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EXPERIMENTAL ARTICLES

Functionality of the *xoxF* Gene in *Methylobacterium dichloromethanicum* DM4

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Abstract—Activation of expression of the *xoxF* gene encoding PQQ-dependent methanol/ethanol dehydrogenase (METDI2492) in dichloromethane (DCM)-grown *Methylobacterium dichloromethanicum* DM4 was first demonstrated. The sequence of the only XoxF homolog found in the genome of strain DM4 exhibited 50% identity to that of the protein (MxaF) of the large subunit of methanol dehydrogenase (MDH). A knockout mutant with inactivated *xoxF* gene (Δ*xoxF*) was found to be unable to grow on methanol due to the absence of the expression of the gene cluster of the classical MDH, as was confirmed by the GFP test. When grown on succinate, the Δ*xoxF* mutant exhibited a lower growth rate on DCM than the original strain and was more sensitive to various stress factors (oxidative, osmotic, and heat shock). Based on these data, the *xoxF* gene was hypothesized to belong to a group of genes affecting the expression of proteins of general stress response.

Keywords: aerobic methylobacteria, dichloromethane, *xoxF*

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Methylobacterium dichloromethanicum DM4 is a gram-negative aerobic methylotrophic bacterium that can utilize as carbon and energy sources C_1 compounds (containing no C–C bonds) and a number of polycarbon substrates. Strain DM4 grows well on methanol and methylamine and is considered a promising strain for bioremediation due to its ability to efficiently mineralize dichloromethane (CH_2Cl_2, DCM), a long-lasting, persistent carcinogenic and mutagenic pollutant, widely used as an industrial solvent. The key enzyme of DCM transformation is cytoplasmic dichloromethane dehalogenase (DCMDH, DcmA), which catalyzes CH_2Cl_2 conversion into formaldehyde and two HCl molecules in a reduced glutathione-dependent reaction (Vuilleumier and Pagni, 2002). The genome of *M. dichloromethanicum* DM4 has been completely sequenced and partially annotated (Vuilleumier, et al., 2009), which provides the basis required for identification and investigation of the genes and enzymes enabling growth of methylobacteria on DCM. In particular, although the key role in the adaptation to DCM consumption belongs to *dcmA*, other genes have been implicated in this process (Muller et al., 2011).

In this respect, an interesting candidate gene is *xoxF* (METDI2492), which presumably encodes a protein homologous to MxaF, the large subunit of the classical methanol dehydrogenase (MDH, MxaFI). Both enzymes catalyze methanol oxidation in a pyrroloquinoline quinone (PQQ)-dependent reaction; however, they differ considerably in their subunit structure and kinetic traits. The classical MDH is a tetramer composed of two large (MxaF) and two small (MxaI) subunits containing Ca^{2+} in the active center, whereas the known XoxF proteins act as monomers upon Ca^{2+} binding, and can form homodimers in the presence of rare-earth elements (lanthanum, cerium, or praseodymium). XoxF–La^{3+} exhibited a 100-fold higher methanol-specific activity than MDH. Moreover, since XoxF can utilize formaldehyde with a high rate and affinity, it was supposed to oxidize methanol directly to formate without intermediate production of free formaldehyde (Keltjens et al., 2014). The *xoxF* genes are widely present in the genomes of methylotrophic and nonmethylotrophic microorganisms. This fact indirectly suggests that XoxF proteins act not only as MDH enzymes, but also perform other, yet unknown functions.

It has previously been shown that XoxF expression is activated in a number of methylobacteria growing on such C_1 substrates as methylamine (*Methylotenera*) and dimethyl sulfide (*Methylophaga*), as well as in those colonizing aerial parts of plants (*Methylobacterium*) (Bosch et al., 2009; Schäfer, 2007; Delmotte et al., 2009). It is known that epiphytic methylotrophs are exposed to such stress factors as UV radiation, reactive oxygen species, and dramatic changes in temperature and humidity (Ochsner et al., 2015).

Bacterial degradation of DCM is accompanied by production of S-chloromethyl glutathione, a geno-

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Table 1. Bacterial strains and plasmids

Strain or plasmid	Characterization	Reference or source
Methylobacterium dichloromethanicum DM4	DCM degrader, wild-type strain	BKM B-2191 = DSM 6343 (Doronina et al., 2000)
Δ*xoxF*	DM4 derivative, Δ*xoxF::aacC1*, Gm^r	This work
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*lac*×74 *recA1 araD*139 Δ(*ara-leu*) 7697 *galU galK rpsL* (Str^r) *endA1 nupG* λ-	Invitrogen
Plasmids:		
pK18mob	Mobilizable multipurpose vector, Km^r	(Schäfer et al., 1994)
p34S-Gm	Source of the Gm^r cassette, Ap^r, Gm^r	(Dennis and Zylstra, 1998)
p*xoxF*	pK18mob containing a 1341-bp fragment of *xoxF* (METDI2492) from *M. dichloromethanicum* DM4 cloned into *Xba*I/*Hin*dIII sites	This work
pΔ*xoxF*-Gm	p*xoxF* containing a 447-bp deletion within the *xoxF* fragment and the Gm^r cassette from p34S-Gm in the direct orientation obtained by cloning into *Sal*I sites	This work
pCM160	Mobilizable vector used for protein expression in methylotrophs controlled by the promoter of large MDH subunit-encoding gene (*mxaF*), Km^r	(Marx and Lidstrom, 2001)
pGreenTIR	Source of the *gfp* cassette, Ap^r	(Miller and Lindow, 1997)
pCM160*gfp*	pCM160 containing the *gfp* gene from pGreenTIR cloned into the *Eco*RI site	Provided by D.N. Fedorov

toxic intermediate of CH_2Cl_2 dehalogenation capable of alkylating nitrogenous bases of DNA, as well as of hydrochloric acid and formaldehyde (Vuilleumier and Pagni, 2002); as a result, the cell is simultaneously exposed to several stress factors, and XoxF might be involved in the associated processes.

The goal of the present work was to elucidate the functional role of the putative methanol dehydrogenase (XoxF, METDI2492) during *M. dichloromethanicum* DM4 growth on DCM and methanol.

MATERIALS AND METHODS

Bacterial cultures and vectors. Bacterial strains and plasmids used in the study are listed in Table 1. Methylobacteria were grown in the MM mineral medium (Torgonskaya et al., 2011) with methanol, *Escherichia coli* was cultured in the Luria–Bertani medium; for mutant strains, the media were supplemented with the relevant antibiotics, as described previously (Firsova et al., 2011).

Isolation of total and membrane-bound proteins of *M. dichloromethanicum* DM4. Cell protein fractions were obtained as described previously (Torgonskaya et al., 2007). The cells were disrupted using a MiSonix S-4000 ultrasound disintegrator (United States); the membrane-bound protein fraction was precipitated in a Beckman L7 ultracentrifuge (United States). Proteins were separated using PAGE according to Laemmli (1970) in a 10% denaturing gel. After electrophoresis, the gels were fixed with a mixture of 40% methanol and 10% acetic acid and stained with $AgNO_3$ (Shevchenko et al., 1996).

Protein identification by MALDI mass spectrometry. Protein bands from the denaturing polyacrylamide gel were identified by analyzing the mass spectra of the peptides obtained by trypsinolysis of the corresponding specimens. Protein trypsinolysis in the gel was performed as proposed by Shevchenko et al. (1996). Peptide specimens were mixed with the matrix directly on the sample target. The MALDI matrix was 20 mg/mL 2,5-dihydroxybenzoic acid (Bruker, Germany) solution in 50% acetonitrile and 0.3% trifluoroacetic acid. Mass spectra of the peptides with molecular mass from 800 to 4000 Da were registered in the reflection mode using a MALDI–TOF Bruker Ultraflex II mass spectrometer (Bruker). Mass calibration was performed using the internal standard (peaks of trypsin autolysis products). The obtained mass spectra were analyzed by searching the NCBI database with the MASCOT engine for peptides with +1 charge, peptide mass tolerance of 70 ppm, and one missed trypsin cleavage site allowed. Proteins with scores higher than the Mascot probability threshold were considered as matches (Perkins et al., 1999).

Obtaining a *xoxF*-knockout mutant of *M. dichloromethanicum* DM4. Isolation of genomic and plasmid DNA, cloning, and competent cell transformation were performed using conventional techniques (Sambrook and Russel, 2001). The deletion mutant

was obtained by double homologous recombination. A 1341-bp-long *xoxF* fragment from *M. dichloromethanicum* DM4 containing two *Sal*I recognition sites (positions 671 and 1124 in the METDI2492 sequence) was cloned into *Xba*I and *Hin*dIII sites of a pK18mob suicide vector using the primers xoxFfor (5'-CGTCTAGAACGAGGGCTCCCCGCTCGT-CG-3') and xoxFrev (5'-ACAAGCTTGGCCT-TCAGGAAGCCTTCGAGC-3'). The resulting p*xoxF* plasmid was treated with *Sal*I restriction endonuclease, and the internal 447-bp-long fragment of *xoxF* was substituted with the gentamycin resistance gene. The obtained pΔ*xoxF*-Gm vector (Table 1) was used to transform *E. coli* S17-1 cells, and the transformants were conjugated with *M. dichloromethanicum* DM4. Transconjugates were selected for gentamycin resistance and kanamycin sensitivity on MM medium with sodium succinate (20 mM). Insertion of the gentamycin cassette was additionally verified by PCR with primers to the sequences flanking the inactivated gene.

Growth dynamics of the strains on DCM. In the first series of experiments, the cells were grown on MM containing DCM (10 mM) or succinate (20 mM) until the early stationary phase, collected by centrifugation (6000 *g*, 30 min), washed twice with fresh sterile medium, and resuspended in the same medium to OD_{600} = 0.15. Next, 50-mL aliquots of the cell suspension were transferred into 300-mL conical glass flasks sealed with screw caps with rubber membranes and cultured on DCM for 56 h, as described previously (Firsova et al., 2011). Aliquots of the bacterial suspension (2 mL) were regularly sampled from the flasks to determine their optical density and the concentration of chloride ions in the medium.

In the second series of experiments, cells grown in MM with succinate (20 mM) to the mid-exponential phase (OD_{600} = 0.5) or to the stationary phase (OD_{600} = 1.0) were collected by centrifugation, washed twice with fresh medium, and resuspended in the same medium to OD_{600} = 1.0. Next, 25-mL aliquots were transferred into 300-mL flasks and cultured on DCM for 7 h, whereas induced DCMDH activity in cell extracts obtained from cell suspension specimens collected at different time points and the concentration of chloride ions in the medium were measured as described previously (Torgonskaya et al., 2011). All experiments were performed in three replicates.

Analysis of GFP expression. The pCM160*gfp* plasmid carrying the gene of green fluorescent protein (GFP) under control of the *mxaF* promoter (the gene encoding the large MDH subunit) was transferred into the wild-type *M. dichloromethanicum* DM4 and the Δ*xoxF* mutant cells by biparental crossing using *E. coli* strain S17-1 (Table 1). The obtained wt/pCM160*gfp* and Δ*xoxF*/pCM160*gfp* transconjugates were grown in MM medium containing succinate (20 mM) and methanol (60 mM) to OD_{600} = 0.5. Fluorescence was measured on a FLUOstar OPTIMA fluorimeter (BMG Labtech, Germany) using excitation and emission wavelengths of 485 and 520 nm, respectively.

Assessment of stress resistance. Wild-type *M. dichloromethanicum* DM4 and the Δ*xoxF* mutant cells were grown in liquid MM medium with sodium succinate (20 mM) to OD_{600} = 1.0. Solid MM medium containing 20 mM succinate was used to cultivate the cells subjected to various stress factors. Heat shock resistance was determined by heating 1-mL aliquots of cell suspensions to 55°C for 5 min in 1.5-mL Eppendorf tubes in a Termit solid-phase thermostat (DNA-Technology, Russia), immediately preparing 10^{-1} to 10^{-8} serial dilutions, and plating 4 μL of each dilution on petri dishes with MM agar. Cell suspension that was not exhibited to heat shock served as a control. The tolerance to salt and ethanol was determined by plating serial dilutions of cell suspensions on MM agar containing 100 mM NaCl or 2% ethanol as described by Gourion et al. (2008). The colonies were grown at 29°C and counted on day 5. The strains' resistance to hydrogen peroxide, formaldehyde, and sodium dodecyl sulfate (SDS) was determined using diffusion assay on cellulose disks (Gourion et al., 2008). For this purpose, cells were mixed with soft MM agar and layered onto solid MM agar containing sodium succinate (20 mM). Diameter of growth retention zones observed after loading 50% H_2O_2, 40% CH_2O, or 10% SDS (5 μL) in the center of a 1-cm disk was measured after 3 days of cultivation at 28°C. All experiments were performed in three replicates.

Analytical techniques. MDH activity was determined by dichlorophenol indophenol reduction (Anthony and Zatman, 1964). Concentrations of chloride ions in the medium were determined using the method of Jörg and Bertau (2004) with ferric nitrate(III), as described previously (Firsova et al., 2011).

RESULTS AND DISCUSSION

Our analysis of the *M. dichloromethanicum* DM4 genome sequence available from the NCBI database showed it to contain one gene homologous to *xoxF*1 from *Methylobacterium extorquens* AM1. The amino acid sequence of the encoded protein showed 50% identity to the MxaF sequence. This gene, *xoxF* (METDI2492), was located in the same cluster as the genes encoding cytochrome *c* and a MxaJ-like protein (METDI2493 and METDI2494) and in relative proximity of the gene clusters responsible for tetrahydrofolate and PQQ synthesis.

Analysis of the amino acid sequence translated from *xoxF* using the PSOTRb, CELLO, HMM-TM, and DAS algorithms predicted XoxF to have periplasmic localization and to contain transmembrane domains. Membrane-bound proteins of *M. dichloromethanicim* DM4 cells cultured on DCM or metha-

nol were studied by one-dimensional denaturing PAGE (Fig. 1b). Protein bands corresponding to molecular mass of approximately 60 kDa and expected to contain MxaF and XoxF were excised for subsequent analysis by mass spectrometry (Fig. 1b, marked by arrows). As a result, it was found that, in addition to the large MDH subunit MxaF (METDI5145), the membrane-bound protein fraction of cells growing on DCM contained PQQ-dependent methanol/ethanol dehydrogenase, XoxF (METDI2492) (Table 2). This protein was not detected in *M. dichloromethanicum* DM4 growing on methanol. Furthermore, irrespective of the substrate used, the specimens were shown to contain a molecular chaperonin GroEL. Thus, *xoxF* expression was activated in *M. dichloromethanicum* DM4 growing on DCM.

To analyze the causes of this activation, we obtained a mutant *M. dichloromethanicum* DM4 strain where *xoxF* was inactivated by deletion of a 447 bp-long coding sequence and insertion of the marker gene of gentamycin resistance. The Δ*xoxF* lost the capacity for growth on methanol-containing medium, although it was able to grow on methylamine, formate, and DCM. Cell-free extracts of the mutant Δ*xoxF* cells grown on MM medium with succinate or succinate and methanol did not exhibit any MDH activity. Denaturing PAGE of cell-free extracts of wild-type *M. dichloromethanicum* DM4 and the mutant Δ*xoxF* cultures grown on the succinate/methanol mixture revealed a major protein band whose molecular mass (60 kDa) corresponded to that of XoxF and MxaF. However, analysis of MALDI-TOF mass spectra of the peptides obtained by trypsinolysis showed that the band derived from Δ*xoxF* did not contain XoxF or MxaF, but contained the GroEL chaperonin (METDI5839) (Mascot score 164). The 60-kDa band from wild-type DM4 consisted of MxaF and GroEL. The lack of MxaF in the Δ*xoxF* mutant may be a consequence of *mxa* promoter suppression, as it was previously observed in *Methylobacterium extorquens* AM1 (Scovran et al., 2011).

To test this hypothesis, we transformed the wild-type and the mutant strains with a pCM160 plasmid carrying the gene of green fluorescent protein (*gfp*) under control of the MDH promoter. It was found that the relative fluorescence of Δ*xoxF*/pCM160*gfp* transconjugate cells was significantly lower and constituted less than 2.0% of the level observed for the wt/pCM160*gfp* transconjugate obtained from wild-type DM4 (1393 and 78163 relative fluorescence units/OD, respectively). These data indicate that the inability of *xoxF*-knockout cells to grow on methanol substrate is due to the loss of *mxaF* expression.

As it was pointed out above, *xoxF* inactivation generally did not impair the ability of *M. dichloromethanicum* DM4 to utilize polycarbon substrates (e.g., succinate and pyruvate), formate, methylamine, or DCM. However, *xoxF*-knockout cells were markedly less

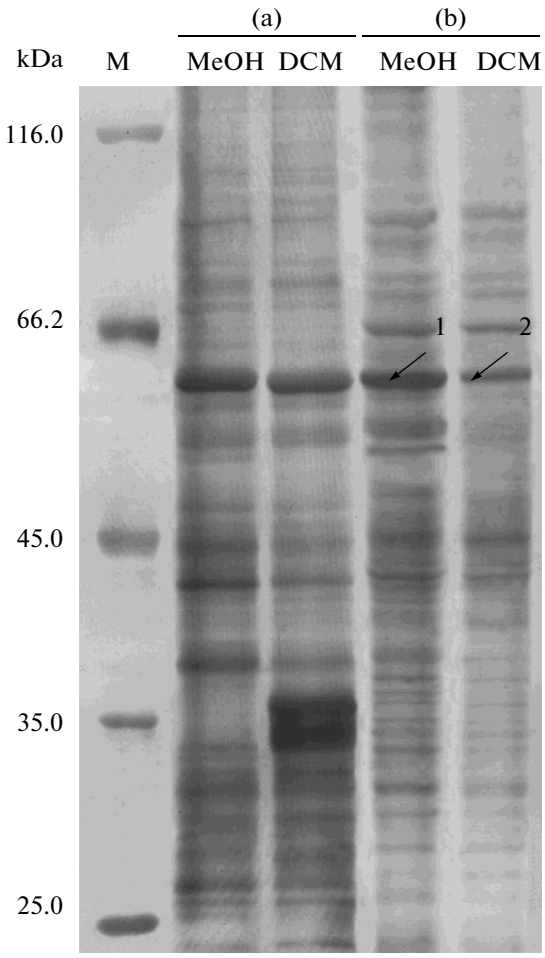

Fig. 1. SDS–PAGE (10% gel) of subcellular protein fractions of *M. dichloromethanicum* DM4 degrader strain growing on methanol and on DCM: total cell proteins (a); and, membrane-bound proteins isolated by ultracentrifugation (b). M, marker proteins. The list of proteins identified in the bands indicated with arrows is provided in Table 2.

adapted to utilization of the most toxic substrate, DCM. In particular, after a succinate-based culture, Δ*xoxF* mutant exhibited significantly lower growth rate on DCM than the original strain (Fig. 2). However, in the course of subsequent passages on DCM, metabolic reorganization required for DCM utilization was completed, and the mutant strain regained the growth rate nearly identical to that of the wild-type strain.

To analyze the causes of retarded Δ*xoxF* growth in more detail, DCMDH activity and chloride ion concentrations in the medium were determined for *M. dichloromethanicum* cells grown in succinate-containing medium to the mid-exponential growth phase or to the stationary phase (Fig. 3). When the cells were collected in the mid-exponential growth phase, wild-type DM4 strain did not differ from the Δ*xoxF* mutant in DCMDH activity or in chloride ion concentration

Table 2. Proteins identified in the membrane fractions of *M. dichloromethanicum* DM4

Substrate	Protein	Gene name and genomic number	MW_{app}*/ MW_{exp}**, kDa	Number of peptide matches	Sequence coverage, %	Mascot Score***
Methanol	Precursor of the large MDH subunit	*mxaF* METDI5145	61.9/65.8	24	44	323/281
	GroEL molecular chaperon	*groL* METDI5839	61.9/57.4	14	27	323/110
DCM	Precursor of the large MDH subunit	*mxaF* METDI5145	61.9/65.8	24	47	333/195
	Putative precursor of PQQ-dependent dehydrogenase	*xoxF* METDI2492	61.9/64.9	21	41	333/160
	GroEL molecular chaperon	*groL* METDI5839	61.9/57.4	18	43	333/125

* Apparent molecular mass in one-dimensional denaturing PAGE.

** Theoretically expected molecular mass according to the NCBI database.

*** Mascot scores are given for total protein/individual proteins in the mixture; scores higher than 85 were considered as significant ($P < 0.05$).

in the medium (Fig. 3a). However, the strains exhibited significant differences when the cells were grown to the stationary phase (Fig. 3b). DCMDH activity in Δ*xoxF* cell extracts was nearly 1.5-fold lower than in wild-type cells. Chloride ion concentration in the culture medium also was significantly lower for Δ*xoxF* than for the wild-type strain.

Our hypothesis is that the cells of both strains grown on succinate to the stationary phase and then transferred into DCM-containing medium had in fact already been exposed to a starvation stress (Bačun-Družina et al., 2011). However, wild-type cells seem to accumulate higher levels of stress-response proteins than Δ*xoxF* mutant. Therefore, they were better adapted to growing on DCM, which is not only a carbon source, but also a stress factor. At the same time, Δ*xoxF* mutant probably accumulated stress response proteins at a lower level, which resulted in slower adaptation to stress conditions, less active DCMDH expression, and lower growth rate on DCM.

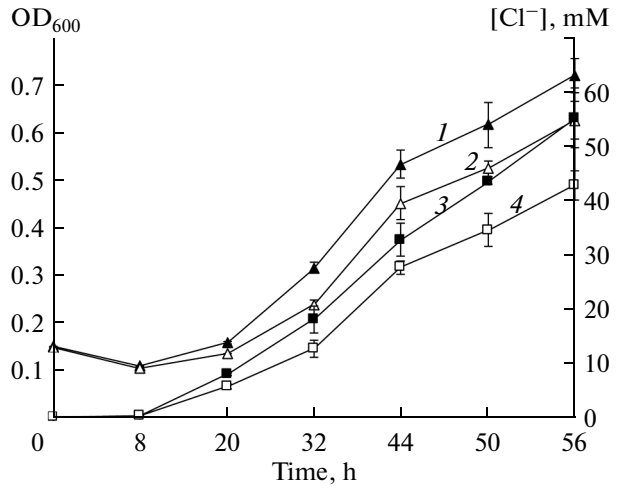

Fig. 2. Growth dynamics exhibited by wild-type DM4 (*1, 3*) and knockout mutant Δ*xoxF* (*2, 4*) on DCM after cultivation in a succinate-containing medium. Optical density of cell suspensions (OD_{600}) (*1*, *2*) and concentration of chloride ions in the medium (*3, 4*).

For these reasons, we evaluated the resistance of the Δ*xoxF* mutant and the wild-type DM4 cells to different types of stress (oxidative, osmotic, and thermal); adaptation to them would result in considerable alteration of cellular metabolism.

It was found that the Δ*xoxF* mutant was more sensitive to increased sodium chloride concentration, ethanol, and heat shock than the wild-type DM4 strain. In the mutant strain, the portion of cells that formed visible colonies on fifth day of growth on solid medium was two times lower for the cells subjected to osmotic stress (100 mM NaCl), nearly 25% lower in the presence of 2% ethanol, and 5.5 times lower after heating to 55°C for 5 min in a thermostat (Table 3). Similar results were obtained in experiments evaluating resistance to hydrogen peroxide, formaldehyde, and SDS using a diffusion assay on cellulose disks: for the Δ*xoxF* mutant, growth inhibition zones were slightly larger than for the control strain (Table 3). Exposure to hydrogen peroxide H_2O_2 models an oxidative stress and SDS is a surface-active compound inducing cell lysis, whereas formaldehyde produces a general toxic effect by nonspecific reacting with proteins and nucleic acids. Thus, the *xoxF*-knockout mutant was more sensitive to different types of stresses than the original strain.

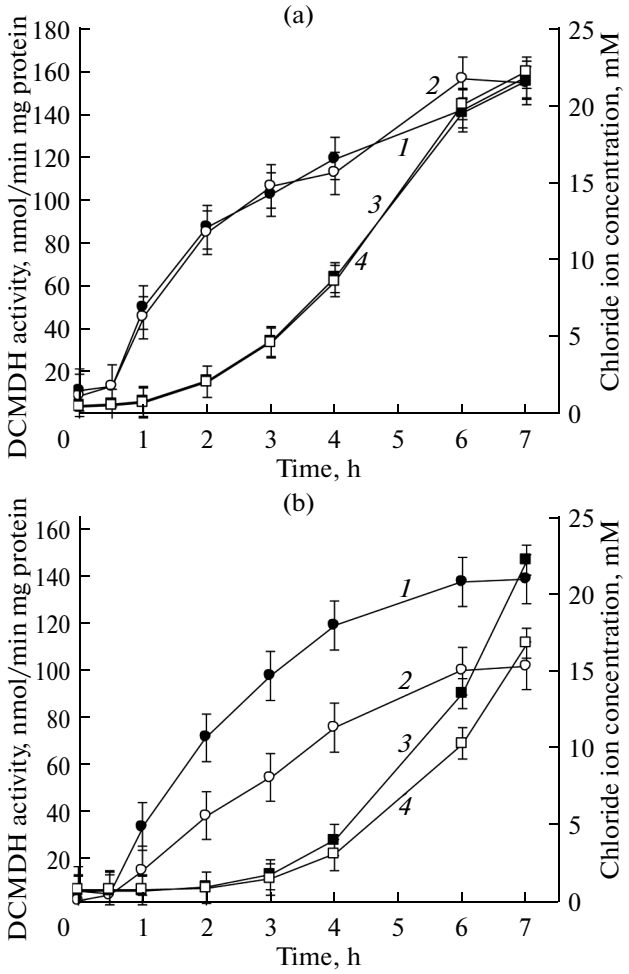

Fig. 3. Dynamics of DCMDH activity and chloride ion concentration in the medium during the growth of wild-type DM4 (*1, 3*) and knockout mutant Δ*xoxF* (*2, 4*). Cultures had grown on succinate to the middle of the exponential growth phase (a) or until the stationary phase (b). DCMDH activity, nmol min^{-1} mg^{-1} total protein (*1, 2*) and chloride ion concentration in the medium (*3, 4*).

When discussing *xoxF* functions, it should be noted that the corresponding protein possesses catalytic activity: recombinant XoxF from *M. extorquens* AM1 exhibited MDH activity in vitro, although the methanol oxidation rate was by an order of magnitude lower than that of MxaFI (Schmidt et al., 2010). It was shown that XoxF1 from *M. extorquens* AM1 functioned as a La^{3+}-dependent MDH, since a Δ*mxaF* mutant was unable to grow on methanol in the presence of Ca^{2+}, but restored this capacity when the medium was supplemented with La^{3+} (Nakagawa et al., 2012).

Apart from that, *xoxF* was shown to act as a regulator of the MDH cluster gene expression (Scovran et al., 2011). The genes required for production of active MDH form a large *mxaFJGIRSACKLDEHB* cluster of 14 genes; such clusters are nearly identical in AM1 and DM4 strains (Vuilleumier et al., 2009). These genes encode structural MDH subunits, a group of proteins required for its functioning, and several proteins with unknown functions (Chistoserdova et al., 2003). The obtained mutant *M. dichloromethanicum* DM4 strain with inactivated *xoxF* was unable to grow on methanol due to suppression of the *mxa* promoter. This conclusion was confirmed by the fact that the GFP-encoding gene controlled by the *mxa* promoter was not expressed in the mutant cells. This implies that XoxF functions are not only catalytic but also regulatory.

Thus, our data suggest that XoxF is required for *M. dichloromethanicum* DM4 growth on methanol. For the first time, we have shown that XoxF expression is also activated in the presence of DCM. Apparently, *xoxF* belongs to the group of genes that regulate the expression of proteins of general stress response. Further research is required to gain a detailed understanding of *xoxF* functioning.

Table 3. Resistance of *M. dichloromethanicum* DM4 and the Δ*xoxF* mutant to different stress factors

Stressor	Evaluation*	% surviving cells (I)/diameter of growth inhibition zone, mm (II)	
		DM4	Δ*xoxF*
NaCl, 100 mM	I	61.17 ± 4.1	33.40 ± 9.0
Ethanol, 2%	I	94.50 ± 6.3	73.35 ± 5.1
Heating to 55°C, 5 min	I	0.11 ± 0.03	0.02 ± 0.01
H_2O_2, 50%	II	39.0 ± 1.6	42.3 ± 0.6
CH_2O, 40%	II	39.3 ± 0.7	42.0 ± 1.0
SDS, 10%	II	44.3 ± 2.8	50.0 ± 1.9

* I, serial dilutions assay; II, diffusion assay using cellulose disks.

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