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Electrophoresis and spectrometric analyses of adaptation-related proteins in thermally stressed *Chromobacterium violaceum*

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ABSTRACT. *Chromobacterium violaceum* is a Gram-negative proteobacteria found in water and soil; it is widely distributed in tropical and subtropical regions, such as the Amazon rainforest. We examined protein expression changes that occur in *C. violaceum* at different growth temperatures using electrophoresis and mass spectrometry. The total number of spots detected was 1985; the number ranged from 99 to 380 in each assay. The proteins that were identified spectrometrically were categorized as chaperones, proteins expressed exclusively under heat stress, enzymes involved in the respiratory and fermentation cycles, ribosomal proteins, and proteins related to transport and secretion. Controlling inverted repeat of chaperone expression and inverted repeat DNA binding sequences, as well as regions recognized by sigma factor 32, elements involved in the genetic regulation of the bacterial stress response, were identified in the promoter regions of several of the genes coding proteins, involved in the *C. violaceum* stress response. We found that 30°C is the optimal growth temperature for *C. violaceum*, whereas 25, 35, and 40°C are stressful temperatures that trigger the expression of chaperones, superoxide dismutase, a probable small heat shock protein, a probable phasing, ferrichrome-iron receptor protein, elongation factor P, and an ornithine carbamoyltransferase catabolite. This information improves our comprehension of the mechanisms involved in stress adaptation by *C. violaceum.*

Key words: CIRCE; Chaperones; Heat shock response; HSP; Differential gene expression

INTRODUCTION

Chromobacterium violaceum (Bergonzini, 1881) is a Gram-negative β-proteobacterium that grows in a variety of ecosystems in tropical and subtropical areas (Lima-Bittencourt et al., 2011). In Brazil, it is largely found in the water and on banks of the Rio Negro in Amazonas State. *C. violaceum* has been largely investigated due to its potential use for biotechnological purposes, and its complete genome was sequenced in 2003 by the Brazilian National Genome Project Consortium. This sequencing revealed a total of 4,751,080 base pairs and 4430 ORFs (open reading frames), many of which suggest that *C. violaceum* could have biotechnological applications. Several ORFs likely participating in the organism's stress response were identified, including ORFs involved in heat shock protein signal transduction, iron metabolism, cell transport, oxidation and UV light responses (Vasconcelos et al., 2003; Hungria et al., 2004). Collectively, this supports the idea that this bacterium is highly adaptable. Although all these

proteins may be involved in the response of *C. violaceum* to stressful environmental conditions, their specific roles are poorly understood.

Many of the stress responses overlap with one another in bacteria (Ishibashi et al., 2010) and adaptive reactions involve general changes in metabolism as well as in gene transcript levels (Jozefczuk et al., 2010; Gomes et al., 2012). The expression of heat shock genes in Gram-negative proteobacteria is positively modulated by the transcriptional regulator RpoH, the σ^{32} subunit of RNA polymerase, which induces the expression of proteins involved in the response to heat and cold stress (Yura et al., 1993; El-Samad et al., 2005). Some of these proteins are chaperones and others heat shock proteins, which are responsible for the folding, assembly, translocation and degradation of proteins in the cell under physiological conditions, and can also assist in cellular protein refolding under stress. In turn, many thermal sensors are components of cellular structures that are damaged or altered by heat, such as ribosomes, DNA, intracellular proteins and, less commonly, membrane components (Rowbury, 2005).

Genome analysis is not sufficient to generate a complete picture of the heat-related stress response as it does not reveal the levels of gene expression. Proteomic studies following genomic and transcriptomic analyses have been described for prokaryotes (Broberg and Clark, 2010; Hamon et al., 2011) and eukaryotes (Rossignol et al., 2009). Together, these studies aim to improve our understanding of numerous diverse cellular processes, including those involving abiotic stress (Hongsthong et al., 2008; Woo et al., 2009). Here, the main goal was to identify proteins involved in the adaptation of *C. violaceum* (ATCC12472) subjected to stressful temperature conditions. To evaluate the response of *C. violaceum* to thermal stress, we tested the hypothesis that 25, 35 and 40°C would be stressful growth temperatures, whereas 30°C would be the optimal growth temperature for this organism. Proteins related to thermal stress were identified by mass spectrometry and their putative roles during the adaptation process are discussed.

MATERIAL AND METHODS

Bacterial cultivation and protein extraction

Individual colonies of *C. violaceum* (ATCC12472) were grown in 1 L LB (Luria-Bertani) medium, pH 7.0, with aeration at 25, 30, 35 and 40°C and at 200 rpm. Absorbance at 590 nm was monitored hourly up to 34 h, and the resulting measurements were plotted to obtain growth curves. Total protein extracts for two-dimensional electrophoresis (2-DE) analysis were obtained after 7 h (exponential phases) and 19 h (stationary phases) of cultivation. *C. violaceum* cells were harvested by centrifugation at 2700 *g* for 15 min at 4°C. The cells were washed twice with 25 mL 0.15 M NaCl, and 100 mg cell pellet (wet weight) were transferred to microtubes and washed twice in 1 mL Milli-Q ultrapure water containing 2 mM PMSF and a protease inhibitor cocktail (Amersham Biosciences, USA). The pelleted cells were processed immediately or stored at -80°C. Cells were resuspended in lysis buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 100 mM DTT and 80 mM citric acid) in the presence of protease inhibitor cocktail and disrupted using a syringe. The resulting cell extract was centrifuged at 28,000 *g* for 20 min at 25°C. The soluble fractions from each tube were mixed to ensure the homogeneity of the samples and redistributed in aliquots of 200 µL. The protein extract was precipitated in 5 volumes of 100% acetone, incubated for 1 h at room temperature and centri-

fuged at 7000 g for 10 min at 25°C. The pellet was washed twice in an 80% acetone solution and then centrifuged under the same conditions described above. The protein extracts were analyzed immediately or stored at -20°C. Extracts were resuspended in 500 µL Tris-glycine buffer and protein concentration was estimated using the BCA protein assay kit (BioAgency, Brazil) according to the manufacturer instructions. Bovine serum albumin (Sigma, USA) was used as the standard.

2-DE gel

2-DE was performed using a standard procedure (O'Farrel, 1975) with modifications. The protein extract (250 µg) was resuspended in 250 µL DeStreak Rehydration Solution (GE Healthcare, Sweden) in the presence of 0.5% IPG buffer 3-11 NL (GE Healthcare) and loaded onto an immobilized pH gradient strip (3-11 NL) by passive in-gel rehydration (10 h). Isoelectric focusing (IEF) was performed at 20°C in IPGPhor3 (GE Healthcare) as follows: 150 V (2 h), 300 V (2 h), 1000 V (gradient for 4 h), 8000 V (gradient for 2 h) and 8000 V (2 h). The proteins on the IPG strips were reduced and alkylated for the second dimension separation, which was performed on 12% SDS polyacrylamide gels in the SE600Rub System (18 x 16 cm - GE Healthcare) at 50 mA. Polyacrylamide gels were fixed using 10% acid acetic and 40% methanol, followed by staining with Colloidal Coomassie Blue (8% ammonium sulfate, 0.8% phosphoric acid, 0.08% Coomassie Blue G-250 and 20% methanol). Destaining was performed with deionized water. Gel images were captured by scanning (Image Scanner - GE Healthcare), and the images were analyzed with the Image Master Platinum software (Version 6). Three reproducible gels (over 70% in similarity) were run corresponding to at least two independent extraction procedures from each experimental condition. Spot counts were obtained according to the saliency and smooth parameters, which were equal to 100 and 2, respectively. The gel with the highest number of spots was regarded as the reference gel. Differentially expressed proteins were determined based on the ImageMaster Platinum software (Version 6) analysis.

MALDI microplate preparation and analysis by mass spectrometry (MALDI Q-TOF/MS/MS)

Spots of interest were subjected to in-gel tryptic digestion (Porcine Pancreas Trypsin, Sigma) and analyzed by mass spectrometry. Protein spot digestion was performed according to the manufacturer recommendations using the Montage In-Gel Digest$_{ZP}$ kit (Millipore, USA) in 96-deep-well Zip Plates (ZipPlate, Millipore). Three microliters of each sample (obtained by tryptic digestion) were applied onto a microplate using the dried droplet method (Karas and Hillenkamp, 1988). The matrix was added to the sample with a total volume of 1.2 µL and was then air dried at room temperature. This matrix solution was prepared using α-cyano-4-hydroxycinnamic acid (Beavis et al., 1992) in a 1:1 (v/v) acetonitrile/H_2O solution containing 0.1% (v/v) TFA. MALDI Q-TOF mass spectra were acquired in a MALDI Q-Tof Premier mass spectrometer (Waters-Micromass, UK). Mass spectra were obtained with a solid state laser operated in positive mode (LDI+). Real-time calibration was performed with lock mass correction using a mixture of PEG oligomers (PEG 600, 1000, 1500 and 2000). Each spectrum was collected over a 1 s scan and the spectra were accumulated over approximately 2 min. The instrument was controlled by MassLynx 4.1v software. All mass spectra were processed into "peak list" files with a *.pkl exten-

sion using the ProteinLynxGlobalServer 2.3v (Waters Corporation, UK). Protein identification was performed by searching a database using the peptide peak list (*.pkl file) mass and intensity files generated by MALDI Q-TOF. The *C. violaceum* databank was compiled with a random databank called 10X_Random_SwissProt via the ProteinLynxGlobal Server 2.3v. Monoisotopic peak lists were processed with the following search parameters: one missed cleavage, tryptic digestion and carbamidomethylation as a cysteine modification. The search error tolerance was set at 5 ppm with a $[M + H]^+$ charge state after spectral deconvolution.

RESULTS

Analyses of the proteomic profiles of *C. violaceum* grown at 25, 30, 35 and 40°C were performed to improve our knowledge about this organism's adaptive responses to thermal stress (Figure 1). The numbers of spots visualized by 2-DE using proteins extracted from cultures maintained at 25, 30, 35 and 40°C in the exponential growth phase were 254, 99, 214 and 336, respectively. For the samples in stationary phase, the numbers of spots were 160, 235, 307 and 380, respectively (Figure 2). Together, the total of spots detected was 1985.

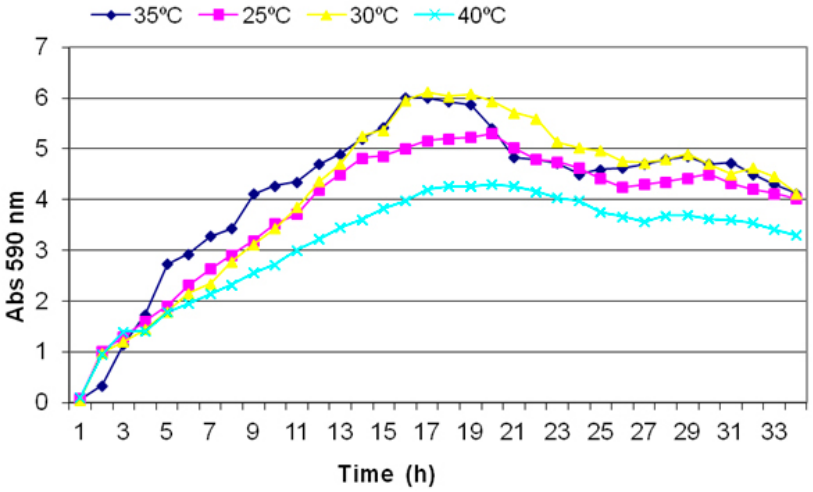

Figure 1. *Chromobacterium violaceum* cultured under different temperatures. Individual colonies of *C. violaceum* (ATCC12472) were grown in 1 L LB (Luria-Bertani) medium, pH 7.0, with aeration at 25, 30, 35, and 40°C at 200 rpm. The absorbance at 590 nm was monitored hourly up to 34 h, and the resulting measurements were plotted to obtain growth curves.

After a comparative analysis of the 2-DE gels from all conditions, 93 spots were selected for mass spectrometry (MS) analysis (Figure 2). Thirty-two proteins were identified as modulated by temperature and/or growth phase. Of those, 21 proteins were observed in all conditions tested, but at different intensities. The other 11 were absent in gels from at least one of conditions tested and showed variation in their intensity when observed (Table 1). Proteins identified were categorized into six groups: chaperones, proteins expressed exclusively under heat stress, enzymes that participate in the respiratory and fermentation cycles, ribosomal proteins and proteins related to transport/secretion and membrane.

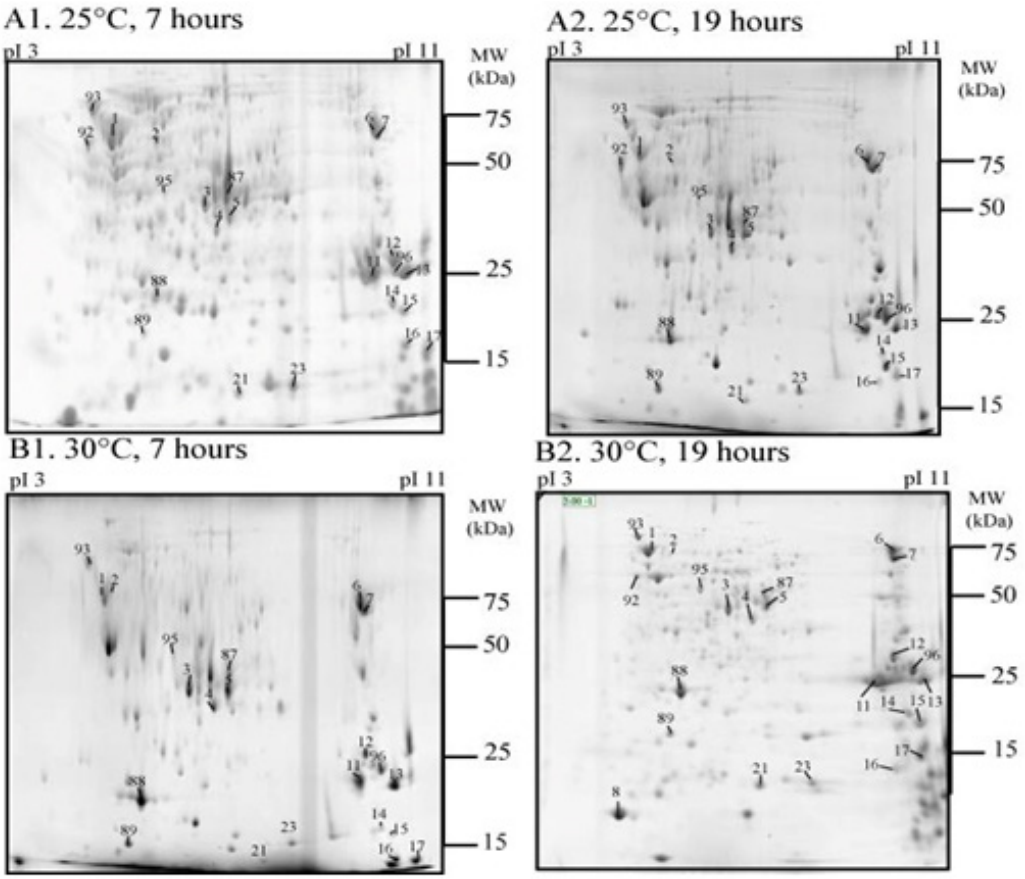

Figure 2. A proteomic map of *Chromobacterium violaceum* grown under different temperatures. For all conditions, 250 µg soluble protein was used. For the first dimension separation, an immobilized pH gradient from 3 to 11 NL was used. For the second dimension, protein separation was carried out by vertical SDS PAGE (12%). The gels were stained with colloidal Coomassie blue. The spots identified were numbered according to their identification by mass spectrometry. Experimental growth conditions are indicated at the top of each gel.

Chaperones

Three chaperones were identified in *C. violaceum* under all assayed conditions (Table 1 and Figure 3): DnaK (CV1643), GroEL1 (CV4014) and GroEL2 (CV3233). Compared to cells growing at the non-stressful temperature of 30°C, a tenfold increase in the expression level of GroEL1 was observed in cells grown at 35°C (stationary phase) and 40°C (exponential phase). Both GroEL2 and DnaK chaperones were expressed at a lower rate compared to GroEL1. The higher expression of GroEL2 was observed at 40°C in the stationary phase, and for DnaK, the variation under different experimental conditions was not considered relevant. In addition, the probable small heat shock protein (CV1177) was identified at 35 and 40°C (exponential and stationary phases) (Table 1).

Table 1. Proteins identified by mass spectrometry. This table shows the open-reading frames (ORFs, genome annotation), protein names, and the theoretical and experimental values for the isoeletric points (pIs) and molecular weights (MW) of each identified protein. The score and likelihood were obtained by the ProteinLynx Global Server.

Spot N°	ORF	Protein name	Theorical		Experimental		Score	Likelihood (%)	Conditions
			pI	MW (Da)	pI	MW (Da)			
Chaperones and proteins expressed exclusively under thermal stress									
1	CV4014	60-kDa chaperonin 1 (GroEL1)	5.25	56.67	5.07	56.64	8.29	100	All*
2	CV3233	60-kDa chaperonin 2 (GroEL2)	5.09	57.41	4.89	57.38	8.29	100	All
9	CV2504	Superoxide dismutase	5.86	21.58	5.85	21.57	8.29	100	35°C (7 and 19 h), 40°C (7 and 19 h)
10	CV1177	Probable small heat shock protein	5.95	15.92	5.56	15.91	8.29	100	35°C (7 and 19 h), 40°C (7 and 19 h)
18	CV1366	Probable phasin	6.84	19.47	7.77	19.40	8.29	100	40°C (7 h)
19	CV2251	Ferrichrome-iron outermembrane receptor protein	9.29	77.98	9.45	77.94	8.29	100	40°C (7 h)
20	CV1378	Elongation factor P	4.79	20.90	4.59	20.89	8.29	100	40°C (7 h)
91	CV3781	Ornithine carbamoyltransferase catabolic	5.96	37.76	5.80	37.73	8.2935	99.94	35°C (7 and 19 h), 40°C (19 h)
93	CV1643	Chaperone protein dnaK	4.94	69.12	4.75	69.07	8.294	100	All
Respiratory and fermentation cycles									
3	CV0187	Fructose biphosphate aldolase	5.66	38.31	5.59	38.29	8.29	100	All
4	CV1062	Malate dehydrogenase	5.92	35.03	5.88	35.01	8.29	100	All
5	CV2728	Probable alcohol dehydrogenase	6.12	39.21	6.12	39.18	8.29	100	All
87	CV2790	Acetyl-CoA acetyltransferase	6.33	40.11	6.35	40.09	8.294	100	All
88	CV2311	Phosphoenolpyruvate-protein phosphotransferase	5.43	89.37	5.29	89.32	8.294	100	All
Ribosomal proteins									
12	CV4196	50S ribosomal protein L1	9.66	23.96	10.11	23.94	8.29	100	All
13	CV4186	50S ribosomal protein L3	9.90	22.24	10.33	22.22	8.29	100	All
14	CV4174	50S ribosomal protein L5	9.44	20.30	9.78	20.29	8.29	100	All
15	CV4171	50S ribosomal protein L6	9.68	18.79	10.15	18.78	8.29	100	All
17	CV4190	30S ribosomal protein S7	10.44	17.69	10.89	17.68	8.29	100	All
21	CV3640	30S ribosomal protein S6	6.14	14.23	6.16	14.22	8.294	100	All, except 35°C (19 h)
23	CV3637	50S ribosomal protein L9	6.84	16.16	7.83	16.15	8.294	100	All, except 35°C (19 h)
90	CV4185	50S ribosomal protein L4	9.93	22.88	10.33	22.87	8.294	100	All
Proteins related to transport/secretion and membrane									
6	CV4329	Probable oligopeptide ABC transport system substrate binding protein	9.15	59.48	9.44	59.44	8.29	100	All
7	CV1097	Probable binding protein component of ABC dipeptide transporter	9.21	59.01	9.52	58.97	8.29	100	All
8	CV0871	Probable amino acid permease transmembrane protein	9.10	59.30	9.22	59.26	8.29	100	30°C (19 h), 35°C (7 h), 40°C (7 h)
11	CV3571	Outer membrane protein A	9.05	24.28	9.31	24.27	8.29	100	All
16	CV3409	Proton/sodium glutamate symport protein	8.81	40.46	8.67	40.47	8.29	100	All
95	CV3524	Probable multidrug efflux protein	9.72	49.22	9.83	49.19	8.294	100	All
96	CV0069	Probable secretion protein	9.97	45.48	10.26	45.48	8.294	100	All, except 40°C (19 h)
Unknown functions									
25	CV2409	Putative uncharacterized protein	4.59	32.82	4.44	32.80	8.294		100 All
89	CV1131	Putative uncharacterized protein	9.50	60.76	9.66	60.73	8.15		86.8 All
92	CV4022	Putative uncharacterized protein	8.43	37.46	8.24	37.47	8.294		100 All, except 30°C (7 h)

*Proteins detected at all cultivation temperatures.

Promoter regions of the chaperone genes *groEL1*, *groEL2, dnaK* and the probable small heat shock protein exhibit regions that are likely recognized by the heat shock σ^{32} sigma factor (Table 2). For *groEL1*, the IR (inverted repeat) sequence (GCACct-N_{12}-agGTGC) represents a potential CIRCE, although it was distinct from the bacterial consensus sequence (GCACTC-N_9-GAGTGC) (Zuber and Schumman, 1994), as indicated by lower case. No CIRCE-related sequence was identified in the *groEL2* and *dnaK* genes.

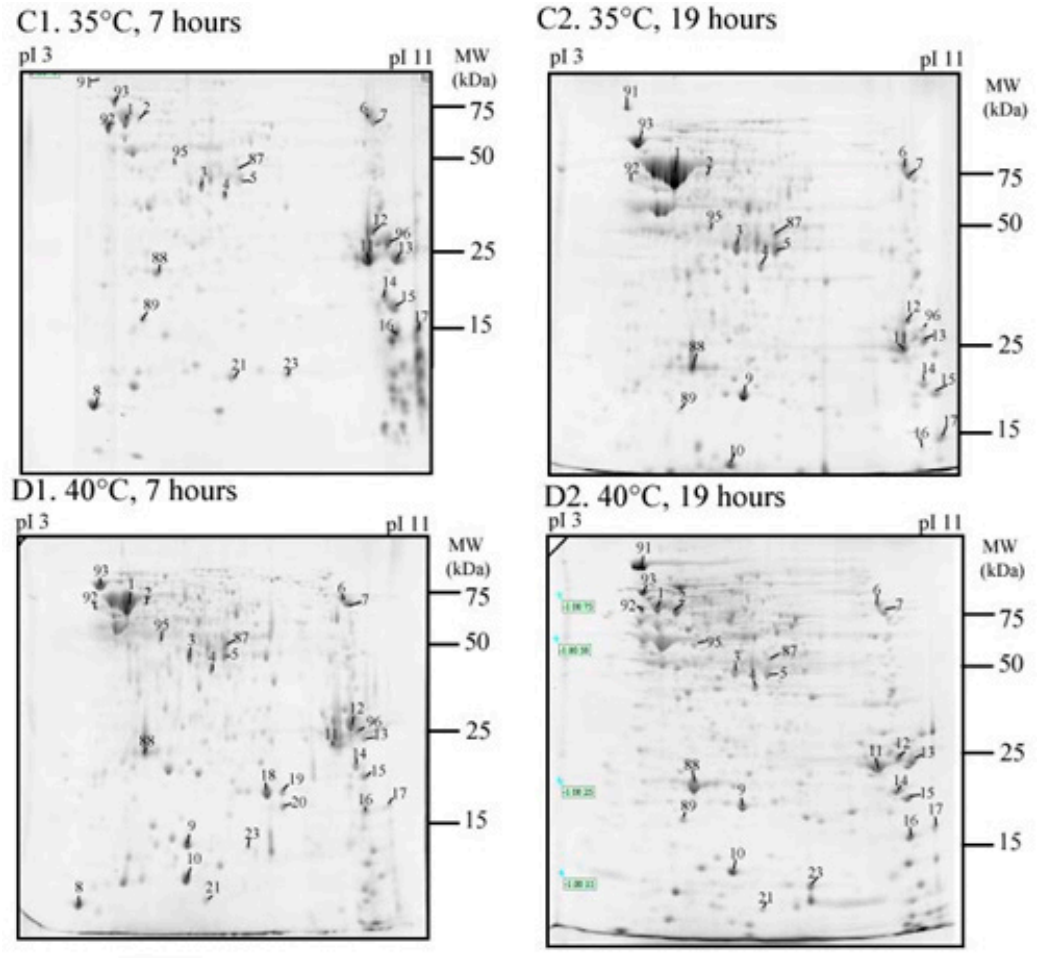

Figure 3. Chaperones of *Chromobacterium violaceum* that are involved in the heat stress response. The values (% vol) were determined by Image Master Platinum. The spots that were identified under each experimental condition are shown in detail.

Proteins expressed exclusively under heat stress

Six proteins showed expression related to stressful temperatures above 30ºC. Three proteins were identified only in cells grown at 40°C in the exponential phase: a probable phasin (CV1366), a ferrichrome-iron outer membrane receptor protein, a siderophore trans-

porter (CV2251, gene *fhuA*) and elongation factor P (CV1378). A less-specific response was observed for ornithine carbamoyltransferase (CV3781) and superoxide dismutase (CV2504). Ornithine carbamoyltransferase was identified in the exponential and stationary phases in cells grown at 35°C, as well as in the stationary phase in cells grown at 40°C. Superoxide dismutase was identified at the exponential and stationary phases in cells grown at 35 and 40°C. The probable regulatory regions of the genes coding for ferrichrome-iron outer membrane receptor protein, phasin and superoxide dismutase had sequences likely recognized by the heat shock σ^{32} sigma factor (Table 2). Probable small heat shock protein, even expressed exclusively under heat stress in this work, is discussed in the section about chaperones.

Table 2. Promoter regions recognized by the σ^{32} sigma factor.

Gene (protein name)	-35 box	Spaced region	-10 box	bp to ATG
shsp (Small heat shock)	TTGAAt	12	GTCCCTATcT	52
	TcGAcA	21	-AagCCcaTga	17
groEL1 (GroEL1)	TTGAAg	14	GGCCgCAagg	37
	TTGAAg	6	aTCCCaATcc	47
groEL2 (GroEL2)	TgtAAt	19	GACCCTtTcc	34
	TTGAgt	5		34
fhuA (Ferrichrome iron)	TaGAtA	12	GAtCaaggTaa	41
cv1366 (Phasin)	TTtAcg	12	GCgCaCAaTa	55
	TcaAcg	13	GACCCCATTT	23
dnaK (DnaK)	TTGAAA	11	GCCaaTATTT	68
sodB1 (SOD)	TTGcAA	11	-TatCatTTTa	90
	TTGgcc	11	-AttCCAgTaa	55
grpE (1642)**	TTGAAA	11	GTCCCTAccT	24
CONSENSUS *E. coli**	TTGAAA	11-12	GNCCCCATWT	Variable
CONSENSUS *C. violaceum*	TTGAAA	Variable	GNCCCYATTT	Variable

*The consensus sequence for the σ^{32} sigma factor of *E. coli* (Nonaka et al., 2006). **Proteins related to the heat shock response that have been described in the *C. violaceum* genome, but were not identified here by mass spectrometry. N = A/C/G/T; Y = C/T; W = A/T.

Enzymes involved in the respiratory and fermentation cycles

Five enzymes related to glycolysis and the tricarboxylic acid cycle, central metabolic pathways, were identified: fructose bisphosphate aldolase (CV0187), malate dehydrogenase (CV1062), acetyl-CoA acetyltransferase (CV2790), a probable alcohol dehydrogenase (CV2728) and phosphoenol pyruvate phosphotransferase (CV2311) (Table 1). These enzymes were expressed by *C. violaceum* under all the experimental conditions, although their levels of expression were affected by the temperature and phase of bacterial growth (Figure 4). Though all proteins displayed a complex expression pattern, the highest expression levels of these proteins were observed in cultures grown at 30°C in the exponential phase.

Ribosomal proteins

Eight ribosomal proteins were detected in all experimental assays, but they differed in their expression level according to the temperature and growth phase conditions (Table 1). The ribosomal proteins identified were the 50S proteins L1 (CV4196), L3 (CV4186), L4 (CV4185), L5 (CV4174), L6 (CV4171), and L9 (CV3637) and the 30S proteins S6 (CV3640) and S7 (CV4190).

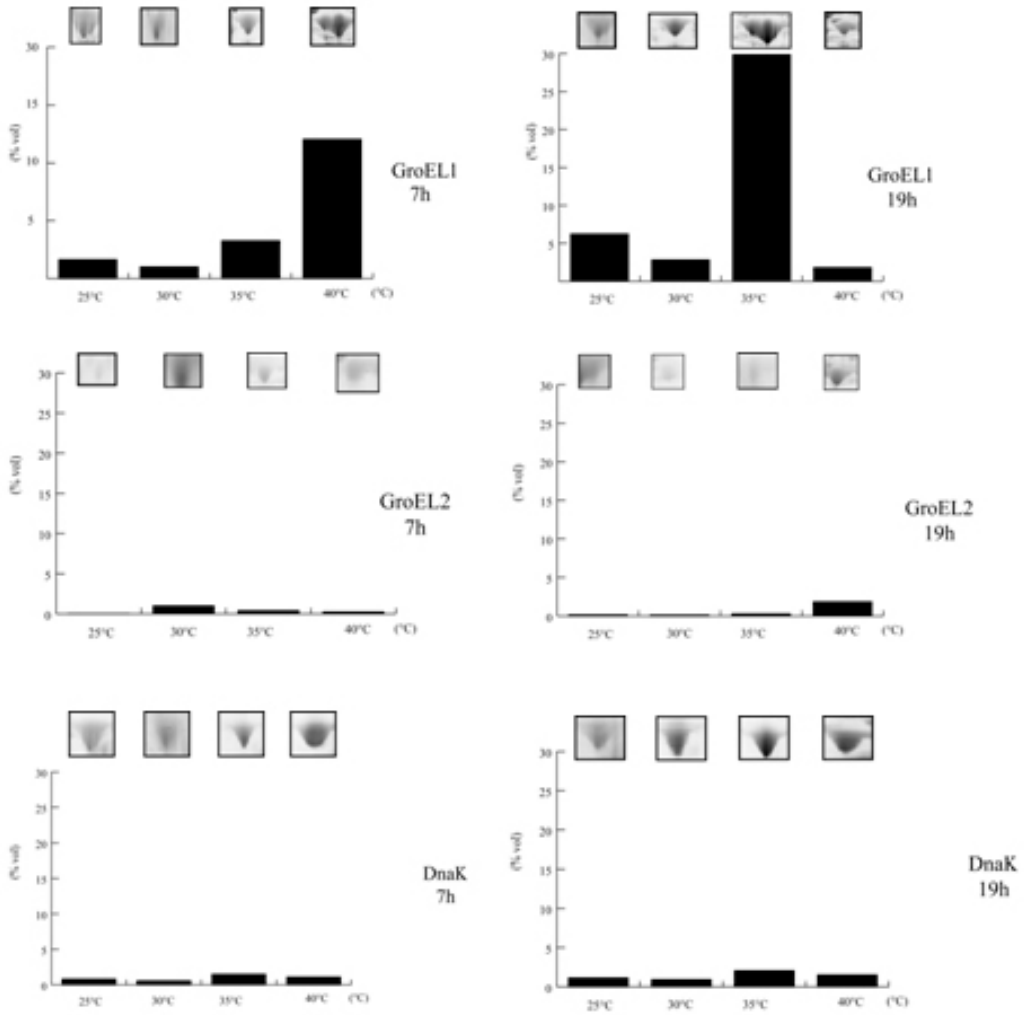

Figure 4. Effect of the temperature and growth phase on the metabolic enzymes of *Chromobacterium violaceum*. The values (% vol) were determined by Image Master Platinum. The spots that were identified under each experimental condition are shown in detail.

Transport/secretion and membrane related proteins

Six proteins that play roles in the transport or secretion of metabolites and one related to membranes were detected: a proton/sodium glutamate symport protein (CV3409), an oligopeptide likely involved in substrate binding in the ABC transporter system (CV4329), a probable multidrug efflux protein (CV3524), an ABC dipeptide transporter (CV1097) and an outer membrane protein A (CV3571) were observed under all conditions tested. Expression was altered by conditions testing for a probable secretion protein (CV0069), which was detected under all conditions tested except at 40ºC (stationary phase), and for a probable amino acid

permease transmembrane protein (CV0871), which was observed in stationary phase (30ºC) and exponential phase (35 and 40°C) (Table 1).

DISCUSSION

Molecular chaperones are essential for the correct folding of proteins (Techtmann and Robb, 2010) and confer protection against the formation of cellular protein aggregates (Henry et al., 2011). The GroEL1 and GroEL2 chaperones that were identified in *C. violaceum* shared 76% amino acid sequence similarity (http://www.brgene.lncc.br/cviolaceum) and are probably expressed from paralogous genes, as described for Mycobateria (Ojha et al., 2005) and *Myxococcus xanthus* (Li et al., 2010). In these microorganisms, the amino acid similarity between the *groEL1* and *groEL2* genes was 60 and 79%, respectively.

DnaK, GroEL1 and GroEL2 chaperones were expressed under all conditions tested, but increased expression of GroEL1 was correlated with heat stress (Figure 3). These results suggest that these temperatures are stressful and that the GroEL1 heat shock protein is the main chaperone required for the adaptation of *C. violaceum* to thermal stress, as described for *M. xanthus* (Li et al., 2010). This result reinforces that 30°C is the optimum growth temperature for *C. violaceum*, in accordance with a review by Hungria et al. (2004). In addition, it was previously demonstrated that, in *Streptococcus mutans*, the expression of proteins related to bacterial cell adhesion was dependent upon the heat shock proteins DnaK and GroEL, suggesting that these chaperones are involved in other cellular processes besides stress response (Ishibashi et al., 2010). This is consistent with the results shown in this work, as heat shock protein chaperones were expressed under all tested conditions.

We observed an increase in the expression of a small heat shock protein (sHSP) of approximately 15 kDa in size at 35 and 40°C in both the exponential and stationary phases. Typically, sHSP plays a role like other chaperones, as it binds to other proteins, enabling protein folding and preventing protein aggregation (Murata et al., 2011). The temperature-driven expression of this sHSP, as well as the presence of a consensus heat shock σ^{32} sigma factor binding site in its promoter, reinforces the notion that temperatures equal to or greater than 35°C represent heat shock conditions for *C. violaceum*.

The operons for chaperones usually contain IRs, and these regulatory sequences may contribute to the heat shock response by two mechanisms. They may function as regulatory sites of the heat shock genes (Zuber and Schumman, 1994) not undergoing transcription, or they may contribute to the stability of the mRNA when transcribed (Segal and Ron, 1995). CIRCE regions may be recognized by the heat shock responsive transcription regulator, HrcA (Techtmann and Robb, 2010). Usually, these sequences are located upstream or downstream of the promoter region, with variation observed among individual organisms, and they may be crucial for understanding the heat shock response in *C. violaceum*. The differences reported here between the promoter regions of *groEL1*, *groEL2* and *dnaK* genes, particularly the lack of probable CIRCE elements in *groEL2* and *dnaK* genes, could explain the higher expression observed for GroEL1 protein under thermal stress.

Polyhydroxyalkanoic acids (PHA) are polyesters naturally produced by microorganisms that are known to serve as sources of energy and to function as carbon reserves (Singh et al., 2009). Phasins are proteins that play roles in PHA granule formation (de Almeida et al., 2007) and stabilization of the PHA granule membranes in the cytoplasm of the cell (Ko-

libachuk et al., 1999), whose expression is thermally regulated (Ting et al., 2010). Although polyhydroxyalkanoate synthase, which has been described in the genome of *C. violaceum* (CV2789, http://www.brgene.lncc.br/cviolaceum), was not identified here, we suggest that the phasin-like protein contributes to the establishment of PHA granules by an unknown mechanism in *C. violaceum* during stress response.

Iron is important in several enzymatic processes (Faraldo-Gómez and Sansom, 2003; Eisenhauer et al., 2005). It was demonstrated that the *fhu* operon contributes to the growth of the Gram-negative bacterium by assisting in the uptake and transport of iron (Morton et al., 2010). In *C. violaceum*, the ferrichrome-iron outer membrane receptor protein and an ATP-dependent transporter are involved in iron acquisition (Hungria et al., 2004). Although the corresponding proteins were not identified in this work, ORFs coding for several proteins associated with the transport of iron-siderophores besides *fhuA* are present in the *C. violaceum* genome. These include CV1487 and CV1488 (inner membrane iron siderophore ABC transporters), CV1489 (a periplasmic solute-binding protein of an iron siderophore ABC transporter) and CV2234 (a putative iron siderophore uptake system ATP-binding component) (Vasconcelos et al., 2003). The presence of a probable promoter region recognized by σ^{32} sigma factor for *fhuA* gene corroborates the hypothesis that the ferrichrome-iron receptor is required for the temperature stress adaptation mechanism of *C. violaceum*, even though by an unknown mechanism.

Exposure of the bacterium to heat stress, even under aerobic conditions, imposes an oxidative stress that can induce the heat shock response in bacteria and eukaryotic organisms (Balamurugan and Dugan, 2010). Thus, it is thought that the two stressors may trigger similar responses. Though SOD is typically related to oxidative stress, in agreement with our results, SOD expression has been observed in *E. coli* cultures grown under high temperature conditions (Aldsworth et al., 1999). Because *C. violaceum* SOD is an iron-dependent enzyme, the observed increase in the expression of the ferrichrome-iron outer membrane receptor protein at high temperatures may account for the observed overexpression of SOD. Two probable elements that are recognized by σ^{32} sigma factor in the promoter region of gene coding for SOD were found, corroborating the proposal that SOD contributes during stress response.

Elongation factor P (EF-P), detected only at 40°C (exponential phase) in the present investigation, directly stimulates protein elongation (Greganova et al., 2011; Pech et al., 2011), yet its precise mode of action at the ribosome is unclear. As previously discussed, many different types of stress elicit a complex series of events that confer adaptability on microorganisms (England et al., 2011). Therefore, we suggest that EF-P is required under higher demand in cells grown under heat stress, where they would be necessary to maintain the translation of myriad different proteins. Other works have shown that ribosome and ribosome-associated factors contribute to stress adaptation (Pech et al., 2011), corroborating our results.

Ornithine carbamoyltransferase participates in the biosynthesis of amino acids through the production of fumarate, which is the final acceptor of electrons in *C. violaceum* under anaerobic conditions (Vasconcelos et al., 2003). Although *C. violaceum* is a facultative anaerobic bacterium, the expression levels of ornithine carbamoyltransferase were comparable to proteins related to thermal stress, suggesting that stressful temperatures could result in the activation of metabolic pathways that are also utilized under anaerobic conditions. Even though ornithine carbamoyltransferase protein was expressed only under stress condition in *C. violaceum*, its regulatory region has been analyzed, and it is likely that σ^{70} sigma factor can

bind to this region (data not shown). Additional investigations are required to identify regulatory regions related to thermal stress response.

As opposed to chaperones and other proteins that are associated with heat stress, the highest expression levels of proteins related to the respiratory and fermentation cycles were observed at 30°C (Figure 4). This reinforces the notion that 30°C is the optimal growth temperature for *C. violaceum* under the conditions investigated. The regulatory regions of the genes coding for the proteins described in this section were analyzed. Their probable promoter regions may interact with σ^{70} sigma factor (data not shown).

Ribosomal proteins have been shown to be involved in the modulation of protein translation under environmentally stressful conditions (Singh et al., 2009; Hamon et al., 2011; Murata et al., 2011). Nevertheless, ribosomal proteins were detected in all conditions tested in this work, though at variable levels of expression. No heat shock-related regulatory motif was found in the promoter sequences of the ribosomal genes and we suggest that ribosomal proteins detected in this investigation were expressed in a constitutive fashion.

Usually, bacteria that have the capacity to adapt to various environments harbor ABC (ATP binding cassette) transport proteins (Higgins, 2001). In the *C. violaceum* genome, 2.7% of all ORFs encode transporter proteins, of which 80% are ABC transporter proteins (Grangeiro et al., 2004). In this work, the ABC transporters identified were highly expressed at 30°C in exponential phase (data not shown). These proteins are likely associated with the increase in primary metabolism that was observed during the exponential growth phase at the optimal growth temperature, as discussed before. The proton/sodium glutamate symport protein (CV3409) is involved in the transport of glutamate, an important carbon source, across the membrane through a system involving the co-transport of protons and sodium (Raunser et al., 2006). This protein is known to be expressed under nutrient-limiting conditions. In the present work, glutamate symport levels increased in cells grown at 25 and 35°C during the exponential growth phase (data not shown) in contrast to that observed for metabolic enzymes. This upregulation may represent a response to a decreased function of the primary metabolic pathways, such as glycolysis, which may also occur during carbon and nitrogen starvation. However, more studies need to be performed to explore the contribution of transport proteins in different adaptation strategies of *C. violaceum* to adverse conditions such as thermal, oxidative and starvation stresses.

In conclusion, even though the complete scenario for stress adaptation for *C. violaceum* was not pictured, the involvement of chaperones and other proteins that typically contribute to adaptation in stressful conditions was demonstrated. To better understand the contribution of these proteins and their regulatory elements, the promoter regions of genes related to heat stress in *C. violaceum* have been isolated and cloned, and studies of their activities under different temperatures are in progress. The work presented here provides new insight into the genome and proteome of *C. violaceum*.

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