptive gene-regulatory processes may cause the differences in gene expression that we have observed. Another possibility is that mutation of an unknown regulator, e.g. a transcription factor, is responsible for the differences in expression patterns observed. Future work will go into more detail in order to investigate these mechanisms.

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Transparency declarations

None to declare.

Supplementary data

Figures S1, S2, S3, S4 and S5 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/). The whole data set from the microarray screens are available at http://www.ipa.vetsuisse.unibe.ch/index.php?option=com_content&task=view&id=56&Itemid=134&lang=DE.

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