Appl. Biochem. Biotechnol.

0273-2289/91/27(1): 1–8/$2.00

Kinetics of Growth and Metabolism of *Clostridium thermosaccharolyticum* Culture

Isolation and Characteristics of Its Plasmids

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Received October 13, 1989; Accepted February 1, 1990

ABSTRACT

The kinetics of growth and metabolism of *Clostridium thermosaccharolyticum* DSM 571 has been studied at varying initial pH and glucose concentration. A weak inhibitory effect of excess glucose on the specific growth rate has been shown. The effect of antibiotics of various classes on culture growth and hydrogen evolution has been studied. Streptomycin and kanamycin resistance of this culture has been revealed as well as the phenomenon of increased hydrogen production in the presence of the above antibiotics. New plasmids, pNB1 (4.9 kb) and pNB2 (2.0 kb), were isolated from *C. thermosaccharolyticum* DSM 571. The restriction analysis of pNB1 and pNB2 has been performed.

Index Entries: *Clostridium thermosaccharolyticum;* glucose; hydrogen; ethanol; acetic; propionic and lactic acids; carbon dioxide; kinetics; specific growth rate; half-saturation constant; inhibition constant; antibiotic-resistance; streptomycin; kanamycin; plasmid; restriction analysis.

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INTRODUCTION

The thermophilic microorganisms of the genus *Clostridium* present a great interest both for theoretical investigations and for industrial microbiology. This is related to the following:

1. Among metabolic products of clostridial microorganisms, there are promising energy-carriers, such as ethanol, hydrogen, butanol, and so on;
2. Many enzymes isolated from thermophilic bacteria feature a notable thermostability; and
3. The plasmids of thermophilic organisms are usually small and can serve as vectors for genetic research (*1–3*).

Clostridium thermosaccharolyticum was first isolated and described in 1935 (*4*). The bacterium grows at 55–60°C and utilizes glucose, producing hydrogen, lactate, butyrate, and carbon dioxide during growth as well as ethanol during spore formation. The strains of this culture isolated later have some distinctions; for instance, during growth, they form ethanol in place of butyrate (*20*). On the whole, *C. thermosaccharolyticum* is close in properties and performance to *Clostridium thermohydrosulfuricum* and *Clostridium thermosulfurogenes* (*6*).

The objects of the intensive study of plasmids of thermophilic microorganisms are aerobic species of *Thermus* (*7*) and *Bacillus* (*1,8*) genera. The information about plasmids from thermophilic anaerobes is quite scanty. *Clostridium thermoaceticum* were reported (*9*) to contain 30 kb plasmid. There was no relation between the presence of the plasmid and the hydrogenase activity of *C. thermoaceticum*.

Screening for plasmids in 150 strains (75 species) of the genus *Clostridium* has been carried out (*10*). Plasmids were found in 26 strains (21 species). The authors failed to detect plasmids in the thermophilic strains, including four *C. thermosaccharolyticum* strains.

A similar screening of 21 mesophilic strains of *Clostridium acetobutyricum, Clostridium butyricum,* and *Clostridium saccharoperbutylacetonicum* showed the presence of cryptic 1.6–133 kb plasmids in 7 strains (*11*). Some strains of *Clostridium perfringens* are also known to carry plasmids resistant to tetracycline and chloramphenicol (*12*).

The objective of this work is to study the kinetics of growth and metabolism of the thermophilic saccharolytic bacterium *C. thermosaccharolyticum* as well as to isolate and study the plasmid DNA from this culture.

MATERIALS AND METHODS

The object under study is the thermophilic anaerobic bacterium *C. thermosaccharolyticum* DSM 571 from the collection of the Institute of Microbiology of the USSR Academy of Sciences (Moscow, USSR). The bacteria

were cultivated under anaerobic conditions at 55°C on the following medium (g/L): NH_4CL, 2.0; K_2HPO_4, 4.0; KH_2PO_4, 2.0; $MgCl_2 \cdot 6H_2O$, 0.42; $CaCl_2$, 0.05; D-glucose, 3.0; yeast extract, 1.0; and 1 mL/L of a solution of microelements (mg/L: $CuSO_4 \cdot 5H_2O$, 6.4; $FeSO_4 \cdot 7H_2O$, 7.9; $MnSO_4 \cdot 4H_2O$, 7.9; $ZnSO_4 \cdot 7H_2O$, 1.5). Depending on experimental purposes, the medium pH and the initial glucose concentration were varied. These experiments were carried out in 4 replicates. In some experiments, the medium was supplemented with filter sterilized antibiotics (streptomycin, kanamycin, ampicillin, tetracycline, chloramphenicol, and cycloserine) in the concentration range of 5–200 μg/mL. The experiments with antibiotics were performed in 10 replicates.

Hydrogen and carbon dioxide in the gaseous phase as well as ethanol and volatile fatty acids in the liquid phase were quantified chromatographically (*13*). Lactic acid and glucose in the medium were determined spectrophotometrically; lactic acid with the aid of lactate oxidase (*14*) and glucose using glucose oxidase and peroxidase (*15*).

Culture growth was monitored by the change in optical density of the medium at 570 nm and by protein concentration. Protein was quantified by the modified Lowry method (*16*). The number of cells/U vol was determined from the optical density of bacterial suspension with the use of the calibration curve for counting of cells in the Goriayev chamber.

To isolate the plasmid DNA, three different lysis procedures were used: The lysis by boiling, the lysis by alkali, and the soft lysis by sodium dodecyl sulfate (*17*). Purer DNA preparations with the highest yield were obtained by the alkali procedure (*18*). The plasmid preparations were treated with 50 μg/mL RNAase A (Sigma) at 30°C for 60 min. Gel electrophoresis of plasmid DNA was performed in 0.8% agarose (Sigma) (*17*). The concentration of ethidium bromide in the gel was 0.5 μg/mL.

RESULTS AND DISCUSSION

Figure 1 presents the plots defining the growth of *C. thermosaccharolyticum* culture and the accumulation of metabolites in the medium at the initial pH 7.5 and 55°C. The culture growth is accompanied by the decrease of pH in the medium because of accumulation of organic acids. The metabolic products of this bacterium are hydrogen, ethanol, acetic and lactic acids, carbon dioxide and an insignificant quantity of propionic acid. Upon counting the cell number in the Goriayev chamber, we inferred that through the culture growth that the cell number/mL increases from $0.4 \cdot 10^9$ to $2.6 \cdot 10^9$, that correlates with a six and one-half fold increase in the optical density. The culture growth is specified by a long lag phase, during which the culture consists of small 1×3 μm rods and numerous spores. During the exponential phase, the cells become longer, up to 2×20 μm, and curved; the spores almost disappear. The type of spore formation is plectridial (a round terminal spore).

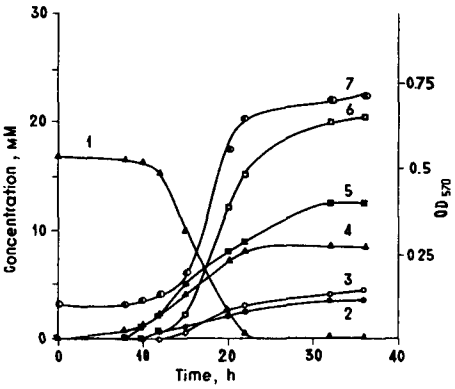

Fig. 1. Kinetics of growth and metabolism of the culture *C. thermosaccharolyticum* DSM 571 at initial pH 7.5 and 55°C: 1, glucose; 2, acetic acid; 3, lactic acid; 4, hydrogen; 5, ethanol; 6, carbon dioxide; 7, optical density.

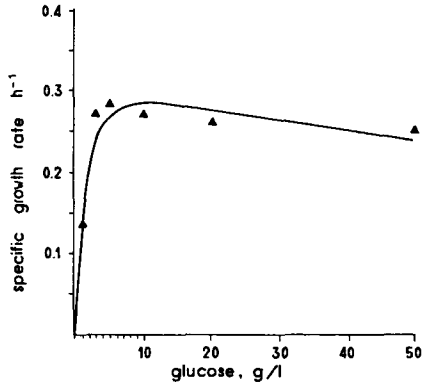

Fig. 2. Inhibition of *C. thermosaccharolyticum* by excess substrate (initial pH 7.5). Dots are the experimental results; solid line is the theoretical curve.

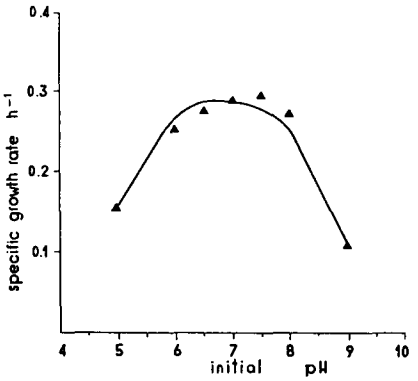

Fig. 3. pH Dependence of *C. thermosaccharolyticum* (glucose, 3 g/L). Dots are the experimental results; solid line is the theoretical curve.

Processing of the experimental results from the culture growth (*19*) showed us to determine the specific growth rate to be 0.3 h^{-1} (the doubling time is about 2 h), which is comparable with literature data (*7*).

Figure 2 shows the plot for the specific growth rate (in the exponential phase) of *C. thermosaccharolyticum* vs the initial glucose concentration in the medium. One can see that the culture is subjected to a weak inhibition or growth by excess substrate. This dependence is described by the equation

$$\mu = \mu_m / (1 + K_s/S_o + S_o/K_i) \quad (1)$$

where μ is the specific growth rate; μ_m is the maximum specific growth rate; S_o is glucose, K_s is the half-saturation constant, and K_i is the inhibition constant by excess glucose.

The experimental results were used to compute a theoretical curve and to calculate the parameters of Eq. (1):

$$\mu_m = 0.29\ h^{-1};\ K_s = 1.15\ g/L;\ K_i = 124.5\ g/L$$

Figure 3 shows the plot for the specific rate of culture growth (initial glucose concentration - 3 g/L) in the exponential phase vs the initial pH of the medium. It was established that bacterial growth is possible over the initial pH range 5.0–9.0; the maximum falls at pH 7.5. The curves of Fig. 3 are described by the equation:

$$\mu = \mu_m / (1 + H^+/K_a + K_b/H^+) \quad (2)$$

where K_a and K_b are the dissociation constants. From the experimental results, the parameters of Eq. (2) were computed to be:

$$\mu_m = 0.29\ h^{-1};\ pK_a = 5.0;\ \text{and}\ pK_b = 8.8$$

We examined the effect of antibiotics of various classes on growth and metabolism of *C. thermosaccharolyticum*. These are cycloserine and ampicillin, affecting the synthesis of the cell wall, tetracycline, streptomycin, kanamycin, and chloramphenicol influencing the translation mechanism. The growth of the cultures was monitored by the change of the optical density and the metabolic activity by the amount of hydrogen evolved in 30 h incubation.

Even minor concentrations of cycloserine, ampicillin, chloramphenicol, and tetracycline (individually added) suppress culture growth and metabolism (Figs. 4 and 5). The effects of streptomycin and kanamycin turned out to be different. First, the culture was resistant to 5–20 μg/mL streptomycin and kanamycin. Second, the increased production of hydrogen was observed in the presence of 5–50 μg/mL of these antibiotics. An interpretation of the observed phenomenon during culture growth on a medium with antibiotics needs additional experiments.

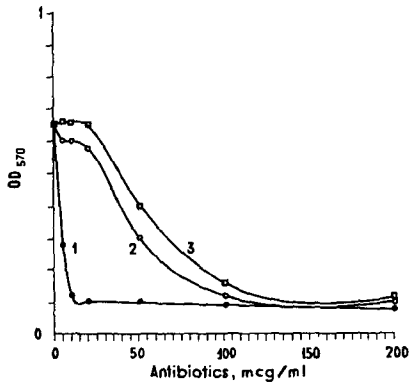

Fig. 4. Effect of antibiotics on *C. thermosaccharolyticum* growth (initial glucose concentration, 3 g/L): 1, ampicillin; tetracycline, chloramphenicol, and cycloserine; 2, streptomycin; and 3, kanamycin.

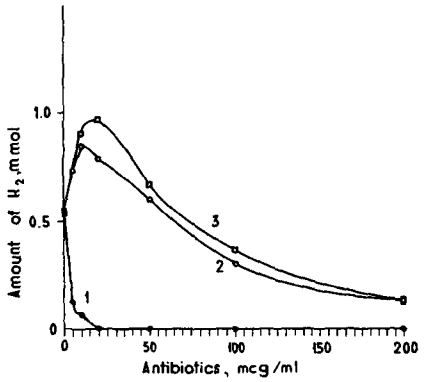

Fig. 5. Effect of antibiotics on hydrogen production by *C. thermosaccharolyticum:* 1, ampicillin, tetracycline, chloramphenicol, and cycloserine; 2, streptomycin; and 3, kanamycin.

Our experiments showed that the strain *C. thermosaccharolyticum* DSM 571 contains the plasmid DNA. Figure 6 presents the results of electrophoresis of a sample of plasmid DNA from *C. thermosaccharolyticum* DSM 571 treated with some restrictases. The plasmid DNA sample untreated with restrictases (lane 1) shows five basic bands. The first upper band is the linear form of the first plasmid. The second is the supercoiled form of the same plasmid. The third is the irreversible denatured DNA (resistant to restrictases). Finally, the fourth and fifth bands belong to the second plasmid. The restriction analysis affords a conclusion about the presence of two plasmids, 4.9 and 2.0 kb in *C. thermosaccharolyticum* DSM 571. We termed them pNB1 and pNB2, respectively. pNB1 has one EcoR I site, one Pst I site, and three Hind III sites. There are no restriction sites for Bgl II and BamH I. pNB2 has two Hind III sites and one Pst I site and has no sites for EcoR I, Bgl II, and BamH I. Figure 7 shows the restriction map of pNB1. Detailed restriction maps and descriptions of pNB1 and pNB2 will be reported elsewhere (manuscript in preparation).

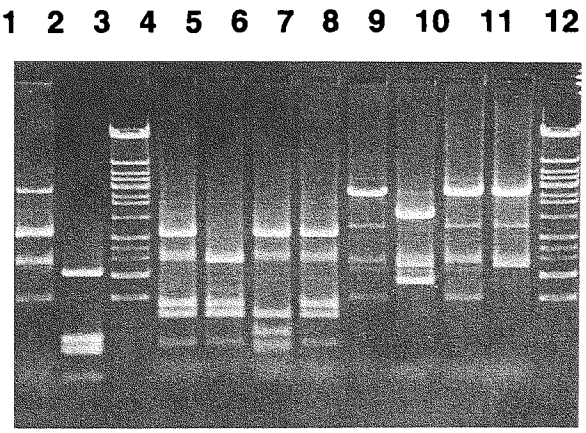

Fig. 6. Gel electrophoresis of a) plasmid DNA from *C. thermosaccharolyticum:* (1) without pretreatment of restrictases; (4)+Hind III; (5)+Hind III+EcoR I; (6)+Hind III+Pst I; (7) the same as in lane 4; (8)+EcoR I; (9)+EcoR I+Pst I; (10)+EcoR II+Bgl II; (11)+Pst I; b) DNA standard marker: (2) pBR 322+Dde I (0.162, 0.465, 0.570); (3) Phage λ+E coR I, Phage λ+Hind III, pUC18+Hind III, pUC18+Bgl I (0.564, 1.118, 1.568, 2.027, 2.322, 2.686, 3.530, 4.361, 4,878, 5.643, 5.804, 6.557, 7.421, 9.416); (12) the same as in lane 3.

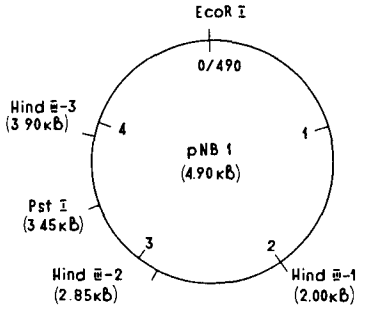

Fig. 7. Restriction map of the plasmid pNB1 from *C. thermosaccharolyticum* DSM 571.

It is necessary to note that the obtained results are at variance with published data (*10*), where no plasmids were found in *C. thermosaccharolyticum* DSM 571. It is possible, however, that this variance is due to the difficulties in detection of small and low copy number plasmids in *C. thermosaccharolyticum* grown in medium without antibiotics. The copy number of pNB1 during cultivation of the bacteria in medium without antibiotics is 1–2 per cell. On selective media containing kanamycin or streptomycin, the plasmid copy number per cell increases up to 5–10. Plasmids pNB1 and pNB2 can be used as vectors for genetic study of thermophilic Gram-positive bacteria.

We thank A. A. Belogurov and E. P. Delver for help in the experiments with plasmids and useful suggestions in the course of this work.

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