ZYMOMONAS MOBILIS LEVAN PRODUCTION IN THE PRESENCE OF ANTIMETABOLIC ANGENTS

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Abstract: Our studies have focused on the screening of *Zymomonas mobilis* bacterial strains capable to produce levan under the action of three antimetabolic agents (sulfafurozol, sulfametaxazol, trimetoprim). The experiments were carried out using spontaneous mutant strains derived from *Z.mobilis* NCIB 11163 and 11163/70, obtained by supplemented media with methotrexate (600 µg/mL) and trimethoprim (1000 µg/mL). Exponential growth profiles of bacterial cells and the production of levan were assessed in the absence and presence of antimetabolites of different concentrations. The studies have shown that *Z.mobilis* 11163/70 strains manifest a progressive growth in the presence of trimethoprim (50 µg/mL), an inhibition growth in the presence of sulfametoxazol (100 µg/mL). and also good resistance in the presence of sulfafurazol (100 µg/mL). Sulphonamides can inhibit the production of levan (*Z.mobilis* CP4PRRif, *Z.mobilis* NCIB 11163/70). On the other hand, a stimulation of levan production has been observed in the presence of trimetoprim (50 µg/mL) (*Z.mobilis* CP4Rif and 10988).

INTRODUCTION

Levans are microbial fructose polymers whose units are linked by type β-(2,6)-fructosyl-fructose. They are produced by several levansucrase microorganisms, among them *Zymomonas mobilis*, a mobile Gram-negative bacterium. Their synthesis is accompanied by FOS (fructooligosacharides) production (Bekers *et al*.,2002). It has been reported that levans produced by Z.mobilis possess antitumour activity (Yoo *et al.,* 2004) (up to 72% inhibition) and that the activity depends on the polysaccharide molecular weight (Calazans *et al*., 2000,1997).

Gram-negative bacteria *Z.mobilis* is a single bacterium in the evolution of bacterial world whose taxonomic position is not fully established (Rogers *et al*, 1984). On the sucrose-based media *Z.mobilis* is producing ethanol and various secondary products, especially high molecular weight levan (1975-2000 kDa) sorbitol, gluconic acid and FOS (Viikari, 1988; Vına *et al,* 2001). *Z.mobilis* bacterium is able to tolerate high concentrations of sucrose than glucose (Swings and De Ley, 1977) and at high concentrations of glucose cell membranes are affected by the rate of 10-15% while the high efficiency of hydrolysis of sucrose concentration is kept constant until 46% (Viikari, 1988).

MATERIALS AND METHODS

Preparation of extract of sweet sorghum. In our study we used sweet sorghum (*Sorghum bicolor*) from the National Institute for Research and Development of Technical Plants: (a) 11100 variety (b) 11061 variety (c) LC 99 variety (d) Prut04 variety (e) Sudan grass. Sweet sorghum extract was achieved by boiling extraction of sucrose from the plant stem and chemical characterized .

Selection of strains with different degrees of resistance/sensitivity to antimetabolite. As antimicrobial agents capable of producing mutant strains were used methotrexate and trimethoprim, compounds with antifolic activity. In our paper we used wild bacterial stains which came from the Laboratory of biochemistry, genetics and molecular biology of Univerităţii of Ioannina, Greece. Cultures were kept in optimal conditions (frozen at -80°C in a mixture composed of growth medium and glycerol in 1:1 ratio). Were tested the resistance of three pure strains belonging to the genus *Zymomonas mobilis* (ATCC 10988, NCIB 11163 and ATCC 31381-CP4RifR) to methotrexate and trimethoprim. Sugars and levan were determined by the method described by Dubois *et al* (1956; Kushwaha and Kates, 1981; Mukhopadhyay, 2005). The compounds with antimetabolite activity: sulfafurazol, sulfamethoxazole and trimethoprim were analyzed.

Growth curves in the presence of sulfafurazole, sulfamethoxazole and trimethoprim. Analysis of levan produced.
In order to study the exoenzimes involved in levan biodegradation produced by *Z.mobilis*, cells in exponential growth phase were used as inoculum to obtain a initial liquid culture (107 cells/ml). The increased turbidity was monitored at wavelength of 600 nm. The optical density at 600 nm (OD600nm) equal to 0.9 corresponds to a weight of 0.35 mg dry material / ml (Douka, 1999). Growth profiles were studied of the four microbial strains in the absence and presence of trimethoprim (50µg/ml, 100µg/ml respectively), sulfafurazol (100µg/ml, 500µg/ml respectively) and sulfamethoxazole (100µg/ml, 500µg/ml respectively) (*Sigma Chemical Co)*. At the end of the experiment the levan produced was extracted and analyzed (Andersone, 2004).

RESULTS AND DISCUSSION

Chemical characterization of sweet sorghum extract. Sorghum extracts used in our studies were chemically characterized in terms of sucrose and polyphenol gallic acid equivalent content (Table 1)

Table 1.Chemically characterization of Sorghum bicolor variety used

Sorghum varty	Sucrose concentration (g/100ml)	Polyphenols concentration (mg gallic acid equivalent/ml)
11100 variety	11.42	67.98
11061variety	10.07	68.5
LC 99 variety	6.75	68.24
Sudan grass	5.81	73.75
Prut04 variety	12.82	66.96
15A variety	29.46	72.47

Chemical analysis of used extracts indicates that 15A sorghum variety contains significantly higher amounts of sucrose and polyphenols. While the other varieties the sugar content varies by species, polyphenols are relatively constant values.

Selection of strains with different degrees of resistance/sensitivity to antimetabolite. Our studies have shown that in the presence of methotrexate or trimethoprim (100-1000 mg/ml liquid culture medium), NCIB11163 *Z.mobilis* strain presented difficulties of growing, are therefore most sensitive compared with strain ATCC 10988, as the deducted low generation times shown (Table 2)

Table 2. *Zymomonas mobilis* generation times tested grown in the minimum liquid medium supplemented with antimetabolite

Bacterial stain	NCIB 11163	ATCC 10988	CP4Rif
Inhibitor concentration (µg/ml)	MTX TMP	MTX TMP	MTX TMP
0	56 56	41 41	37 37
100	49 50	39 40	36 35
125	137 137	47 82	52 43
250	134 87	60 85	67 56
500	429 155	259 94	75 60
750	448 168	270 101	107 75
1000	585 211	352 127	120 76

Existence of R plasmid which confers resistance to rifampicin in CP4RifR strain has as results in induction of repair mechanisms (Miller, 1996; Darste, 2001), that counters the toxic effects of methotrexate or trimethoprim. NCIB 11163 *Z.mobilis* strain is the most affected by the presence of antimetabolic agents (TD1000 µg/ml being about five times increased from CP4 RifR strain and two times respectively compared with ATCC 10988 strain). This can be made in relation to inhibition of dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP$^+$ oxidoreductase, EC 1.5.1.3) (Stoian, 1996, Rao and Venkatachalam, 2000) key enzyme in the metabolism of folic acid and transfer of a carbon atom involved in the "de novo" biosynthesis of purine and pyrimidine nitrogenous bases or of amino acids.

Judging by the growth rates, increasing concentrations of methotrexate goes to a profound alterations of cellular metabolism. Inhibitory capacity of two compounds with antipholic activity on solid medium to determine the minimum inhibitory concentration (MIC) showed the same characteristic in terms of resistance / susceptibility for *Z.mobilis* strains. Thus, experiments performed on solid medium in the presence of MTX (100-1000 µg/ml MTX) showed that NCIB 11163 *Z.mobilis* strain are most susceptible, with MIC located around 300 µg/ml MTX. ATCC 10988 *Z.mobilis* strain is less sensitive (MIC = 500 µg / ml MTX) while CP4RifR *Z.mobilis* strain was resistant to the maximum concentration tested. Resistance profile of the three strains tested against trimethoprim *Z.mobilis* was similar.

Studies regarding the MIC for trimethoprim are similar to those obtained by other authors (Sprenger *et al*, 1993) who reported a natural resistance (> 95% survival) equal to 50 µg/ml TMP, while MIC values were 250, respectively 280 and 400 mg/ml TMP NCIB 11163 for *Z.mobilis* strains, ATCC10988 and CP4. The big difference (4 times) between the two experiments is probably due to the mode of administration (as ion) antifolic compound. Moreover Sprenger and associates (1995) used a wild CP4 strain compared to us who have tested the mutant strain resistant to rifampicin CP4. However the order of susceptibility to trimethoprim is stored in both experiments (NCIB 11 163 <ATCC 10988 <CP4).

Trimethoprim like as sulfisoxazolul is often used as a selective agent for isolation of resistant populations to the cytotoxic effects of the drug (Wang, 1994). Flowing growth in medium supplemented with trimethoprim, were isolated many spontaneous mutants from strains n case of NCIB ATCC 10 988 Z.mobilis and 11163. As with methotrexate, wild *Zyomonas* stain grown in presence of trimethoprim, formed spontaneous mutant colonies (1000 µg /ml TMP and 1200 µg /ml TMP)

Growth curves in the presence of sulfafurazole, sulfamethoxazole and trimethoprim. Analysis of levan produced

A variety of chemical compounds (aromatic compounds, xenobiotics, alcohols, alkanes.) are generally known as a basic induction for the structural and functional complex changes, that are related to the ability of a compound to partition into the cytoplasmic membrane of bacteria (Denich *et al*., 2003; Sikkema *et al*., 1995). These are the most amphiphilic substances, for example, each contain in their molecule both a hydrophilic region and a region waterproofing, and thus they replicate similarly glicerofosfolipidelor properties and membrane proteins (Denich *et al*., 2003).

For this reason, affinity for membrane of amphiphilic compounds depends on molecular size and degree of hidrofobicity (Zikmanis *et al*., 2005). Thus, aliphatic alcohols containing a polar hydroxyl group, they are increasing hidrofobicity with increasing alkyl chain length, non-polar (Denich *et al*., 2003; Sikkema *et al*., 1995, Dwyer and Bradley, 2000; Mc Karns *et al*., 1997).

In addition, it may be noted that all the processes of protein secretion to Z.mobilis remain almost unknown except on scientific reports on the process of TAT-dependent glucose processing fructoso oxidoreductaze (EC1.1.1.99) (Wiegert *et al*., 1996, Halbig *et al*., 1999), confirmed the absence of N-terminal signal in peptides and secretion of levansucrase due to interaction of specific intracellular proteins and and external cell membranes (Kyono *et al*., 1995, Oda *et al*. , 1994). Zikmanis *et al*. (2005) analyzed the effect of aliphatic alcohols, aromatic and surfactants on levansucraze secretion by 113S *Z.mobilis* strain in terms of structure and concentration of these amphiphilic compounds. Thus, *Z.mobilis* levansucrase appear not to be the typical N-terminal region sequences (Kyono *et al*., 1995, Oda *et al*., 1994), and can be compulsive for general secretion path (Pugsley, 1993). Therefore seems unlikely the ATP involvement in a secondary transport system ATP-dependent (type II path) is more appropriate to the existence of a type III transport pathways associated with membrane associated ATPase or ATP binding system (system ABC), type I in *Z.mobilis* cells. For achievement tests were first performed the exponential growth profiles of bacterial strains in the absence of inhibitors (Figure 1)
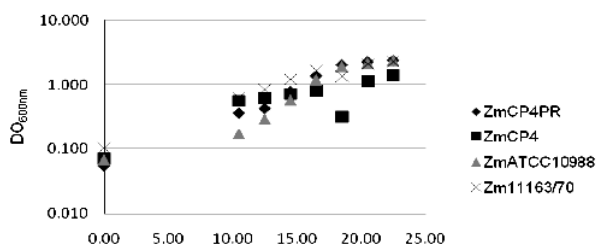

Figure 1.Exponential growth profiles of bacterial strains in the absence of inhibitors

In the presence of trimethoprim at a concentration of 50 mg /ml we observed a significant increase of lag period, except NCIB 11163 /70 *Z.mobilis* strain, the selected strain by spontaneous mutagenesis. At a concentration of 100 mg / ml trimethoprim NCIB 11163/70 and CP4RifPR *Z.mobilis* strains appear to reach the initial plateau phase.

The other two strains, the growth of ATCC 10988 and CP4Rif are damaged. In the presence of 100 mg/ml and respectively 500 mg/ml sulfamethoxazole Z.mobilis strains growth is alterate and the duplication time in so exaggerate. At 100 mg/ml and 500 mg/ml sulfafurazol NCIB 11163/70 *Z.mobilis*, methotrexate resistant, appears not to be affected by the presence of sulfonamide (Figure 2). Figure 3 shows the levan variation depending on production by treatment CP4RifPR *Z.mobilis*, CP4Rif *Z.mobilis*, ATCC 10988 *Z.mobilis* and NCIB 11163/70 compared with control.
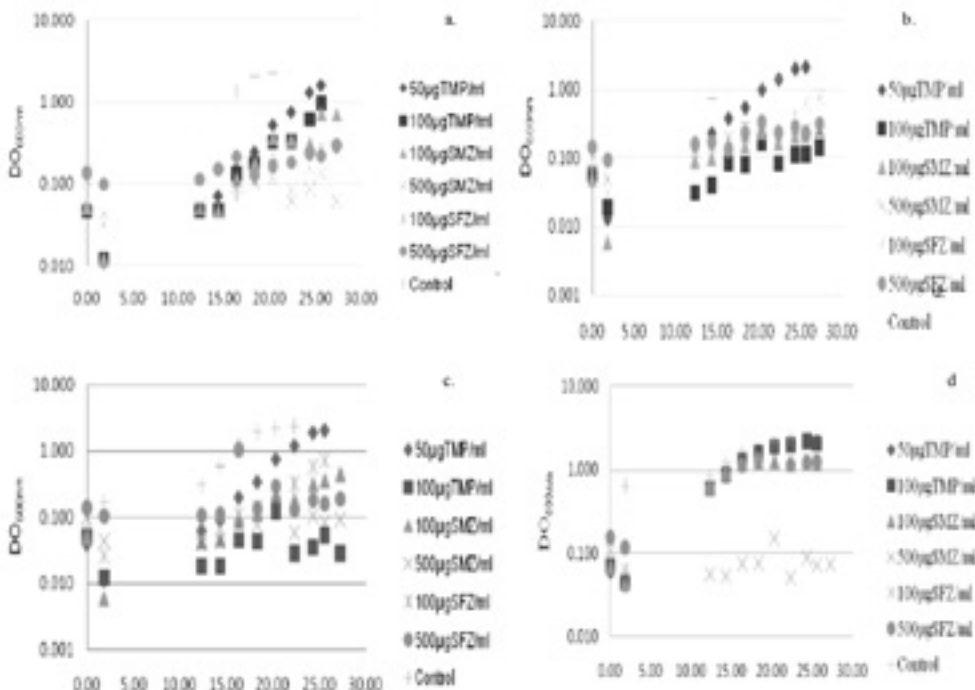
Figure 2. Growth curves of bacterial strains in the absence and presence of inhibitors. a. Growth curves of CP4PR bacterial strains in the absence and presence of inhibitors, b. Growth curves of CP4Rif bacterial strains in the absence and presence of inhibitors, c. Growth curves of ATCC 10988 bacterial strains in the absence and presence of inhibitor, d. Growth curves of NCIB11163/70 bacterial strains in the absence and presence of inhibitors
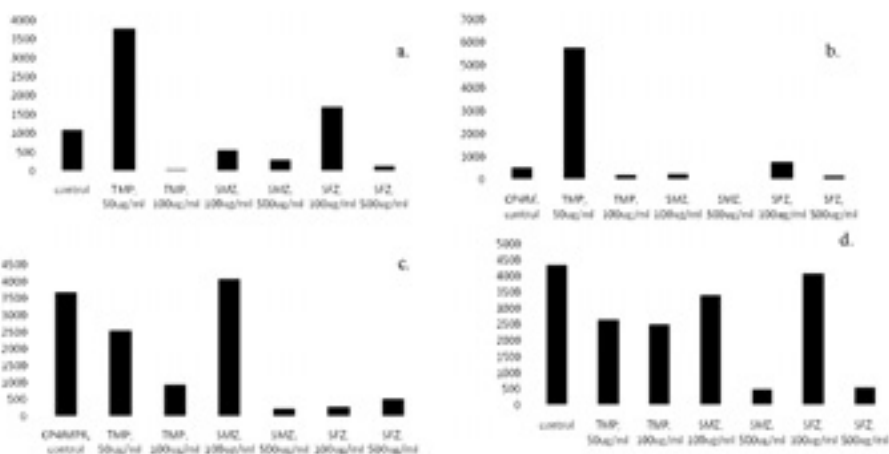

Figure 3. Levan variation depending on production by strains treatment (for a.Cp4 Rif, b. ATCC 10988, c.Cp4PR, d.11163/70 *Z.mobilis* stains

Levan production is profoundly affected by the concentration of nutrients in the culture medium and environmental conditions for growth. Thus, assessment of mineral salts on the formation of fructooligosacharide and products such as gluconic acid and sorbitol by 113S *Z. mobils* strain showed that key factors are produced in the synthesis of growth medium osmolarity in the context of the industrial substrates containing sucrose have a significantly increased level of salts as in the case of sugar beet molasses (Bekers *et al*, 2000).

It was noted that levan production is stimulated by the presence of sodium and potassium ions (Vigants *et al*, 1996), showing a direct activation by NaCl and KCl levansucrase *in vitro* in cell extracts (Vigants *et a*l, 1998). However, recent studies show that the stimulatory effect of mineral salts on the biosynthesis of oligosaccharides occur only at low concentrations of substrate (Bekers *et a*l, 2000). It was also found that if ethanol production is inhibited at high osmotic pressure, activity of levan biosynthesis is stimulated (Vigants *et al*, 1996).

CONCLUSIONS

As salts affect the performance of fermentative bacteria, their presence in the culture medium affects cell morphology (Vriesekoop *et al*, 2002). As NaCl is common agent growth inhibition for *Z.mobilis*, in data literature, consumption of glucose and ethanol production is attributed to both anion and cation as the last having a major effect. In contrast, filament formation is given utmost by chloride ion (Vriesekoop *et al*, 2002). Sulfonamides (sulfafurazol, sulfamethoxazole) as sulfizoxazolul antimetabolite agent were selected as because of the synergy with p-aminosalicilic acid.

Assuming that p-aminosalicilic acid may act on metabolic pathway involving p-amino benzoic acid at microorganisms, it can be speculate that the two sulfonamides are involved in inhibition of synthesis of folic acid and its derivatives, dihydrofolate and tetrahydrofolic acid. Trimethoprim, a compound nonsulfonamidic is a substitute which inhibits pyrimidine dihydrofolate reductase activity and thus interfere in dihidrofolat conversion to tetrahydrofolate. In addition, our previous data indicated involvement of trimethoprim in inhibition of NAD(P)H dehydrogenase to *Z.mobilis*. Spontaneously induced resistance to antifolice led to impaired cell wall biosynthesis with implications in biosynthesis of a complex NAD(P)H oxidase altered.

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