ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 2, pp. 253–255. © Pleiades Publishing, Ltd., 2013.
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SHORT COMMUNICATIONS

Construction and Characterization of *Methylomicrobium alcaliphilum* 20Z Knockout Mutants Defective in Sucrose and Ectoine Biosynthesis Genes

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Received August 27, 2012

DOI: 10.1134/S0026261713020021

Sucrose, the key plant carbohydrate, is synthesized by many phototrophic microorganisms and is supposed to act as an osmoprotectant in cyanobacteria [1]. Sucrose was also found in halotolerant methylotrophic bacteria, where it accumulated in response to increasing salinity of the growth medium, which is in agreement with a potential osmoprotective function of this disaccharide [2]. It was also hypothesized that in some methanotrophs sucrose biosynthesis could act as a sink for excessively toxic formaldehyde [3].

Higher plants and cyanobacteria synthesize sucrose from UDP-glucose and fructose-6-phosphate with intermediate formation of sucrose-6-phosphate by sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP) and/or from UDP-glucose and fructose with the involvement of sucrose synthase [4]. Recently we have revealed a cluster of the *sps-spp-fruK-ams* genes in the genome of the halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z. The products of these genes were homologous to the characterized SPS and SPP of plants and cyanobacteria, as well as to fructokinases (FruK) and amylosucrases (Ams) of heterotrophic bacteria utilizing sucrose as a growth substrate [5]. Although the arrangement of the sucrose metabolism genes within a single locus on the chromosome of *Mm. alcaliphilum* 20Z suggested the biochemical pathway of its biosynthesis and subsequent decomposition, the role of sucrose was not quite clear, especially since ectoine is the predominant osmoprotectant in this organism [2]. In order to answer this intriguing question, we have obtained and characterized the *Mm. alcaliphilum* 20Z mutants defective in the *sps* and *ams* genes. We have also obtained the mutants with insertion of the kanamycin cassette in the *ectBC* genes encoding the enzymes of ectoine biosynthesis; their properties demonstrated the osmoprotective function of sucrose.

Mm. alcaliphilum 20Z (VKM B-2133 = NCIMB 14124) was grown in 750-mL shaker flasks containing 200 mL of the 2P medium [2] with different salt concentrations (1–7% NaCl). The sterile solution of 1 M $NaHCO_3$ (5 mL per 100 mL of the medium) was added as well. Methanol at a final concentration of 0.2% (vol/vol) was added as a carbon source.

DNA was isolated from the cells by the standard methods [6]. The knockout mutants were obtained by double homologous recombination [7]. DNA fragments with the nucleotide sequences flanking the *sps*, *ams*, *ectB*, and *ectC* genes were cloned in the suicidal vector pCM184. The obtained plasmids pCMsps, pCMams, or pCMectBC were used to transform the cells of *Escherichia coli* S17-1 [8]; the transformants were used for conjugation with *Mm. alcaliphilum* 20Z. The transconjugates were selected by kanamycin resistance and tetracycline sensitivity. The insertion of the kanamycin cassette was additionally confirmed by PCR with the primers targeting the sequences flanking the inactivated gene. Sucrose and ectoine were extracted from the cells of the methanotroph with methanol [2], and their content in the extracts was determined using the anthrone reagent [9] and HPLC method [10], respectively.

It was shown that the strain with deletion of the hypothetical *sps* (Δsps) gene did not accumulate sucrose during growth at different NaCl concentrations; this finding indicated the key role of the *sps* gene product in sucrose biosynthesis in *Mm. alcaliphilum* 20Z and existence of the single pathway involved in sucrose synthesis: via sucrose phosphate synthase and sucrose phosphate phosphatase. The Δsps mutant did not differ from the initial strain in the degree of halotolerance, since both strains grew at the same rate in the presence of 1, 3, or 5% NaCl. On the other hand, the level of the osmoprotectant ectoine in the mutant cells growing in the presence of ≥5% NaCl was higher than in the parental strain (Fig. 1).

In *Mm. alcaliphilum* 20Z, ectoine is synthesized from aspartate, and these reactions are catalyzed by specific enzymes whose genes are located at the

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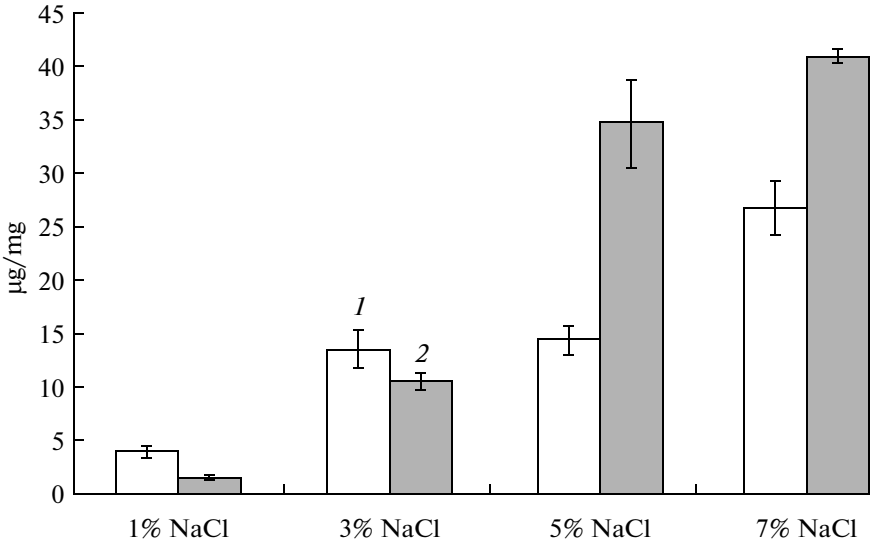

Fig. 1. Ectoine accumulation in the cells of the strains 20Z (*1*) and Δsps (*2*) grown at different concentrations of NaCl.

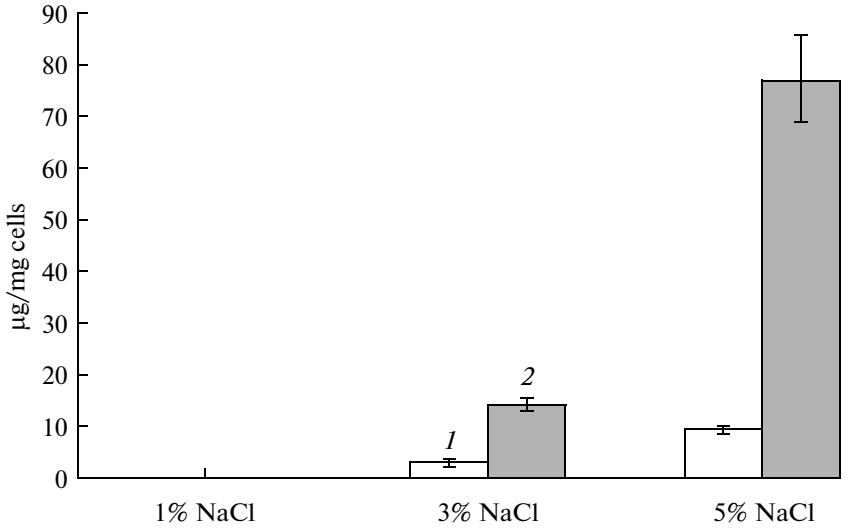

Fig. 2. Sucrose accumulation in the cells of the strains 20Z (*1*) and ΔectBC (*2*) grown at different concentrations of NaCl.

ectABC-asK operon [7]. As a result of inactivation of the *ectB* and *ectC* genes coding for diaminobutyrate aminotransferase and ectoine synthase, we obtained a mutant strain ΔectBC. Analysis of the methanol extracts of the strains 20Z and ΔectBC showed that the mutant did not accumulate ectoine. The intracellular level of sucrose in the mutant ΔectBC increased ~8-fold compared to the wild type strain (Fig. 2). The mutant demonstrated slower growth in media with relatively high salinity but did not grow at more than 5% NaCl (Fig. 3).

Earlier, a similar effect has been shown for *Synechocystis* sp. PCC 6803 [11]. While cyanobacterial cells with the inactivated *sps* gene did not synthesize sucrose, their salt tolerance actually was not changed due to accumulation of another osmoprotectant, glycosyl glycerol. On the contrary, lower halotolerance and higher level of sucrose were observed in the mutant with impaired biosynthesis of glycosyl glycerol [12]. Thus, the methanotroph and cyanobacteria have similar compensatory mechanisms for osmoadaptation, with sucrose as an additional osmoprotective compound.

Inactivation of the *ams* gene leads to a visible (1.3×) increase in the intracellular sucrose concentration, implying the involvement of amylosucrase in the deg-

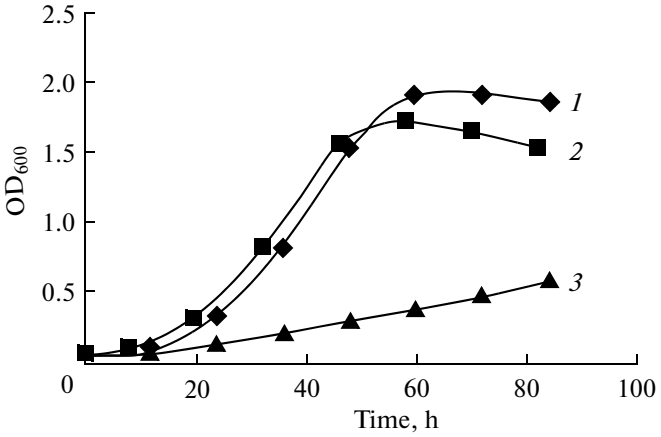

Fig. 3. Growth of the strains 20Z (*1*), Δsps (*2*), and ΔectBC (*3*) in the presence of 5% NaCl.

radation of the disaccharide via hydrolysis to fructose and glucose, as well as the transfer of the glucopyranose residue of sucrose to glycogen. Thus, *Mm. alcaliphilum* 20Z is able to synthesize and metabolize sucrose, e.g., to convert it into an osmotically inert form (glycogen), which is especially relevant in case of fluctuations in salinity of the medium.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (projects nos. 12-04-32122-a and 11-04-00801-a) and State Contract no. 16.740.11.0615.

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