Biochemical Characteristics of a Mutant of the Methanoarchaeon *Methanothermobacter thermautotrophicus* Resistant to the Protonophoric Uncoupler TCS

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**ABSTRACT.** In an attempt to more closely define a protein basis of differences in ATPase and ATP synthase activities in a mutant of the methanoarchaeon *Methanothermobacter thermautotrophicus* resistant to the protonophoric uncoupler TCS (3,3’,4’,5-tetrachlorosalicylanilide), the composition of membrane associated proteins from the wild-type and mutant strains has been compared. The uncoupler-resistance in the mutant strain was not accompanied by changes in a protein size or changes in the level of subunits A, B and C (proteolipid) of the A₁A₀-type ATPase–synthase. On the other hand, we revealed a 670-kDa membrane-associated protein complex that is abundantly present only in the mutant strain; it is composed of at least 5 different subunits of 95, 52, 42, 29 and 22 kDa.

Based on original ideas published over three decades ago (Kováč *et al.* 1967; Butlin *et al.* 1971), a systematic genetic approach has proved to be very valuable to the study of problems associated with energy conservation processes in yeast and bacteria. Especially a combination of such approaches with an exploitation of uncouplers of oxidative phosphorylation has resulted in a powerful tool that has given insight into energy coupling. Generally, the uncouplers are various compounds (protonophores, respiration or ATP synthesis inhibitors, etc.) that are able to uncouple respiratory and photosynthetic chain redox reactions from ADP phosphorylation (Hanstein 1976; Skulachev 1998). Many mitochondrial, chloroplastic and bacterial mutants resistant to different uncouplers have been isolated during the past 20 years. Studies of these mutants significantly contributed to understanding of the molecular mechanisms of energy coupling, but also to the molecular mechanisms of uncoupling in bacterial and eukaryotic domains of life (Kruwicht *et al.* 1990; Lewis *et al.* 1994). On the other hand, there are only very scarce data about energy coupling in the *Archaea*, the so-called third domain of life. Especially methanoarchaeae, which exhibit several unique features such as the very rigid pseudomurein-containing cell wall, a cytoplasmic membrane formed by a monolayer of unusual ether-linked isoprenoid lipids (Sprott 1992), or a specific energy pathway – methanogenesis, which together provide a challenging model for understanding of the effect of uncouplers. Isolation and characterization of uncoupler-resistant mutants among these organisms could be of great importance to our knowledge of energetics of methanogens. The methanogens utilize a conversion of CO₂ and H₂ to methane as their only energy-conserving pathway. The bioenergetics of this process has some unique features; in the pathway of CO₂ reduction to methane, two primary electrochemical ion gradients are formed by two distinct primary ion pumps. Heterosulfide reductase (EC 1.12.99.2) has been characterized as a proton-translocating system (Deppenmeier *et al.* 1990) and N⁵-methyltetrahydromethanopterin:coenzyme M methyltransferase (EC 2.1.1.86) has been shown to act as a primary sodium ion translocating enzyme (Becher *et al.* 1992). Both of these gradients are directly coupled to ATP synthesis via two specific H⁺- and Na⁺-dependent processes. It is believed that the proton-dependent synthesis is catalyzed by the A₁A₀-ATPase–synthase. A genetic approach to the problem of energy coupling in methanoarchaea, started in our laboratory in 1997, has resulted in the isolation and characterization of several interesting mutants (Šmigáň *et al.* 1997; Majerník *et al.* 2003; Čuiboňová *et al.* 2003). A preliminary characterization of a 3,3’,4’,5-tetrachlorosalicylanilide (TCS)-resistant mutant of *M. thermautotrophicus* has revealed that this mutant shows an increased ATPase activity and a diminished ability to synthesize ATP compared to the wild type (Čuiboňová *et al.* 2003). Here we present an analysis of a TCS-resistant mutant of *M. thermautotrophicus* that focuses on the membrane-associated protein spectra and subunits of the A₁A₀-ATPase–synthase.
MATERIAL AND METHODS

Proteins from membrane vesicles were analyzed by SDS-PAGE using standard techniques (Laemmli 1970). Native PAGE and ATPase activity staining were performed according to Kakinuma and Igarashi (1990). Separated proteins were electroblotted onto nitrocellulose and probed with antibodies against the A and B subunits of $A_1A_0$-ATPase from *Halobacterium salinarium*. Visualization was performed with pig anti-rabbit IgG secondary antibodies conjugated with alkaline phosphatase. Selective extraction of subunit $c$ of the $A_1A_0$-ATPase from cytoplasmic membranes of *M. thermautotrophicus* and its uncoupler-resistant mutant by chloroform–methanol extraction was done according to Ruppert *et al.* (2001). Protein was quantified according to Lowry with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

We isolated and partly characterized a mutant of *Methanothermobacter thermautotrophicus* resistant to the protonophoric uncoupler TCS (Čuboňová *et al.* 2003). In an attempt to more closely define the biochemical basis of this mutation, the composition of membrane-associated proteins from both wild-type and mutant strains has been compared. An abundant, membrane-associated protein complex with molar mass $\approx 670$ kDa was detected in the mutant but not in the wild type (Čuboňová *et al.* 2003). This protein complex did not exert ATPase activity (*not shown*). After elution of this complex from native gel and re-analysis by SDS-PAGE, we observed at least five putative subunits (Fig. 1). Calculated molar mass for each band was estimated at 95, 52, 42, 29 and 22 kDa in order of decreasing size. These results indicate that the mutant cells contain a membrane-bound protein complex with an as yet unidentified physiological function.

![Fig. 1](image1.png)

Markedly diminished ATP synthesis driven by methanogenic electron transport or potassium diffusion potential in the presence of either $H^+$ or $Na^+$ ions and elevated ATPase activity in the mutant cells (Čuboňová *et al.* 2003). Western blot of protein fractions derived from membrane vesicles of wild-type and TCS resistant-mutant cells of *M. thermautotrophicus* with antibodies against the A and B subunits of the $A_1A_0$-ATPase from *H. salinarium*; arrows indicate cross-reactive zones; subunit A (left) and B (right); 1 – wild type, 2 – mutant.

![Fig. 2](image2.png)