Antonie van Leeuwenhoek (2020) 113:101–116
https://doi.org/10.1007/s10482-019-01320-5

ORIGINAL PAPER

Different roles of two *groEL* homologues in methylotrophic utiliser of dichloromethane *Methylorubrum extorquens* DM4

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Received: 12 July 2019 / Accepted: 19 August 2019 / Published online: 28 August 2019

Abstract The genome of methylotrophic bacteria *Methylorubrum extorquens* DM4 contains two homologous *groESL* operons encoding the 60-kDa and 10-kDa subunits of GroE heat shock chaperones with highly similar amino acid sequences. To test a possible functional redundancy of corresponding GroEL proteins we attempted to disrupt the *groEL1* and *groEL2* genes. Despite the large number of recombinants analysed and the gentle culture conditions the *groEL1*-lacking mutant was not constructed suggesting that the loss of GroEL1 was lethal for cells. At the same time the Δ*groEL2* strain was viable and varied from the wild-type by increased sensitivity to acid, salt and desiccation stresses as well as by the impaired growth with a toxic halogenated compound—dichloromethane (DCM). The evaluation of activity of putative P_{groE1} and P_{groE2} promoters using the reporter gene of green fluorescent protein (GFP) showed that the expression of *groESL1* operon greatly prevails (about two orders of magnitude) over those of *groESL2* under all tested conditions. However the above promoters demonstrated differential regulation in response to stresses. The expression from P_{groE1} was heat-inducible, while the activity of P_{groE2} was upregulated upon acid shock and cultivation with DCM. Based on these results we conclude that the highly conservative *groESL1* operon (old locus tags METDI5839-5840) encodes the housekeeping chaperone essential for fundamental cellular processes. On the contrary the second pair of paralogues (METDI4129-4130) is dispensable, but corresponding GroE2 chaperone promotes the tolerance to acid and salt stresses, in particular, during the growth with DCM.

Keywords Aerobic methylotrophic bacteria · Chaperonins · Dichloromethane · 60-kDa heat shock protein · Stress response

Introduction

Chaperonins GroEL (Cpn60, Hsp60) and GroES (Cpn10, Hsp10) represent one of the most important groups of molecular chaperones and are necessary for proper folding and refolding of many cell proteins (Hayer-Hartl et al. 2016; Mizobata and Kawata 2018). For the assembly of functionally active GroE chaperone 14 GroEL oligomers form a barrel-shaped

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10482-019-01320-5) contains supplementary material, which is available to authorized users.

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structure with a lid consisting from 7 GroES subunits. The resulting macromolecular complex possesses a central cavity, hydrophobic amino acid residues of which are able to interact with denatured protein (Motojima 2015 for a review; Mizobata and Kawata 2018). The GroE chaperone mediates folding process by transient trapping of polypeptide in the inner cage of complex, thus preventing aggregation of target protein and its incorrect interactions with other cell structures. The passage of GroE trough the folding cycle is regulated by binding and hydrolysis of ATP (Xu et al. 1997; Motojima 2015; Mizobata and Kawata 2018).

In *Escherichia coli* both GroEL and GroES are essential for viability and represent 1 to 2% of total cellular proteins even under optimal growth conditions (Hemmingsen et al. 1988; Fayet et al. 1989; Zügel and Kaufmann 1999). These chaperonins greatly increase the yield of correctly folded proteins, especially for polypeptides tending to form aggregates (like Rubisco) (Goloubinoff et al. 1989; Lee et al. 1997; Hayer-Hartl et al. 2016 for a review) and/or intended for transport across the membrane (Li and Wong 1992; Kumar et al. 2015). Biochemical studies also demonstrated that GroEL co-purifies with some proteins (transcription factors NodD in *Sinorhizobium meliloti*) and is able to modulate their activities (Kumar et al. 2015 for a review). Besides this molecular chaperonins play an important role by preventing of a lethal nonspecific association of proteins under stress conditions (Zügel and Kaufmann 1999; Mizobata and Kawata 2018). It was shown that under stress conditions the amount of GroEL protein in *E. coli* cells significantly raises (Hemmingsen et al. 1988; Zügel and Kaufmann 1999; Kumar et al. 2015), and interruptions of corresponding gene expression may lead to cell death (Fayet et al. 1989; Walter 2002).

Aerobic methylotrophic bacteria, growing with toxic C_1-compounds (methane, methanol, formaldehyde, methylamines, halomethanes, etc.) as the sole carbon and energy sources, are widespread in the environment and face a variety of extra- and intracellular stresses (Trotsenko and Khmelenina 2002; Vuilleumier 2002; De Marco et al. 2004; Kolb 2009; Torgonskaya et al. 2011; Vorholt 2012). In addition, a number of their enzymes essential for primary metabolism of C_1-substrates (methane monooxygenases, quinoprotein dehydrogenases of methanol, methylamine and formaldehyde) are associated with cell membranes or required to be transported into periplasm for functioning. The misfolding of such proteins, which are usually predominant during methylotrophic growth, can lead to their aggregation and loss of metabolic activity. Nevertheless until present the studies of diversity and functions of GroE chaperones in methylotrophs were limited mostly to accidental genomic or proteomic findings (Csáki et al. 2003; Ward et al. 2004; Chongcharoen et al. 2005; Bosch et al. 2008; Hendrickson et al. 2010; Muller et al. 2011). The only specialised study was devoted to cloning and molecular characterisation of operon encoding GroE in *β-Proteobacteria Methylovorus* sp. SS1 DSM 11726 (Eom et al. 2005). Meanwhile chaperonins GroEL and GroES were predominant or induced proteins in proteomes of some representatives of genera *Methylorubrum* (formerly "*Methylobacterium*" (Green and Ardley 2018)) and *Methylobacillus* grown with methanol, methylamine, formaldehyde or dichloromethane (Chongcharoen et al. 2005; Hendrickson et al. 2010; Muller et al. 2011; Firsova et al. 2015). Furthermore the homologue of *groEL* gene revealed in the cluster encoding methane monooxygenase in methanotroph *Methylococcus capsulatus* Bath turned to be indispensable for correct synthesis of this key enzyme of methanotrophic lifestyle (Csáki et al. 2003). All these findings suggest that GroE chaperones may play important roles during destruction of C_1-toxicants and cell responses to accompanying stresses.

In most of studied bacteria chaperonin GroEL and co-chaperonin GroES are encoded by only one bicistronic operon *groESL*, which is necessary for living. However, the presence of multiple *groESL* operons is found in an increasing number (already $\sim$ 30%) of bacterial genomes (Lund 2009; Kumar et al. 2015). Some strains additionally possess separately located *groEL* (*cpn60*) and *groES* (*cpn10*) genes (Eom et al. 2005; Lund 2009). The reasons for maintaining of multiple *groESL* copies are not completely clear, but it was hypothesised that such genes may be differentially regulated and/or encode chaperonins with more specialised functions (Lund 2009). Some evidences supporting these assumptions were already obtained for root nodulating bacteria within the order *Rhizobiales* and some nitrogen-fixing *β-Proteobacteria* (Kumar et al. 2015 for a review). For example, among 4 *groESL* operons and separately located *groEL* gene of *S. meliloti* Rm1021 only one

determinant (*groEL1* or *groEL2*) is necessary for growth and phytosymbiosis, whereas the others are likely specialised for stress response (Bittner et al. 2007). In *Bradyrhizobium japonicum*, all *groESL* operons are individually non-essential, but the loss of two of them causes significant decrease of nitrogenase activity important for symbiosis with plants (Kumar et al. 2015 for a review).

By genome-wide search in a well-studied dichloromethane utiliser *Methylorubrum extorquens* (formerly "*Methylobacterium dichloromethanicum*" (Doronina et al. 2000; Kato et al. 2005; Green and Ardley 2018)) DM4 we also revealed the presence of two *groESL* operons—*groESL1* (old locus tags METDI5840-5839) and *groESL2* (old locus tags METDI4130-4129), which encode highly similar (83–84% of identity) proteins. Herewith, the ability to mineralise dichloromethane (DCM) inherent to this strain implies the metabolic processes associated with a complex of challenges for cells. This compound affects cell membrane integrity as a solvent (Torgonskaya et al. 2011), but acts also as a mutagen (Firsova et al. 2005). Enzymatic dehalogenation of DCM catalysed by dichloromethane dehalogenase occurs in cytoplasm and leads to formation of highly reactive toxic metabolites—S-chloromethylglutathione and formaldehyde (Kayser and Vuilleumier 2001; Vuilleumier 2002). Besides this, dechlorination process is acidogenic and includes intracellular production of chloride ions (Vuilleumier 2002). Taken together these features make *M. extorquens* DM4 a good model for analysis of functioning of homologous genes of GroE chaperones. Accordingly, the present study was aimed to check a possible functional redundancy of *groESL1* and *groESL2* operons and uncover their specific roles during growth of *M. extorquens* DM4 with methanol and dichloromethane.

Materials and methods

Strains and culture conditions

The strains and plasmids used in the study are listed in Table 1. Methylotrophic bacteria *Methylorubrum extorquens* DM4 (Doronina et al. 2000; Kato et al. 2005; Green and Ardley 2018) were grown at 29 °C in a minimal medium (MM) (pH 7.2) with 120 mM methanol, 20 mM succinate or 10 mM DCM, as described earlier (Torgonskaya et al. 2011; Firsova et al. 2015). For cultivation with DCM 300 ml glass flasks closed by Supelco gas-tight Mininert™ caps (Bellefonte, USA) were used. DCM was added to the medium through a membrane with a syringe. *E. coli* strains were cultured at 37 °C in Luria–Bertani (LB) medium. For *E. coli* transformants and mutant *Methylorubrum* strains the media were additionally supplemented with corresponding antibiotics, as indicated previously (Firsova et al. 2011).

DNA manipulation and genetic techniques

The genomic DNA from *M. extorquens* DM4 was purified by Zymo Research Fungal/Bacterial DNA MiniPrep™ kit (Irvine, USA) according to the manufacturer's instructions. Isolation of plasmid DNA was carried out using GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific, Vilnius, Lithuania). The DNA fragments from PCR amplification and restriction reactions were purified and concentrated using Zymo Research Zymoclean™ Gel DNA Recovery Kit (Irvine, USA). Genetic manipulations with DNA including restriction and cloning, competent cells preparation and transformation were performed according to the standard protocols (Sambrook and Russel 2001). For DNA sequencing the BigDye Terminator v. 3.1 reaction kit with subsequent analysis of reaction products with an automated sequencer "3730 DNA Analyzer" (Applied Biosystems, USA) was used.

The search of GroEL and GroES homologues in the database of the National Center for Biotechnology Information (NCBI) was conducted using online interface of BLASTX program (Altschul et al. 1990). Phylogenetic analysis of translated amino acid sequences was carried out by MEGA X software (Kumar et al. 2018). The sequences were aligned by built-in ClustalW function using default settings and all positions containing gaps or missing data were eliminated. The phylogram was generated using Jones–Taylor–Thornton (JTT) model based maximum likelihood method (Jones et al. 1992). Evaluation of topology of resulting tree was done by bootstrap resampling method (Felsenstein 1985) with 1000 replicates. Phylogenetic trees generated using neighbor-joining, minimum-evolution and UPGMA methods had similar topologies.

Table 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
Bacterial strains		
Methylorubrum extorquens DM4	Aerobic methylotrophic DCM-utilising bacterium, formerly "*Methylobacterium dichloromethanicum*" DM4, wild-type strain (VKM B-2191 = DSM 6343)	Doronina et al. (2000), Kato et al. (2005), Green and Ardley (2018)
Escherichia coli S17-1	F$^-$ *thi pro recA hsdR* [RP4-2Tc::Mu-Km::Tn*7*] Tp^r Sm^r	Simon et al. (1983)
Escherichia coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX*74 *recA1 araD*139 Δ(*ara-leu*) 7697 *galU galK rpsL* (Str^r) *endA1 nupG* λ-	Invitrogen, USA
DM4 Δ*groEL2*	DM4 derivative, Δ*groEL2*::*aacC1*, Gm^r	This study
DM4 pCM*gfp*	DM4 derivative with promoterless pCM*gfp* vector, Km^r	This study
DM4 pCM*gfp*:P$_{groE1}$	DM4 derivative with pCM*gfp*:P$_{groE1}$ vector, Km^r	This study
DM4 pCM*gfp*:P$_{groE2}$	DM4 derivative with pCM*gfp*:P$_{groE2}$ vector, Km^r	This study
DM4 Δ*groEL2* pCM*gfp*	DM4 Δ*groEL2* derivative with pCM*gfp* vector, Km^r, Gm^r	This study
DM4 Δ*groEL2* pCM*gfp*:P$_{groE1}$	DM4 Δ*groEL2* derivative with pCM*gfp*:P$_{groE1}$ vector, Km^r, Gm^r	This study
DM4 Δ*groEL2* pCM*gfp*:P$_{groE2}$	DM4 Δ*groEL2* derivative with pCM*gfp*:P$_{groE2}$ vector, Km^r, Gm^r	This study
Plasmids:		
pK18mob	Mobilisable multi-purpose cloning vector, Km^r	Schäfer et al. (1994)
p34S-Gm	Plasmid source of the Gm^r cassette, Ap^r, Gm^r	Dennis and Zylstra (1998)
pK*groEL1*	pK18mob containing a 1691-bp region with *groEL1* gene and its flanking sequences from *M. extorquens* DM4, Km^r	This study
pK*groEL1*-Gm	pK*groEL1* containing Gm^r-cassette cloned into *Pst*I sites, Km^r, Gm^r	This study
pK*groEL2*	pK18mob containing a 2043-bp region with *groEL2* gene and its flanking sequences from *M. extorquens* DM4, Km^r	This study
pKΔ*groEL2*-Gm	pK*groEL2* containing 456-bp deletion within the *groEL2* gene and Gm^r-cassette cloned into *Sal*I sites, Km^r, Gm^r	This study
pGreenTIR	Plasmid source of the *gfpmut1* cassette, Ap^r	Miller and Lindow (1997)
pCM132	Low-background broad-host-range promoter-probe vector with transcription terminator $t_{rrn\ B}$ from *E. coli* and *lacZ* reporter gene, Km^r	Marx and Lidstrom (2001)
pCM*gfp*	pCM132 with reporter *gfpmut1* gene from pGreenTIR replacing *lacZ*, Km^r	This study
pCM*gfp*:P$_{groE1}$	pCM*gfp* with *gfpmut1* gene under control of *groESL1* operon promoter from *M. extorquens* DM4, Km^r	This study
pCM*gfp*:P$_{groE2}$	pCM*gfp* with *gfpmut1* gene under control of *groESL2* operon promoter from *M. extorquens* DM4, Km^r	This study

Average nucleotide identity (ANI) for genomes was evaluated using the Integrated Microbial Genomes & Microbiome System v. 5.0 (Chen et al. 2019). DNA–DNA homology (DDH) for strains was assessed by in silico hybridisation using online Genome-to-Genome Distance Calculator v. 2.1 (Meier-Kolthoff et al. 2013).

Disruption of *groEL1* and *groEL2* genes

The *groEL1* and *groEL2* genes of *M. extorquens* DM4 were knocked out by insertion of gentamicin resistance cassette using site-specific homologous recombination. For this purpose the sequences of *groEL* encoding regions were amplified from genomic DNA

of DM4 strain by PCR with primers matching highly variable flanking regions of corresponding reading frames. The pair of primers 5839f (5′-AAAAA*TCTAGA*CTTCAGGGCCCCTTCCAT-3′) and 5839r (5′-ATACT*AAGCTT*GAATTCCCGGGTGCGTGGACT TAG-3′) was used for *groEL1* (METDI5839) gene amplification, whereas the pair 4129f (5′-TGC*GAATTC*AGCAAGCTCTGACGTCATCG-3′) and 4129r (5′-CGG*AAGCTT*ATCGGTCGATCT-CATCGGAG-3′) was specific for *groEL2* (METDI4129) sequence. The given primers were designed based on the available genome sequence for *M. extorquens* DM4 (GenBank accession number FP103042) and contained artificial restriction sites (italicised) for cloning of amplicons into mobilisable suicide vector pK18mob (Schäfer et al. 1994)—*Xba*I/*Hind*III and *Eco*RI/*Hind*III, respectively. The cloning of amplified DNA fragments into pK18mob plasmid resulted in pK*groEL1* and pK*groEL2* constructs (Table 1). The vectors pK*groEL1* and pK*groEL2* were subsequently cleaved by *Pst*I or *Sal*I restriction site within the *groEL* sequences and ligated with gentamicin resistance gene from p34S-Gm (Dennis and Zylstra 1998) in direct orientation. The obtained pK*groEL1*-Gm and pK*groEL2*-Gm plasmids contained corresponding mutant *groEL1* or *groEL2* genes disrupted by insertion of Gm^r-cassette. Furthermore, in the pK*groEL2*-Gm vector the target *groEL2* gene had also a 456-bp deletion caused by the presence of two *Sal*I restriction sites in the initial sequence (Table 1). The resulting length of homologous sites for recombination located upstream and downstream of Gm^r-cassette insertion amounted 733/900 bp for *groEL1* gene and 1032/543 bp for *groEL2* gene.

The constructs pK*groEL1*-Gm and pK*groEL2*-Gm were transferred into *M. extorquens* DM4 cells by biparental mating using *E. coli* S17-1 as a donor strain, as described earlier (Firsova et al. 2011). Among the transconjugants only gentamicin resistant and kanamycin sensitive (Gm^r, Km^s) double recombinants were selected for further work, since the Km^r phenotype marked single crossovers and cells still carrying the introduced plasmids (Table 1). All obtained constructs were verified by PCR amplification and sequencing.

Phenotypic characterisation of Δ*groEL2* mutant

The growth rates of the wild-type *M. extorquens* DM4 and its *groEL2*-deficient derivative with DCM were determined by measuring optical density at 600 nm (OD_{600}) and chloride ions release in bacterial cultures. For this purpose the strains were grown in MM medium with 10 mM DCM, cells were harvested (6000 g, 30 min) in late log phase, washed twice with a fresh sterile medium and resuspended in it up to OD_{600} = 0.17. The resulting bacterial suspensions (50 ml) were transferred into 300 ml glass flasks closed by Supelco gas-tight Mininert^{TM} caps (Bellefonte, USA) and cultivated with DCM for 42 h, as described earlier (Firsova et al. 2011). The samples of cultures for measurements of OD_{600} and chloride production were taken every 6 h of incubation. Chloride concentrations in supernatants of cell suspensions were determined by a thiol-tolerant method (Jörg and Bertau 2004), as previously described (Firsova et al. 2011; Torgonskaya et al. 2011). All experiments were carried out in triplicate.

For comparative analysis of stress tolerance of DM4 wild-type and Δ*groEL2* strains the cells were grown in MM medium with 120 mM methanol to OD_{600} = 1.0. The resistance of bacteria to hydrogen peroxide, methylglyoxal, formaldehyde, and sodium dodecyl sulphate (SDS) was determined by diffusion method with cellulose discs, and the tolerance to ethanol, desiccation, heat shock and high salinity was assessed by serial dilutions technique. Experimental conditions, which have been used for both approaches, were analogously to earlier described (Gourion et al. 2008; Firsova et al. 2017). Acid stress was induced by addition of 5 M HCl or 8 M CH_3COOH to 50 ml cell suspensions (OD_{600} = 0.5) up to pH 5.0 followed by 2 h incubation at 29 °C on the rotary shaker (180 rpm). After subsequent neutralisation of the media up to pH = 7.0 by 5 M NaOH solution, the serial dilutions (10^{-1}–10^{-8}) of cultures were plated onto agarised MM medium with methanol. The cell suspensions unexposed to acids were used as the control. All experiments were also performed in triplicate.

Construction of transcriptional fusions of *groESL* promoters with reporter GFP gene

To assess the expression activities from promoters of *groESL* operons in *M. extorquens* DM4 the reporter plasmids were constructed basing on the low-background promoter-probe vector pCM132 (Marx and Lidstrom 2001) kindly provided by Mary Lidstrom (Addgene plasmid #45829). The plasmid pCM132 contains transcription terminator sequence $t_{rrn\ B}$ from *E. coli* and the *lacZ* gene of β-galactosidase subunit as a reporter (Table 1). For our study we firstly replaced the *lacZ* repoter in pCM132 by significantly shorter (717 vs. 3060 bp) *gfpmut1* gene of green fluorescent protein (GFP) with two mutations (F64L and S65T), which increase its solubility and shift an excitation maximum from 395 to 490 nm (Miller and Lindow 1997). The *gfpmut1* gene was amplified by PCR from pGreenTIR plasmid (Miller and Lindow 1997) using primers GFPinsf (5′-TGCT*GGTACC*GCTC GAATTCTGATTAA-3′) and GFPinsr (5′-CCCA *GCATGC*CTATTTGTATAGTTCATCCA-3′) containing restriction sites *Acc*65I and *Sph*I (italicised) for directional cloning. The resulting 775-bp DNA fragment was ligated into the pCM132 backbone instead of *lacZ* to construct a new reporter vector pCM*gfp* (8822 bp) (Table 1).

The supposed promoter regions of *groESL1* and *groESL2* operons (P_{groE1} and P_{groE2}) were amplified from genomic DNA of *M. extorquens* DM4 by PCR using primers including *Eco*RI and *Acc*65I restriction sites for subsequent cloning. Among primers intended to clone the P_{groE1} sequence the forward probe p5840f (5′-AAGA*GAATTC*GAGGTGGTCCGCGTTGAG-3′) corresponded to nucleotide positions 497 to 514 upstream of the *groESL1* transcription start site, whereas the reverse one—p5840r (5′-TCTT*GGTACC*CTTG CGGCTTCTCCTTGG-3′) was complementary to 92–109 positions within the *groES1* gene. Similarly, the primers p4130f (5′-ATAA*GAATTC*CAAGCCGT-CACCGTGGTG-3′) and p4130r (5′-AAAT*GGTAC C*CCTCCTGCGGCTTCTCCT-3′) for P_{groE2} amplification matched the 471-488 nucleotides upstream and 95–112 positions downstream of *groESL2* transcription start. The obtained P_{groE1} and P_{groE2} amplicons (623 and 600 bp, respectively) were ligated into pCM*gfp* plasmid between *Eco*RI and *Acc*65I restriction sites (italicised) directly upstream of *gfpmut1* reporter. The resulting transcriptional fusion vectors pCM*gfp*:P_{groE1} and pCM*gfp*:P_{groE2} (Table 1) were transferred into *M. extorquens* DM4 cells by biparental mating using *E. coli* S17-1 as a donor strain, as described earlier (Firsova et al. 2011). The correctness of all obtained constructs was verified by PCR amplification and sequencing.

Assessing of *groESL* promoters activity by GFP fluorescence

The activities of cloned *groESL* promoters were estimated in *M. extorquens* DM4 transconjugants expressing *gfpmut1* reporter under control of P_{groE1} and P_{groE2} (Table 1). For evaluation of a background level of GFP expression the DM4 strain carrying the promoterless pCM*gfp* plasmid was used as a reference. Corresponding cultures were grown to mid-exponential log-phase (OD_{600} = 0.5) with methanol (120 mM), DCM (10 mM) or succinate (20 mM) in 50 ml of MM medium with kanamycin (25 μg/ml). The cells from 10 ml of cultures were pelleted by centrifugation (8000 g for 15 min at 4 °C), washed with 100 mM potassium phosphate buffer (pH 8.0), resuspended in 1 ml of the same buffer and disrupted by 150 W sonication (S-4000, MiSonix, USA) using 50 × 2 s pulses at 40 kHz on ice. Cell debris was removed by centrifugation (13,000 g for 15 min at 4 °C) and cell-free extracts were used for GFP assay. Protein concentrations in the extracts were determined by Bradford method (Bradford 1976). All experiments were carried out in triplicate.

GFP fluorescence was measured in black 96-well non-binding microplates (Greiner Bio-One, Germany) using fluorimeter FLUOstar OPTIMA (BMG Labtech, Germany) at excitation and emission wavelengths of 485 and 510 nm, respectively. All samples were analysed in triplicate. Specific fluorescence intensities were determined by dividing the raw data by protein amounts found in each sample and subtracting of background fluorescence from resulting values. The reported GFP concentrations (μg/mg of total protein) in extracts were estimated according to calibration curves plotted for each measurement using purified GFP standard.

To assess the expected influence of external adverse factors to expression activities from P_{groE1} and P_{groE2} promoters the cells grown with methanol (OD_{600} = 0.5) were exposed to acid, thermal and saline stresses. For acid stresses the experimental conditions

were analogous to described above. Saline stress was induced by addition of 5 M NaCl to 50 ml of cell suspensions up to final concentration of 100 mM. For a thermal stress all tested strains were incubated at 37 °C for 2 h. The reported relative values of GFP production (RP_{GFP}) were determined by subtracting of difference between GFP concentrations in reference DM4 pCM*gfp* strain before (timepoint "0") and after (timepoint "t") induction from those in cells with pCM*gfp*:P_{groE1} and pCM*gfp*:P_{groE2} reporter plasmids, analogously to previously described (Cha et al. 1999; Seo et al. 2003):

$$RP_{GFP} = (GFP_t - GFP_0)_{\text{stress probe}} - (GFP_t - GFP_0)_{\text{ref}}.$$

Results and discussion

Functional *groESL1* operon is essential for viability of *M. extorquens* DM4

To estimate a significance of two distinct GroE chaperones in *M. extorquens* DM4 we attempted to generate strains lacking *groEL1* and *groEL2* determinants by replacing of these genes with their nonfunctional copies. To distinguish between highly similar *groEL1* and *groEL2* reading frames (83 and 82% of nucleotide and amino acid identity, respectively) for amplification of corresponding fragments the primers matching their flanking sequences were used. As a result, the suicide plasmid pK*groEL1*-Gm transferred into cells of *M. extorquens* DM4 harbored the full-length *groEL1* gene disrupted by insertion of a gentamicin resistance cassette (see Materials and methods and Table 1). However the analysis of more than 500 clones of transconjugants did not reveal double recombinants with impaired GroEL1 synthesis. This amount tenfold exceeded the required minimum for *M. extorquens* DM4, for which the expected double crossover event frequency usually equals $\sim 2\%$. The obtained single recombinants (Gm^R, Km^R) had arisen by integration of the entire plasmid into the chromosome and contained intact *groEL1* gene along with disrupted copy (data not shown). Assuming that the inactivation of *groEL1* gene can lead to reduced viability of cells in the presence of toxic C_1-compounds, the selection of transconjugants in repeated experiments was carried out using succinate instead of methanol as a carbon source. Also we attempted to cultivate the cells at a lowered temperature (16 °C). Nevertheless under all these conditions no double recombinants were obtained. On the contrary, the inactivation of *groEL2* reading frame using suicide vector pK*groEL2*-Gm was successful and the observed frequency of double recombinants carrying a 456-bp deletion and Gm^R cassette insertion within the target gene sequence was usual. This result suggests that unlike GroEL2 the functionality of GroEL1 can be crucial for growth of *M. extorquens* DM4, and the corresponding *groESL1* operon may encode a major housekeeping GroE chaperone necessary for fundamental cellular processes.

To further assess this possibility we analysed the expression from promoters of *groESL1* and *groESL2* operons using their transcriptional fusions with GFP encoding gene in low-background vectors (see Materials and methods and Table 1). It was revealed that under all tested conditions in cells of a wild-type *M. extorquens* DM4 the activity of P_{groE1} was about two orders of magnitude higher than those of P_{groE2} (Fig. 1). According to previous reports, such

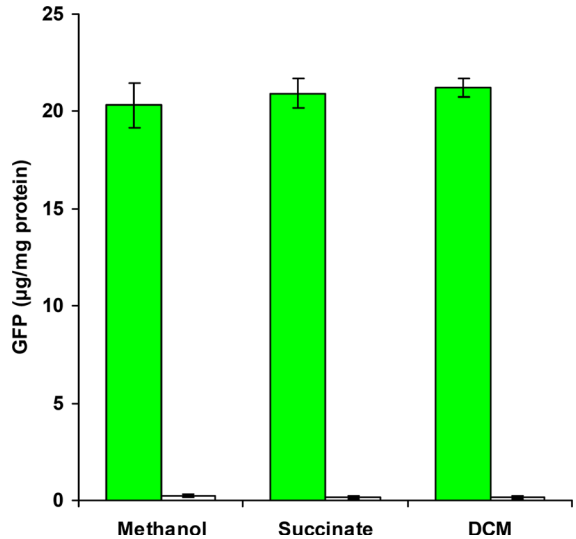

Fig. 1 Expression activity of promoters of two *groESL* operons in *M. extorquens* DM4. The expression levels from P_{groE1} (filled bars) and P_{groE2} (open bars) promoters were estimated in cells grown with methanol (120 mM), succinate (20 mM) or DCM (10 mM) using GFP as a transcriptional reporter. The reported values of concentrations of produced GFP represent the averages from three biological replicates. The error bars show standard deviations from the means

significant predominance in expression of a certain *groESL* operon can also point on the special importance of encoded chaperonins for viability of cells. For example, it was demonstrated that among 3 *groESL* operons of *Rhizobium leguminosarum* two gene sets with substantially lesser expression were not mandatory for normal growth (Rodríguez-Quiñones et al. 2005). Similarly in *Rhodobacter sphaeroides* only one *groESL* operon turned to be essential for living, whereas the products of a second pair of genes were even not found, leading the authors to assumption about pseudogene nature of the latter *groES* reading frame (Lee et al. 1997).

Finally, the search conducted using BLASTX program showed that among GroES and GroEL of *M. extorquens* DM4 the pair encoded by *groESL1* operon is characterised by highest similarity to proteins of closely related representatives of *Methylorubrum*. Phylogenetic analysis of translated amino acid sequences of *groEL* genes in these strains revealed that the proteins similar to GroEL1 and GroEL2 of *M. extorquens* DM4 form distinct clades in the dendrogram (Fig. 2). The GroEL1 group is represented by 100% identical determinants from all available genomes of *M. extorquens* except the data for the type strain TK 0001^T, in which *groESL*-encoding regions seem to contain sequencing errors and need to be rechecked. Since the phylogenetic positioning of many bacteria based on GroEL sequences of their only GroE chaperones is in a good agreement with distribution in the 16S rDNA tree (Goyal et al. 2006), the observed conservativeness of GroEL1 structure within a species also supports the hypothesis on housekeeping function of the *groESL1* operon. In this regard the unexpected presence of *Methylorubrum zatmanii* PSBB041 and several strains of *Methylobacterium* sp. among the closest "neighbors" of *M. extorquens* could be explained by high similarity between corresponding genomes. Indeed, the values of ANI (97.25–99.58%) and digital DDH (72.40–95.20%) (Supplementary material, Table 2, 3) assessed for sequences of mentioned strains and those of representatives of *M. extorquens* were above the criteria for assignment to separate species (95–96% for ANI and 70% for DDH) (Chun et al. 2018).

groESL2 operon of *M. extorquens* DM4 is specialised for non-heat stress response

The GroEL2-like proteins, which are more phylogenetically diverse within the clade (98.4–100.0% of identical amino acid residues), display lesser similarity with GroEL1 homologues (80.8–82.0%) (Fig. 2) and represent another group of GroEL chaperonins in *M. extorquens*. However the comparatively low level of *groESL2* operon expression in *M. extorquens* DM4 (Fig. 1) makes doubtful the necessity of GroE2 chaperone functioning for cell growth. To shed light on the role of GroE2 in *M. extorquens* DM4 we constructed a knockout-mutant lacking functional *groEL2* gene and analysed the expression of *groESL1* and *groESL2* operons under different cultivation conditions.

As expected, the strain DM4 Δ*groEL2* retained the ability to grow with methanol and succinate, however its growth rate with DCM was significantly reduced (by 42.9 ± 12.6%) compared to those of the wild-type (Fig. 3). The chloride production used for estimation of activity of DCM dehalogenation was declined in GroEL2-deficient culture in the similar extent (by 32.9 ± 9.5%) (Fig. 3). At the same time the profiles of expression from *groESL* promoters in the wild-type strain of *M. extorquens* DM4 showed that the synthesis of GFP reporter controlled by P_{groE2} is upregulated during the growth of cells with DCM (by 84.6 ± 7.4%) (Fig. 4). Hence we conclude that despite the huge predominance of GroE1 expression, the functional GroE2 chaperone can be important under certain cultivation conditions.

The need in accessory chaperones usually arise in cells if the main GroE complex does not provide for some reason the proper folding of necessary proteins. In this connection we assumed that the observed growth defect of the GroEL2-deficient strain was associated with the loss of specialised GroE2 machinery functions required under stresses, accompanying DCM mineralisation. To determine possible factors promoting the functional activity of GroE2 chaperone, we analysed the resistance of methanol-grown mutant cells to a range of individual adverse factors. The exposure of the DM4 Δ*groEL2* and wild-type bacteria to formaldehyde, SDS, hydrogen peroxide and ethanol did not reveal significant differences in their viability (Figs. 5, 6). The heat shock (55 °C for 5 min) was lethal for the most of cells however its impact on both

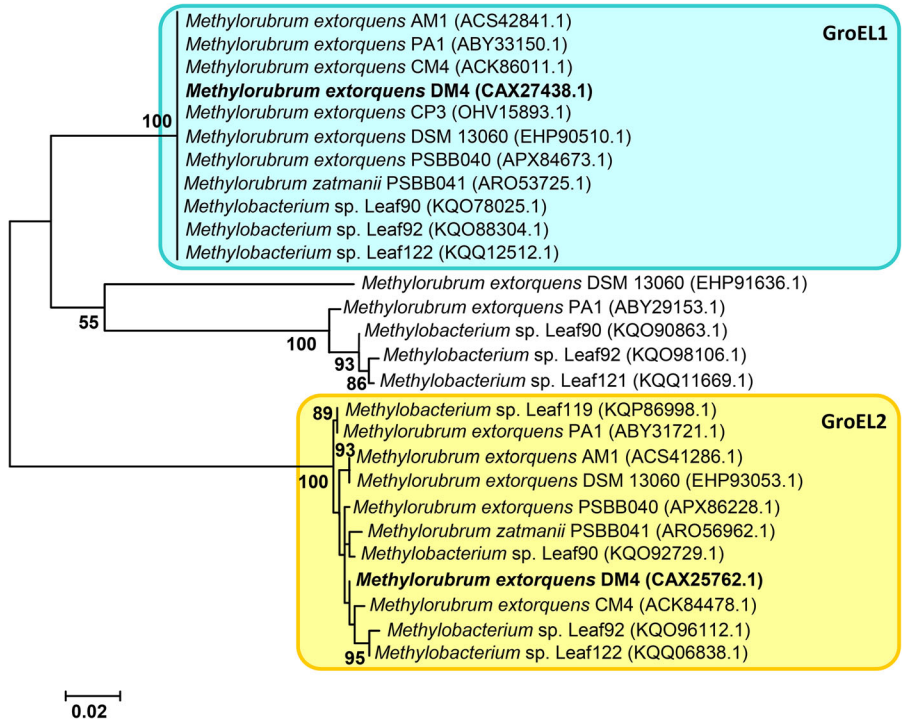

Fig. 2 Phylogenetic analysis of homologues of GroEL chaperonins in strains closely related to *Methylorubrum extorquens* DM4 using JTT-model based maximum likelihood method. The evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). The tree with the highest log likelihood (− 2829.74) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BIONJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The final dataset included 548 positions

strains was also the same (Fig. 6). On the contrary the sensitivity of the Δ*groEL2* mutant to 100 mM NaCl, acidic pH (5.0), desiccation, and methylglyoxal treatment turned to be higher than those of the wild-type strain (Figs. 5, 6). This find is hardly accidental, as acidic, osmotic and oxidative stresses are characteristic for DCM metabolism, due to intracellular production of HCl and reactive intermediate—S-chloromethylglutathione. Thus, considering the similarity of modeled pH and salinity conditions with those acting on cells during dehalogenation, the main reason for the observed decrease of the growth rate with DCM in the GroEL2-lacking culture can be a combination of impaired tolerance to these factors.

The changes registered in expression from promoters of *groESL1* and *groESL2* operons under acid and salt shocks also testify in favor of importance of GroE2 chaperone for responses to these stresses. Unlike slightly decreasing activity of P_{groE1} (for ~ 4–7%), those of P_{groE2} remained stable or even demonstrated induction (up to 140%) after 2 h exposure to HCl and CH_3COOH (pH 5.0) or 100 mM NaCl (Fig. 7). Herewith the greatest effects caused by acetic acid can be explained by its faster permeation into bacterial cytoplasm (Lund et al. 2014). On the contrary the cultivation of cells at the elevated temperature (37 °C) promoted the activation (up to ~ 11%) of GFP synthesis only under P_{groE1} control (Fig. 7), suggesting the insensitivity of P_{groE2} to thermal stress. Altogether the observed regulatory differences do not only imply a functional divergence of two GroE chaperones in *M. extorquens* DM4, but also point out to dissimilarity in molecular mechanisms controlling their expression.

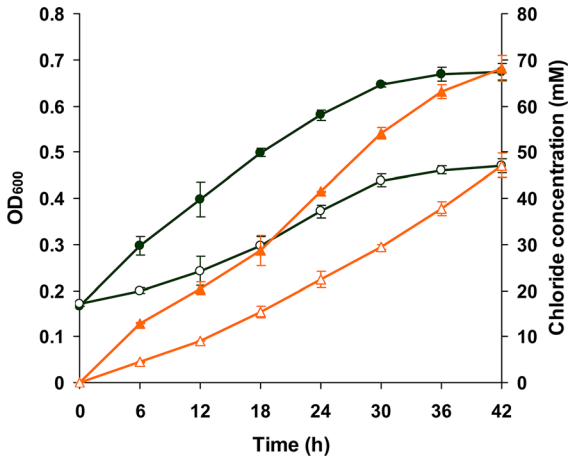

Fig. 3 Comparison of the growth of *M. extorquens* DM4 wild-type and Δ*groEL2* strains with dichloromethane. The values of OD_{600} (circles) of cultures and chloride concentrations (triangles) in medium represent averages from three independent experiments with cells possessing (filled symbols) or lacking (open symbols) functional *groEL2* gene. The error bars indicate standard deviations from the means

In silico analysis predicts differences in regulatory mechanisms for two *groESL* operons

Transcriptional regulation of synthesis of GroES and GroEL chaperonins in bacteria varies among species, although the co-transcription of genes in the order *groES-groEL* represents the common feature for studied *groESL* operons (Fayet et al. 1989). The known systems of positive control for the latter include two types of promoters located upstream *groES* gene and recognised by RNA polymerase in cooperation with corresponding sigma factors. The vegetative (σ^{70}-dependent) promoter provides the synthesis of GroES and GroEL chaperonins under normal growth conditions, and the alternative (σ^{32}-dependent) sequences are used for induction of expression upon heat-shock and other stresses (Zhou et al. 1988; Gruber and Gross 2003). The temperature-sensitive mechanisms of negative regulation represent transcriptional repression of *groESL* operons by proteins interacting with specific *cis*-acting elements—CIRCE (Controlling Inverted Repeat of Chaperone Expression) and ROSE (Repression Of heat Shock gene Expression). The CIRCE determinants and corresponding HrcA repressor operate in most Gram-positive and some Gram-negative bacteria (Zuber and Schumann 1994; Hecker et al. 1996),

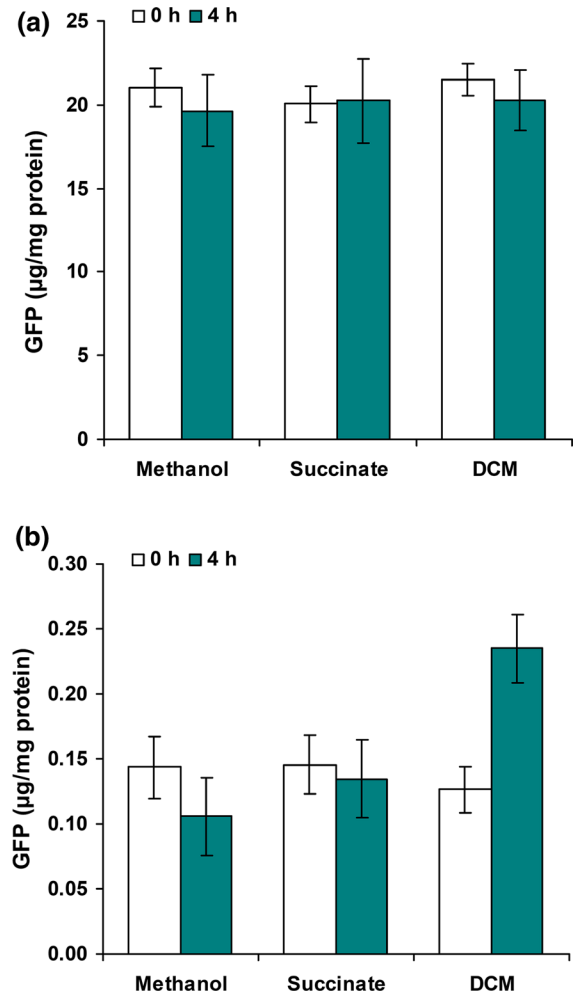

Fig. 4 Effects of cultivation with methanol, succinate and dichloromethane to activity of the promoters of *groESL* operons in *M. extorquens* DM4. The expression levels from P_{groE1} (**a**) and P_{groE2} (**b**) promoters were assessed in exponentially-grown cultures of cells preliminary adapted to corresponding substrates for three generations. The data represent average values of produced GFP concentrations from three biological replicates. The error bars show standard deviations from the means

whereas the ROSE system was found to date in a limited number of rhizobia (Narberhaus et al. 1998a; Nocker et al. 2001).

The comparison of DNA regions preceding *groESL* operons in *M. extorquens* DM4 with consensus sequences for σ^{70}- and σ^{32}-dependent promoters from *S. meliloti*, *Rhizobium etli* and *E. coli* (Barnett et al. 2012; López-Leal et al. 2014; Roncarati and Scarlato 2017) revealed putative regulatory elements upstream of both *groES* genes (Fig. 8). The detected − 10 and

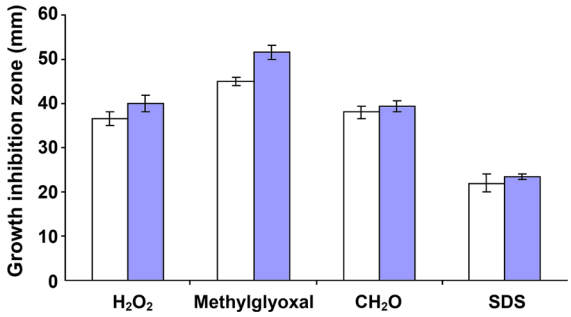

Fig. 5 Hydrogen peroxide, methylglyoxal, formaldehyde and sodium dodecyl sulphate resistance of the wild-type and Δ*groEL2* strains of *M. extorquens* DM4 determined by disk diffusion assay. The cells were mixed with MM soft agar, overlaid onto MM agar plates, exposed to 5 µl of 50% v./v. H_2O_2, 5.55 M methylglyoxal, 37% v./v. formaldehyde, 10% w./v. SDS and incubated at 29 °C for 3 days. The data represent the average values of diameters of inhibition halos for three independent experiments with wild-type (open bars) and mutant (filled bars) strains. The error bars show standard deviations from the means

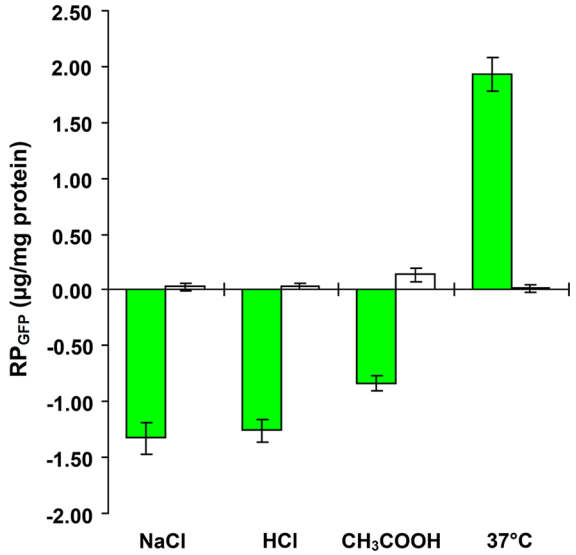

Fig. 7 Effects of high salinity, acid and thermal stresses to expression from the promoters of *groESL* operons in *M. extorquens* DM4. The activities of expression from P_{groE1} (filled bars) and P_{groE2} (open bars) promoters are reported as relative values of GFP production (RP_{GFP}) during 2 h cultivation of cells at elevated temperature (37 °C), in the presence of NaCl (100 mM) or acids (pH 5.0). The data represent the averages from three biological replicates. The error bars indicate standard deviations from the means

− 35 motifs share high similarity with sites recognised by alternative sigma factors, however corresponding boxes near *groESL1* and *groESL2* operons significantly differ between themselves (by 2–3 positions in each hexamer). The analysis of analogous candidate promoters of closely related representatives of *Methylorubrum* showed that these form two distinct similarity groups in agreement with localisation (Fig. 8). The members of the first group, which precede the highly conserved *groESL1* operon, are identical to each other and display equal degree of

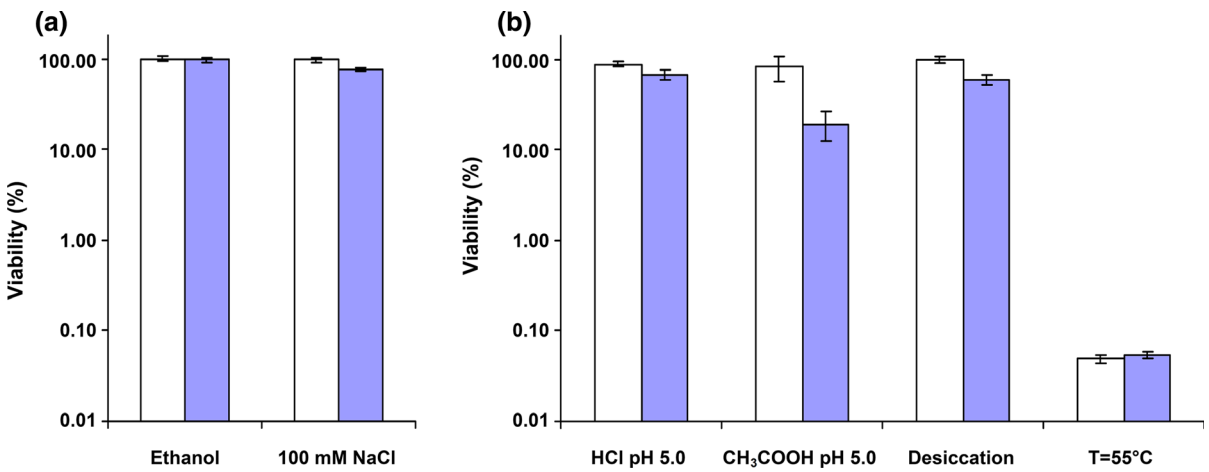

Fig. 6 Saline, ethanol, acid, thermal and desiccation resistance of *M. extorquens* DM4 wild-type and Δ*groEL2* strains. (**a**) The cells were plated on MM (control) or MM supplemented by 100 mM NaCl or 2% ethanol. (**b**) The cultures were plated onto MM medium after exposure to stresses: 2 h incubation at pH 5.0 (caused by 5 M HCl or 8 M CH_3COOH), 5 min heating at 55 °C or 7-day drying on filters at 29 °C. Untreated cultures served as the controls. The data represent the average values of cell viabilities for three independent experiments with wild-type (open bars) and mutant (filled bars) strains determined by serial dilutions method. The error bars indicate standard deviations from the means

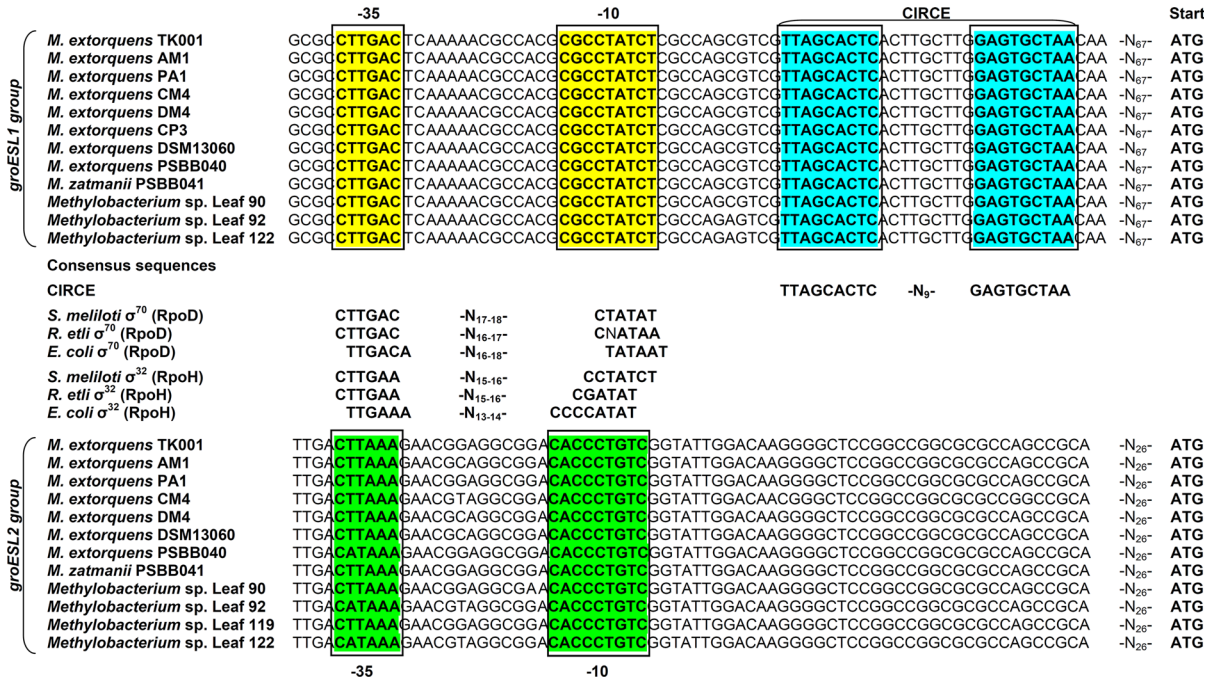

Fig. 8 Alignment of upstream regions of *groESL1* and *groESL2* operons of *M. extorquens* strains with consensus sequences of CIRCE element, RpoH- and RpoD-regulated promoters. The motifs of putative regulatory elements are shaded and indicated by boxes. The consensus sequences for CIRCE and promoters of *Sinorhizobium meliloti*, *Rhizobium etli* and *Eschericia coli* were taken from the literature (Hecker et al. 1996; Barnett et al. 2012; López-Leal et al. 2014; Roncarati and Scarlato 2017)

homology with both σ^{70}- and σ^{32}-dependent elements. On the contrary the more variable motifs found upstream of *groES2* gene align better with sites interacting with alternative sigma factors, than with those for binding of vegetative ones. The consensus sequences of σ^{70}- and σ^{32}-regulated promoters are usually very similar in the same organism, thus not allowing correctly predict RNA polymerase subunits required for their recognition (Barnett et al. 2012; Roncarati and Scarlato 2017). Nevertheless the observed divergence of the − 10 and − 35 boxes detected upstream of *groES1* and *groES2* genes in *M. extorquens* DM4 can suggest participation of different types of sigma factors in transcription of corresponding operons. On the other hand, it is known that the genomes of some representatives of α-*Proteobacteria* can harbor two or more genes encoding σ^{32} subunits (*rpoH*). In representatives of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium* and *Rhodobacter* these multiple RpoH homologues were shown to be functionally unequal and specialised for response to particular stresses (Narberhaus et al. 1998b; Tittabutr et al. 2006; Bittner et al. 2007; Martínez-Salazar et al. 2009). Methylotrophic bacteria *M. extorquens* DM4 also posses two genes of alternative sigma factors (old locus tags METDI1149 and METDI4867), however to confirm or disprove the roles of their products in regulation of expression of *groESL* operons additional studies are necessary.

It was also found that unlike the DNA regions preceding *groES2* gene those upstream of *groES1* are characterised by the presence of sites for a negative control of GroE synthesis. In all tested strains the putative promoters of *groESL1* operons were followed by sequences containing perfect matches with the CIRCE consensus reported earlier for a broad range of bacteria (Hecker et al. 1996) (Fig. 8). Operation of corresponding regulatory mechanism could explain, at least partially, the observed difference in thermal sensitivity between the P_{groE1} and P_{groE2} fragments cloned in our work. However despite the availability of both target sequences and *hrcA* genes in genomes of

M. extorquens DM4 (old locus tag METDI0465) and its closest relatives, the functionality of CIRCE/HrcA system in these microorganisms requires more rigorous evidences. At the same time in should be noted, that such complex regulation involving σ^{32}-dependent promoter along with the heat-inducible HrcA repressor is already known. In particular, in rhizobia strains this variant is characteristic for the main *groESL* operons essential for cell growth (Kumar 2017). Thus besides the likely role in response to thermal stress the presence of CIRCE element upstream of *groES1* gene also indirectly indicates the housekeeping nature of GroE1 chaperone. On the contrary, the *groESL2* operon, which is not subjected to the GroEL-dependent transcriptional repression by HrcA, apparently implements accessory functions.

Conclusion

Considering the often extreme living conditions (chemically contaminated soils and waters, epiphytic growth) and the toxicity of used substrates, methylotrophic bacteria are surprisingly poorly studied in terms of the functions of the main cellular chaperones. Meanwhile, the latter are specialised not only for protection of cells against a variety of external and internal stressors, but also for folding of unique proteins, providing metabolic versatility to their hosts. Nevertheless the reasons for studies of the chaperone systems in C_1-utilisers are not limited to the above aspects. It should not forget that many of these microorganisms are also phytosymbionts. And, whereas in the most known phytosymbiotic organisms—rhizobia the roles of multiple copies of *groESL* operons in interactions with plants and stress responses are intensively studied, the homologous genes in their "relatives" growing with C_1-substrates undeservedly remain out of scope of researches.

Being a first report on this subject for representatives of *Methylobacteriaceae* family our study of two pairs of genes encoding 60-kDa and 10-kDa chaperonins in *M. extorquens* DM4 demonstrates that its homologous *groESL* operons are functionally unequal similarly to found in rhizobia. The *groESL1* operon (old locus tags METDI5839-5840) is highly conservative, actively expressed and indispensable for cells even under non-stress conditions. The second pair of genes (*groESL2*, old locus tags METDI4129-4130) is characterised by more variable sequences and low-leveled expression, but corresponding GroL chaperonin promotes the tolerance of the host to acid, salt stress and growth with toxic halogenated compound—dichloromethane. Thus one can expect that investigations of regulation and functions of multiple homologues of chaperonins in methylotrophic bacteria can lead to uncover of their hitherto unknown adaptation features and optimisation of biotechnological processes based on such strains.

Acknowledgements This work was supported by Russian Foundation for Basic Research (Grants 15-01-04458-a and 18-04-01148-a).

Author's contribution ML Torgonskaya designed the experiments, coordinated the study, analysed the DNA sequences of *groESL* operons and their upstream regions. YE Firsova carried out the generation of the mutant and reporter strains, characterised the phenotype of Δ*groEL2* mutant and registered the GFP expression from promoters. Both authors contributed to data analysis and manuscript preparation. The final manuscript was reviewed and approved by both authors.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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