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Methylovorus sp. MP688 exopolysaccharides contribute to oxidative defense and bacterial survival under adverse condition

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Abstract *Methylovorus* sp. MP688 is an aerobic bacterium that can grow on reduced C1 compounds such as methanol, being regarded as an attractive producer for many commercial materials including polysaccharides. The aim of the study was to learn more information about the biochemical and physiological functions of extracellular polysaccharides (EPS) produced by *Methylovorus* sp. MP688. Firstly, gene clusters involved in EPS synthesis were identified by whole genome sequence analysis. Then EPS produced by *Methylovorus* sp. MP688 were isolated and purified by centrifugation, precipitation and deproteinization. Purified EPS displayed antioxidant activity towards DPPH free radical, hydroxyl radical and superoxide anion radical. Glucose, galactose and mannose were identified to be main component monosaccharides in EPS. One mutant with defect in EPS production was obtained by knocking out *eps*A gene within EPS synthesis cluster. Strain with deletion of *eps*A exhibited compromised growth ability in the presence of oxidative stress due to the sharp reduction in EPS synthesis. Meanwhile, the intracellular antioxidant scavengers were activated to a higher level in order to counteract with the excess harmful radicals. In addition, EPS were assimilated by *Methylovorus* sp. MP688 to survive under disadvantage condition when the preferred carbon source was exhausted. It was reasonable to conclude that EPS produced by *Methylovorus* sp. MP688 contributed to oxidative defense and bacterial survival under adverse condition.

Keywords Exopolysaccharides · Methylotrophic bacteria · Oxidative stress · Survival

Introduction

Production of exopolysaccharides (EPS) is a widespread character among bacteria, particularly in pathogenic and symbiotic bacteria (Guerry et al. 2012; Poli et al. 2011). Exopolysaccharides comprise surface high-molecular-weight polymers designated as capsular polysaccharides (CPS) that form an adherent cohesive layer on cell surface, as well as free soluble extracellular polysaccharides that dissociated from cell surface and are secreted into the culture medium (Yoshida et al. 2003). EPS biosynthesis pathway and genes participating in this process have been proposed and studied in several bacteria by means of random mutagenesis and site-specific gene disruption (Roberts 1996; Yoshida et al. 2000, 2003). Genes directly responsible for the complex process including synthesis of sugar precursors, unit assembly, chain modification and polymerization of repeating units and product transportation have also been identified and characterized in different strains (Huang and Schell 1995; Yoshida et al. 2003; Janczarek 2011; Ayabe-Chujo et al. 2012). However, up to date, few studies focused on the regulation of EPS production, even

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on the definite functions of specific genes coding regulator in EPS cluster.

In the past years, a growing attention has been paid to the antioxidative properties of purified microbial EPS by typical biochemical method (Jin et al. 2011; Liu et al. 2011; Xu et al. 2011; Bylund et al. 2006), as well as the multifarious applications of EPS in food, pharmaceutical and other industries (Kumar et al. 2007). Moreover, being an inherited physical barrier surrounding the cells against diffusion of toxic components, microbial EPS are proved to play crucial roles in protecting cells from variable environmental stress (Poli et al. 2011; Ferreira et al. 2011). EPS constitute the major components of matrix of pathogenic bacteria, and therefore participate in the biofilm formation (Ryder et al. 2007). EPS also facilitated adhesion and colonization and were relevant to the pathogenic persistence (Ryder et al. 2007). Methylotrophic bacteria, being found in a variety of terrestrial and aquatic habitats, represent a diverse group of microorganisms that can utilize carbon substrates with no carbon–carbon bonds (Kolb 2009). EPS production from methanol in methylotrophic bacteria is considered to be a faster and more efficient way of methanol (formaldehyde) removal than oxidation, therefore efficiently converting the toxic metabolic intermediate into harmless molecules (Yoshida et al. 2003). Despite the important characters of bacterial EPS have been reported, relatively little is known regarding the detailed direct physiological function for EPS under oxidative conditions with growing cells and the necessity of the bacteria to produce it during the long-time evolution.

Methylovorus sp. MP688 is a gram negative, aerobic bacterium capable of synthesizing exopolysaccharides when methanol is used as sole carbon and energy source (Xiong et al. 2011), making it a perfect candidate for studying EPS function in methylotrophic bacteria. In this paper, we report the in vitro antioxidant properties of EPS produced by *Methylovorus* sp. MP688. As well, we further knocked out a putative regulator gene within EPS synthesis cluster to investigate the physiological function of EPS under induced stress conditions.

Materials and methods

Bacterial strains, plasmids, growth conditions and analytical methods

The strains and plasmids used in this study were all listed in Table 1. Primers used in this study were listed in Online Resource 1. *Methylovorus* sp. MP688 (CGMCC4096) was used as wild-type strain and was cultured in MP medium containing per liter 0.2 g of $MgSO_4$, 3 g of $(NH_4)_2SO_4$, 1.4 g of KH_2PO_4, 3 g of Na_2HPO_4, 30 mg of $FeCl_3$, 30 mg of $CaCl_2$, 5 mg of $MnCl_2$, 5 mg of $ZnCl_2$, and 0.5 mg of $CuCl_2$. 2 % methanol (vol/vol) was used as sole carbon and energy source if not specially mentioned. All incubations for *Methylovorus* sp. MP688 were done in 250 ml flasks containing 100 ml medium with shaking (200 rpm) at 30 °C. *Escherichia coli* strains were grown at 37 °C in Luria–Bertani medium supplemented with the appropriate antibiotics. For the purpose of examining the behaviors of wild-type and mutant strains under inadequate carbon condition, bacteria were cultured in MP medium with 0.4 % methanol. Determination of carbon source assimilation was carried out by a methanol concentration detector (FC2002, produced by East China University of Science and Technology). Extracellular pyrroloquinoline quinone (PQQ) concentration was determined by glucose dehydrogenase assay (Geiger and Gorisch 1987). All chemicals and reagents used in this study were obtained from Sigma Aldrich and SCRC.

Sequence analysis

Sequence information, primary analysis and ORF search were performed using BLAST from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov). Domain structures of proteins coded by EPS synthesis genes were predicted by SMART (http://smart.embl-heidelberg.de/).

Purification and determination of EPS

The cultures of *Methylovorus* sp. MP688 strains were collected by centrifugation at 12,000 rpm for 10 min at 4 °C. To remove lipid and protein contaminants, the supernatants were washed with the same volume of chloroform for three times. Then two volume of cold ethanol was added into the aqueous solution followed by been kept at 4 °C overnight. Polysaccharides were precipitated by centrifugation at 12,000 rpm for 10 min at 4 °C and redissolved in appropriate volume distilled water. The crude EPS solution was dialysed against distilled water for 48 h. The phenol–sulfuric acid method was used to determine the EPS concentration with glucose as a standard (Yoshida et al. 2003). Putative capsule polysaccharides surrounding the cells were detected by centrifugating 1.5 ml of bacterial culture and directly observing the surface of cell pellets.

Analysis of monosaccharide composition

A 5 mg aliquot of purified EPS was completely hydrolyzed with 2 ml of 2 M trifluoroacetic acid in a sealed ampule at 110 °C for 3 h. The residual acid in the sample was removed by evaporation in vacuum after the hydrolysis was completed. The hydrolysate was redissolved in 1 ml

Table 1 Bacteria strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source
Strains		
Methylovorus sp. Strains		
MP688	Wild type strain	Laboratory stock
Δ*epsA*	MP688 Δ*epsA* strain (*mpq1843* deletion), Gm^r	This study
C*epsA*	MP688 Δ*epsA* strain overexpression of *epsA* (*mpq1843*) under its native promoter, Gm^r, Km^r	This study
E. coli strains		
DH5α	F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 *pho*A *sup*E44 λ– *thi*$^-$1 *gyr*A96 *rel*A1	Laboratory stock
DH5α λ*pir*	λ*pir* lysogen of DH5α	Laboratory stock
WM3064	*thrB1004 pro thi rpsL hsdS lacZ ΔM15 RP4–1360 Δ(araBAD)567 ΔdapA1341*::*[erm pir(wt)]*	Laboratory stock
Plasmids		
pAK1	Suicide vector, Gm^r, Km^r	Laboratory stock
pAK1-1843	*pqq*A1 deletion vector, Gm^r, Km^r	This study
pBBR1MCS-2	Broad host range plasmid, Km^r	Laboratory stock
pBBR1843	For overexpression of *epsA* with its native promoter, Km^r	This study

distilled water. Then 50 μl of the released monosaccharides as well as six monosaccharide standards including glucose, galactose, mannose, rhamnose, glucuronic acid and galacturonic acid were derivatized with 0.5 M of 1-phenyl-3-methyl-5-pyrazolone (PMP) in 0.3 M aqueous NaOH (75 μl) at 70 °C for 45 min. After neutralization with 0.3 M HCl (75 μl), 2.5 ml chloroform was added into the above solutions and the resultant mixtures were shaken vigorously to extract the PMP-labeled samples in water phase. The extraction process was performed for at least three times to remove the excess organic reagent.

The obtained samples were analyzed on Agilent 1100 HPLC system, equipped with a Phenomenex Prodigy ODS-3 column (250 mm × 4.6 mm, 5 μm). After 5 μl of sample was injected, the column was eluted with mobile phase consisted of 20 mM ammonium acetate (A) and acetonitrile (B), and was delivered at a flow rate of 1.2 ml/min. A gradient elution was adopted, starting with 12 % B, reaching 26 % B until 25 min, and 60 % B at 30 min, followed by maintaining 60 % B for 10 min. The UV detection was done at 254 nm and the percentage (concentration) of compositional monosaccharides in the sample was calculated from peak areas using response factors.

Antioxidation assay of EPS in vitro and in vivo

The α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging activities of purified EPS were evaluated according to the method described by Kao and Chen (Kao and Chen 2006). 1.0 ml of purified EPS solution at varying concentrations was added to 3.0 ml of ethanolic DPPH radical solution. The reduction of OD_{517} after incubation in dark for 30 min was measured in triplicate. The scavenging activity of DPPH radical was calculated as follows: scavenging activity (%) = $[1 - (A_{sample} - A_{blank})/A_{control}] \times 100$ %, where A_{sample}, A_{blank}, $A_{control}$ were defined as absorbances of samples, without DPPH and without EPS, respectively.

The hydroxyl radical scavenging activity of purified EPS was determined as described before (He et al. 2012). 1.0 ml of purified EPS solution at varying concentrations was added to 3 ml of the reaction mixture containing 1 ml of phenanthroline solution (7.5 mM), 1.5 ml $FeSO_4$ (0.75 mM) and 0.5 ml H_2O_2 (3 %) was placed at 37 °C for 30 min. The scavenging activity of hydroxyl radical was calculated using the following formula: scavenging activity (%) = $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100$ %.

The superoxide anion radical activity was examined as described elsewhere (Liu et al. 1997). In brief, a series dilution of EPS was added to 3.0 ml of reaction mixture which contained 16 mM Tris–HCl (pH 8.0), 80 mM NADH, 50 μM nitrotetrazolium blue chloride (NBT), and 10 μM phenazine methosulfate (PMS). The color reaction was detected at 560 nm using a spectrophomerter. The scavenging activity of superoxide anion radical was thus calculated with the equation: scavenging activity (%) = $(A_{blank} - A_{sample})/A_{blank} \times 100$ %.

For antioxidation assay in vivo, MP medium containing 5 μM hydrogen peroxides was inoculated with wild-type, *ΔepsA* and complementary strain, and the growth was

allowed for the indicated times at 30 °C. Growth curves were obtained by measuring OD_{600} every 12 h after inoculation. For plate growth assay, cells with serial dilutions were spotted onto MP agar also containing 0, 2.5 and 5 μM hydrogen peroxides respectively.

Construction of mutant and complementary strains about *eps*A

A suicide vector pAK1 was used to create the allelic exchange mutants (Ge et al. 2011). About 1,500 bp upstream and downstream of *eps*A locus (*mpq_1843*) were amplified and ligated flanking the gentamycin sequence of pAK1. The resultant plasmid, pAK1-1843 was then conjugated into wild-type of *Methylovorus* sp. MP688 by the donor strain WM3064 to generate a knock-out mutant (Ge et al. 2011). To confirm the target genes were successfully replaced by antibiotic makers, the transconjugants obtained from gentamycin plates were selected against kanamycin. Clones sensitive to kanamycin were further verified by PCR. Coding sequence of *mpq_1843* as well as 200 bp upstream of it was ligated into pBBR1MCS-2. The obtained plasmid, pBBR1843, was transferred into *eps*A mutant strain by the donor strain WM3064.

Detection the expression of the main antioxidant enzymes

Wild-type and *Δeps*A strains were cultured in MP medium with 2 % methanol at 30 °C. Cells were harvested after 48 h by centrifugation at 12,000 rpm for 10 min. Total RNA from samples were extracted using SV total RNA isolation system (Promega). Reverse transcription and quantitative real-time PCR for the main antioxidant enzyme including *mpq_0026*, *mpq_0554* and *mpq_1043* as well as a control gene *mpq_2164* were performed as previously described (Bo et al. 2012). The level of target gene transcripts in wild-type and *Δeps*A strains were then normalized to the 16S rRNA gene. And the ratio between two normalized values was defined as relative fold expression (Couesnon et al. 2006).

Results

Genetic organization of EPS synthesis cluster in *Methylovorus* sp. MP688

Gene clusters responsible for polysaccharide synthesis have been discovered in various kinds of bacteria (Krol et al. 2007; Janczarek 2011). A comprehensive analysis of genome information revealed that *Methylovorus* sp. MP688 posses a large genomic region spanning from 1880597 to 1901296 with the respective genes found in other species (Fig. 1). The overall organization of this large region was similar to that of other methylotrophic bacteria. Two clusters designated *epsAB* and *epsKLDEFGHIMNOPQSTU* were identified based on transcriptional direction of each individual gene and sequence comparison with identified EPS clusters, although the later cluster was separated by three open reading frames transcribed oppositely coding three unknown small peptides. Deduced amino acid sequences of *epsABKLDEFGHIMNOPQSTU* displayed very high identity with the corresponding proteins among three methylotrophic bacteria including *Methylobacillus* sp. 12S, *Methylotenera versatilis* 301 and *Methylotenera mobilis* JLW8 (Online Resource 2). In addition, the BLAST hit with convincing E score for each gene in the two clusters was presented in Table S2 (Online Resource 3). These bioinformatics analysis indicated the identified genes in *Methylovorus* sp. MP688 may exert similar functions in EPS synthesis. Notably, two putative transcriptional regulators (*mpq_1842* and *mpq_1843*) and three glycosyl transferases, belonging to family GT26 (*mpq_1828*), family GT2 (*mpq_1831*) and family GT1 (*mpq_1832*) respectively, were predicted within the two clusters. However, function of each gene relative to polysaccharide synthesis was poorly understood. As well, markedly differ from reported EPS synthesis cluster in *Methylobacillus* sp. 12S and *Methylotenera versatilis* 301 (Yoshida et al. 2003), three ORFs whose products were EpsC, EpsJ and EpsR were absent in *Methylovorus* sp. MP688.

Purified EPS of *Methylovorus* sp. MP688 exhibit antioxidant activity in vitro

Free radicals, hydroxyl radicals and superoxide radicals are very harmful and can thus cause irreversible injury to bacteria (Bo et al. 2012; Ge et al. 2011). The three kinds of radicals have been widely adopted as tools for evaluating the abilities of antioxidants. To investigate the antioxidant properties of purified EPS of *Methylovorus* sp. MP688, EPS in culture supernatant was isolated by centrifugation, deproteinization and ethanol precipitation (Fig. 2a). The EPS solution was then diluted to a final concentration of 1.0 mg/ml after purification. In general, as seen in Fig. 2b–d, the scavenging activities on DPPH, hydroxyl radical and superoxide radical were all in a concentration dependent manner. For DPPH, the scavenging ability of EPS was weaker than ascorbic acid, which had reached a plateau of 90 % at 0.5 mg/ml. However, the hydroxyl radical scavenging abilities of EPS were more excellent than ascorbic acid and the maximum scavenging efficiency was respectively 95 and 60 % at the range of 0.1–1 mg/ml. Notably, purified EPS were found to exhibited two fold higher scavenging ability to that of ascorbic acid at low concentration. For superoxide radical scavenging, only slight

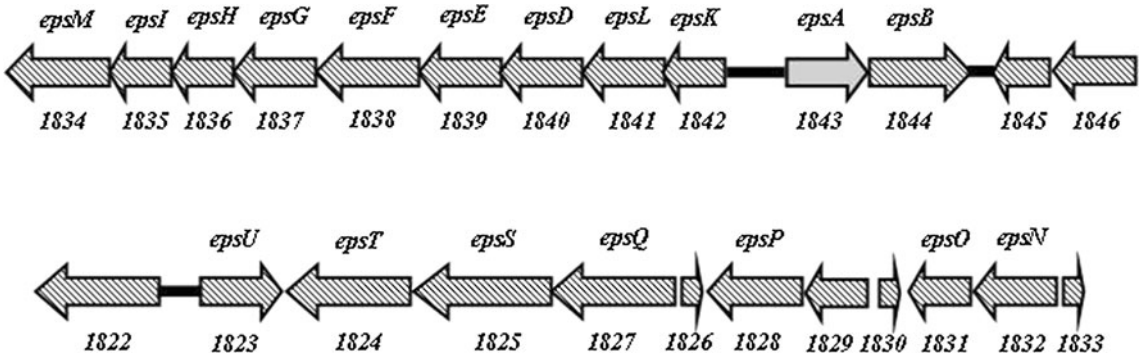

Fig. 1 Genetic organization of genes responsible for extracellular polysaccharides synthesis in *Methylovorus* sp. MP688. *Large arrows* denote individual ORF and transcriptional direction identified by sequence alignment

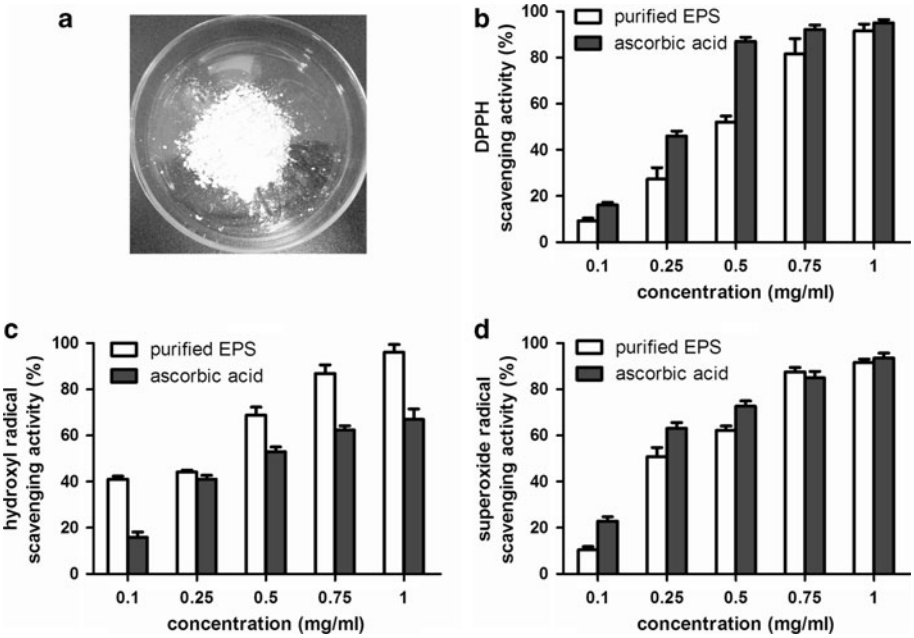

Fig. 2 Purified exopolysaccharides of *Methylovorus* sp. MP688 carry antioxidant activity in vitro. **a** Purified exopolysaccharides; **b** DPPH scavenging activity. **c** Hydroxyl radical scavenging activity. **d** Superoxide radical scavenging activity. Data shown are means of three independent experiments. Ascorbic acid (*filled column*), purified EPS (*open column*)

difference in scavenging effect was seen between purified EPS and ascorbic acid. Taken together, our results indicated that EPS produced by *Methylovorus* sp. MP688 confer antioxidant activity in vitro (Fig. 2b–d).

EPS of *Methylovorus* sp. MP688 consist mainly of glucosyl, galactosyl and mannosyl residues

To identify and quantify the monosaccharide unit of EPS produced by *Methylovorus* sp. MP688, purified EPS were completely hydrolyzed, labeled with PMS and then separated by HPLC. As a result, EPS from MP688 were heteropolysaccharides composed mainly of glucose, galactose and mannose in a molar ratio of 3:3:1 (Figs. 3a, b). Besides the three predominant monosaccharides, an unknown component appeared at 20 min was also detected. The compositional analysis of EPS also provided chemical basis for the radical scavenging activity in vitro.

Deletion of *eps*A gene results in deficiency of EPS production

In order to obtain an EPS-producing deficient strain, we constructed a series of gene deletion mutants within two

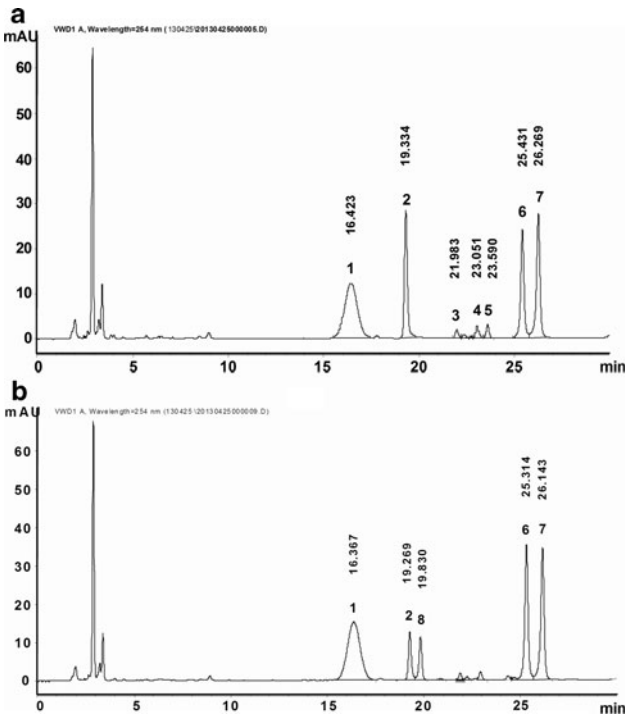

Fig. 3 Exopolysaccharides of *Methylovorus* sp. MP688 are mainly composed of three monosaccharides. **a** HPLC–UV chromatogram for the reference standards; **b** chromatogram for hydrolysate of purified exopolysaccharides. *1* PMP; *2* mannose; *3* rhamnose; *4* glucuronic acid; *5* galacturonic acid; *6* glucose; *7* galactose; *8* unknown component

identified *eps* clusters of *Methylovorus* sp. MP688, using double cross-over recombination to avoid incurring polar effect. Anchor PCR confirmed the removal of the expected coding sequence of target gene while leaving the flanking sequences intact (Fig. 4a, b). Consequently, deletion of *mpq_1843*, the amino acid sequence of which was completely identical to EpsA (a predicted transcriptional regulator) from *Methylobacillus* sp. 12S, caused severe effects on EPS production. Results in Fig. 4d, e demonstrate that, while the wild-type strain and the *Δeps*A strain exhibited no apparent difference in growth curve, the absence of *eps*A resulted in a dramatic decrease in the amount of soluble extracellular polysaccharides, which could be restored to a comparable level to that of wild-type by expression of *eps*A. The production of EPS in the wild-type strain started to increase in the early exponential phase and reached a plateau up to 650 mg/l when it entered into the stationary state. However, the content of EPS in the culture supernatant was less than 30 mg/l without *eps*A throughout the growing process (Fig. 4e). Otherwise, distinct from the wild-type cell pellets forming an amorphous layer which appeared loose and flocculent after centrifugation, the *Δeps*A cells could be pelleted compactly without any viscous substance on the surface of cells (Fig. 4c). Taken together, these findings indicated that disruption of *Δeps*A affected not only the soluble polysaccharides secreted into the growth medium but also the capsule polysaccharides surrounding the cells.

EPS help *Methylovorus* sp. MP688 to survive under induced oxidative stress condition

To probe further into the in vivo functions of EPS, wild-type and *Δeps*A strains of *Methylovorus* sp. MP688 were cultured in liquid medium in the presence of certain amount of hydrogen peroxide, generating an induced oxidative environment. As we expected, both the growth rate and the final biomass of *Δeps*A strain were compromised to approximately 50 % to that of the wild-type strain when hydrogen peroxide was added to a final concentration of 5 μM (Fig. 5a). To further test the effect of changes in EPS on the ability of *Methylovorus* sp. MP688 cells to propagate under stress, cell of wild-type and EPS mutant with serial dilution were simultaneously spotted onto plates containing 0–5 μM of hydrogen peroxide. In contrast to the wild-type and complementary cells grown well on the MP agar with no more than 2.5 μM hydrogen peroxide, growth of *Δeps*A in the same stress condition was severely affected, with barely small colonies seen at higher cell concentration. Moreover, no obvious colony was seen after 2 days in the presence of 5 μM hydrogen peroxide on the plates (Fig. 5b). The significant difference in growth pattern indicated that deletion of *eps*A have reduced the ability of surviving under the oxidative environment.

Since the growth was not completely deprived by exogenous oxidative stress, in this case, we asked whether the cells of mutant would combat with the oxidants in an alternative way to survive. The main intracellular antioxidant enzymes were studied. As we predicted, in the presence of oxidants, the transcription of peroxiredoxin (*mpq_0026*), catalase (*mpq_0554*) and superoxide dismutase (*mpq_1043*) genes were all up-regulated in *eps*A deletion strain, which is 8-10 times to the wild-type (Fig. 5c). A putative glucose dehydrogenase gene (*mpq_2164*), whose product is situated across cell membrane and takes charge of first step of glucose utilization, was used as a control gene. Conversely, the mRNA level of this gene remained almost constant in wild-type and mutant strains. The results suggested that these enzymes responsible for scavenging oxidative radicals in vivo have been activated to function efficiently in the strain lacking the ability of EPS production, enabling the bacteria to propagate under oxidative stress.

Methylovorus sp. MP688 secreted a certain amount of pyrroquinoline quinone (PQQ), a potential reducing agent. To exclude the possibility that the growth defect in mutant strain was due to the reduction in PQQ synthesis. The concentrations of secreted PQQ in the supernatant of both

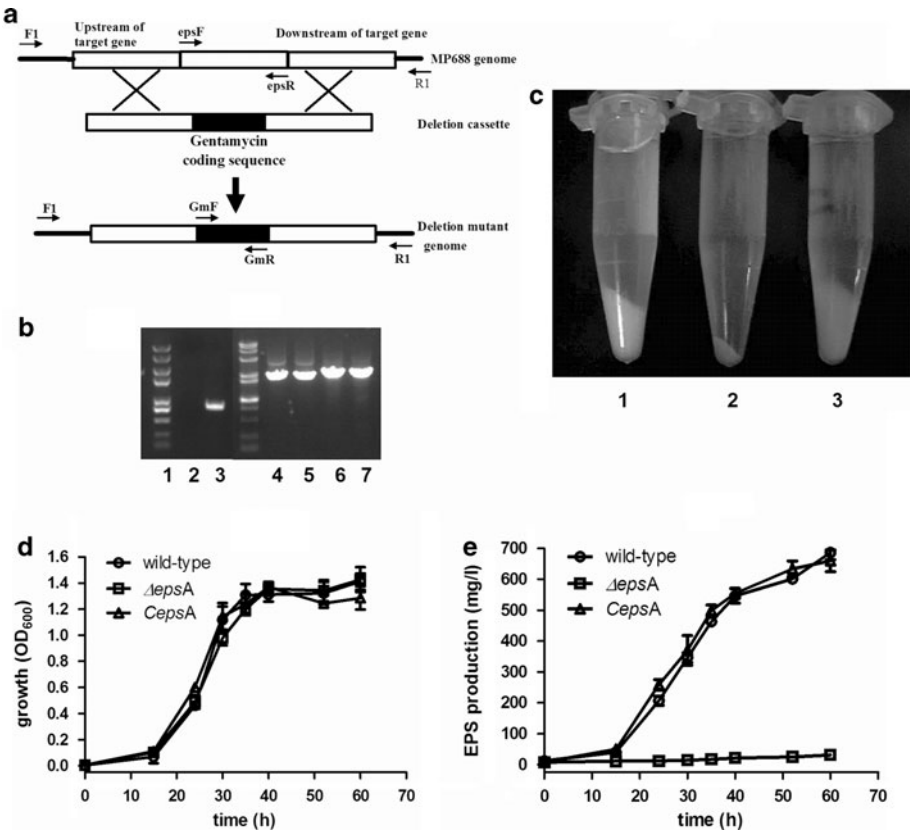

Fig. 4 Deletion of *eps*A abolished exopolysaccharides production in *Methylovorus* sp. MP688. **a** Schematic illustration of homologous recombination for deletion in *eps*A locus. **b** PCR confirmation of the deletion event that *eps*A was successfully replaced by gentamycin coding sequence in *Δeps*A strain, leaving the flanking sequence of *eps*A intact. *Lanes 2* and *3* showed PCR results using primers epsF and epsR (*lane 2*, *Δeps*A strain; *lane 3*, wild-type strain). *Lanes 4* and *5* showed anchored PCR results using primers F1/GmR and GmF/R1 in *Δeps*A strain. *Lanes 6* and *7* showed PCR results using primers F1/epsR and epsF/R1 in wild-type strain. **c** Capsular polysaccharide produced by wild-type (*1*), *Δeps*A (*2*) and complementary strains (*3*). For each strain, 1.5 ml of culture was centrifuged at 12,000 rpm for 10 min. **d** Growth curves of wild-type (*open circle*), *Δeps*A (*open square*) and complementary strains (*open triangle*). **e** Time course of exopolysaccharides production in the soluble form for wild-type (*open circle*), *Δeps*A (*open square*) and complementary strains (*open triangle*). Data shown are means of three independent experiments

wild-type and *eps*A deletion strains were determined. No apparent difference could be observed between the two strains in PQQ producing ability (Fig. 5d). In other words, deletion of *eps*A has not influenced the PQQ production, and therefore it is EPS other than extracellular PQQ that contributed mostly to protect the bacteria from oxidative damage. Together with the results above, we can conclude that EPS secreted by *Methylovorus* sp. MP688 help it to survive under induced oxidative stress conditions.

EPS can support bacterial growth when methanol is almost exhausted

It has been reported that EPS could protect cells from desiccation and thus help to survive under stress condition (Roberts 1996). To test the contribution of EPS for *Methylovorus* sp. MP688 to survive under disadvantage condition, wild-type and *Δeps*A strains were grown with 0.4 % (vol/vol) methanol as sole carbon source, instead of supplying sufficient methanol (no less than 1 %). The growth curves for the two strains, although exhibiting hardly any difference in the early 40 h, went separately in the stationary phase. In brief, wild-type strain reached a plateau of OD_{600} 1.4 while the EPS mutant decreased slowly 40 h after inoculation. Except for this, the maximum biomass of mutant strain was lower than wild-type (Fig. 6a). To further find out the reason for this unexpected phenomenon, methanol utilization and residual EPS in the culture supernatant were measured. It was the time when methanol in the medium was used up that the curve began to decrease for mutant strain and to enter into the stationary phase with only slight increase for wild-type strain. Meantime, the EPS concentration in the supernatant of wild-type strain was sharply reduced to half of the maximum level after methanol was exhausted (Fig. 6b). Furthermore, seen from Fig. 5d, the capsule polysaccharides monitored by centrifugation also started to decrease after 48 h. In order to clarify whether the secreted

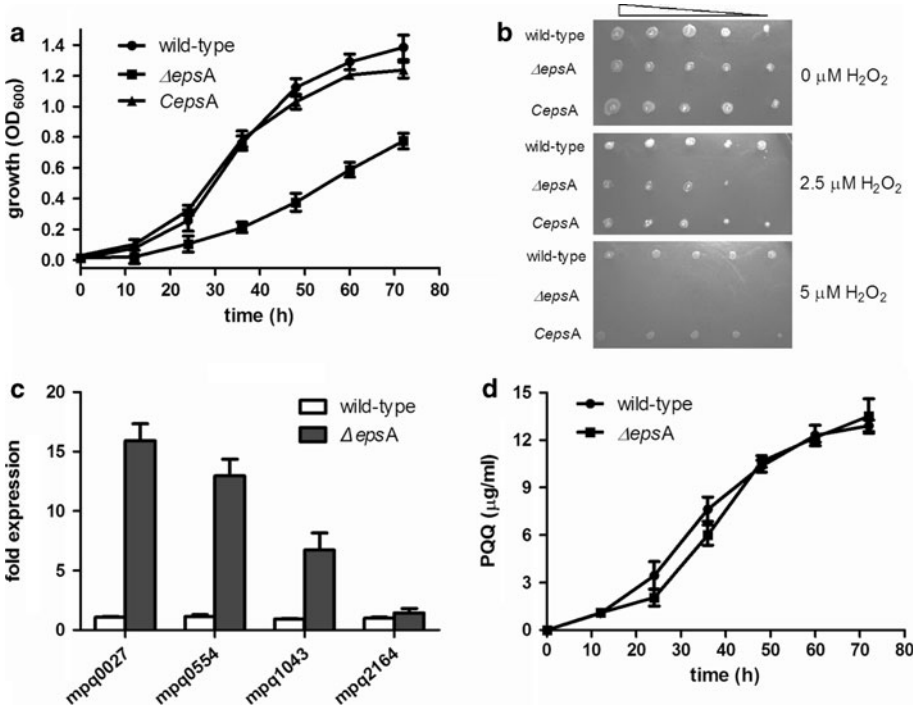

Fig. 5 Deletion of *eps*A resulted in growth defect and antioxidase activation in the presence of oxidative stress. **a** Growth pattern of wild-type (*closed circle*), *Δeps*A (*closed square*) and complementary strains (*closed triangle*) when 5 μM H_2O_2 were added into the culture medium. **b** Ten-fold series dilutions of wild-type, *Δeps*A and complementary strains were spotted onto MP plates containing different concentrations of H_2O_2. **c** Transcription of the main antioxidant enzyme genes in wild-type (*filled column*) and *Δeps*A strains (*open column*) in the presence of 5 μM H_2O_2. **d** PQQ secreted in the culture medium of wild-type (*closed circle*) and *Δeps*A strains (*closed square*)

polysaccharides were consumed by bacteria to support growth, wild-type and mutant strains cultured using the purified EPS as sole carbon source. Ultimately, the bacteria obtained a similar level of growth although lower than when methanol was used (Fig. 6c). To summarize, it is reasonable to conclude from our results that EPS produced by *Methylovorus* sp. MP688 could serve for growth when the preferred carbon source was almost exhausted, also indicating that bacteria were flexible enough to alter the carbon assimilation pathway to adapt the changing environment.

Discussion

In this paper, we report, for the first time, that EPS from *Methylovorus* sp. MP688 protected bacteria from oxidative damage and served for growth under adverse condition

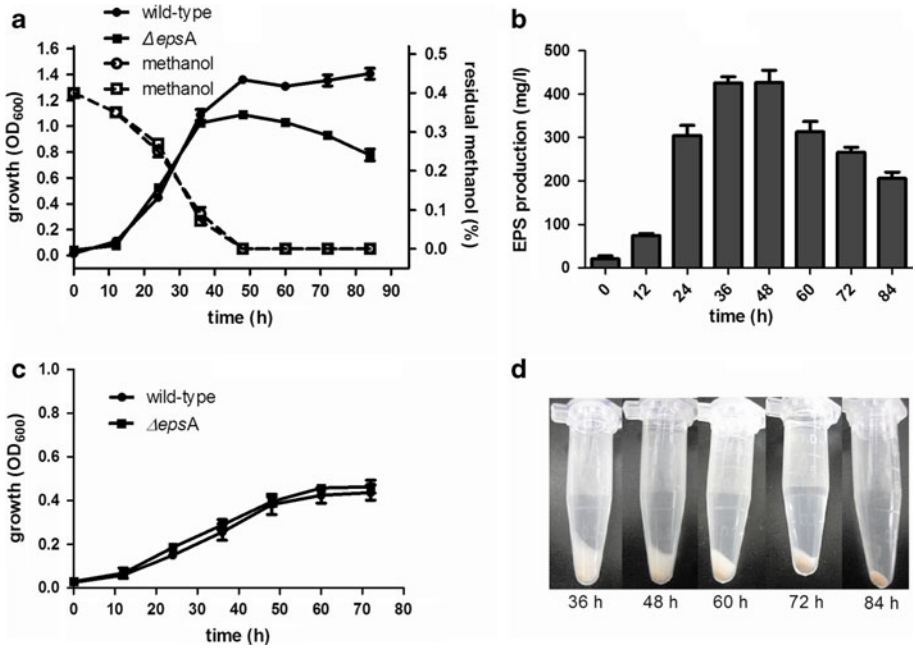

Fig. 6 Exopolysaccharides could be used as an alternative carbon source in methanol limited condition. **a** Growth and methanol consumption for wild-type and *Δeps*A strains. Growth of wild-type (*closed circle*) and *Δeps*A (*closed square*); methanol consumption for wild-type (*open circle*) and *Δeps*A (*open square*); **b** Concentrations of soluble exopolysaccharides when cultured with limited methanol. **c** Growth curves of wild-type (*closed circle*) and *Δeps*A (*closed square*) with purified exopolysaccharides as sole carbon source. **d** Changes in capsular polysaccharide attached to cells when methanol was almost exhausted (36–84 h)

such as lack of methanol. Based on these results, it will be acceptable that EPS either surrounding cells or secreted into environment play an indispensable role in bacterial survival in natural environment other than only being a metabolic product.

EpsA is predicted as a LuxR family regulator and have been proposed to positively regulate EPS production (Yoshida et al. 2003). LuxR transcriptional factor regulates a wide range of genes involving quorum sensing, biofilm formation and virulence production (Chen and Xie 2011). From the result of sequence alignment and domain analysis for the best-characterized LuxR regulators (Online Resource 4), we found that MPQ1843 included a conserved helix-turn-helix DNA binding domain at C-terminal (aa 200–260) and a non-conserved region with no predicted domain at N-terminal (aa 1–150). However, the candidate LuxR transcriptional factor binding sites (Lux box) were not obviously presented within the upstream region of the EPS synthesis cluster, indicating that EpsA may regulate additional genes affecting EPS production outside *eps* operon other than binding directly to regulatory DNA sequence of the cluster. As such, disruption of *eps*A caused dramatically decrease in EPS production in *Methylobacillus* sp. 12S (Yoshida et al. 2003). The behavior of *eps*A deletion mutant in our study was in well accordance with the previous results.

Purified bacterial EPS have been proved to display potential antioxidant activities in vitro by biochemical method, largely due to their reducing group which can donate electrons to react with the oxidative radicals (Yan et al. 2012). Similar to other results, EPS isolated from *Methylovorus* sp. MP688 also could reduce DPPH, hydroxyl radical and superoxide radical comparable to ascorbic acid, entailing it a potential oxidative radical scavenger outside the cells during bacterial growth. The composition of EPS produced by *Methylovorus* sp. MP688 was identified to be glucose, galactose and mannose. The reducing groups in these monosaccharides allowed the macromolecules to act as antioxidants. The sugar components from MP688 are remarkably similar with those from *Methylobacillus* sp. 12S, only in altered molar ratio. Moreover, the EPS production and composition seemed not to be affected by the absence of *epsC*, *epsJ* and *epsR* found in other methylotrophic bacteria.

However, little information was available on the physiological function. Our data showed that, although scarcely any difference in growing ability was observed between wild-type and an EPS-producing defective mutant in the presence of <3 μM hydrogen peroxide (data not shown), the mutant strain exhibited compromised growth with 5 μM hydrogen peroxide. In addition, enzymes involved in antioxidation intracellular were all up-regulated to a relatively high level in the absence of producing EPS. It could be speculated that EPS constitute the extracellular line of defense preventing the oxidative attack at the initial time. Mutant strain lacking EPS exposed to the environment was urgent to choose an alternative way to counteract with the oxidative substance. As a result, the intracellular antioxidant enzymes such as peroxiredoxin, catalase and superoxide dismutase were activated to a higher level meeting the requirement of reducing power, enable the bacteria to survive and propagate.

Bacteria are well equipped with self-protection system during long-time evolution, including antioxidation as well as other stress resistance (Wang et al. 2006; Giuliodori et al. 2007). Many microorganisms produce exopolysaccharides as a strategy for facilitating growth, adhering to solid surfaces, and surviving adverse conditions (Poli et al. 2011). Methylotrophic bacteria represent a diverse group of microorganism that can use C1 compounds as sole carbon and energy source (Kolb 2009). Inhabited in water or soil, methylotrophic bacteria are inevitable to encounter various adverse changes caused by oxidation, reduction, nutrition starvation, and so on. On this point, they are assumed to alter their metabolic pathway to adapt the changing environment as soon as possible in order to avoid death. Our results also proved that *Methylovorus* sp. MP688 could take EPS as carbon source when its preferred methanol was almost exhausted. In the other words, synthesizing EPS from methanol by *Methylovorus* sp. MP688 is, to a certain extent, one way to reserve and store carbon and energy coping with methanol starvation. This hypothesis is also supported by the fact that there are no EPS synthesis genes found in *Methylobacterium extorquens* AM1, another best-studied facultative methylotrophic bacterium that can use a wide range of carbon source in addition to methanol and succinic acid (Laukel et al. 2004).

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