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Growth Characteristics of a Novel Nitrogen-Fixing Cellulolytic Bacterium

RICHARD V. GREENE* AND SHELBY N. FREER

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

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Growth characteristics of a cellulolytic nitrogen-fixing bacterium isolated from a marine shipworm by Waterbury et al. (J. B. Waterbury, C. B. Calloway, and R. D. Turner, Science 221:1401–1403, 1983) are described. When grown microaerobically, the bacterium exhibited doubling times of about 2 days in cellulose-supplemented synthetic medium devoid of combined nitrogen. Maximum growth was reached 12 to 16 days after inoculation. Growth optima for pH, temperature, and NaCl concentration were 8.5, 30 to 35°C, and 0.3 M, respectively. During growth the bacterium produced succinic acid (0.026%) and acetic acid (0.010%). Formic acid (0.010%) was produced during the stationary growth phase. No growth was observed when glucose was the sole carbon source. Cellobiose supported weak growth, while longer-chain-length cellodextrins supported extensive growth. Analysis of residual carbohydrates in the medium during growth indicated that the bacterium catabolized a terminal glucose moiety from the cellodextrin chain.

Gould (3–5) has shown that treatment of lignocellulosic materials, such as crop residues, with a dilute solution of alkaline hydrogen peroxide solubilizes 50% or more of the lignin present. The treatment also appears to significantly decrease the crystallinity of the residual cellulose, making it particularly susceptible to microbial digestion (4, 6). Further, in vivo studies indicate that the alkaline peroxide treatment increases the digestibility of wheat straw to a degree that is suitable for production of ruminant animals (9). However, alkaline peroxide-treated wheat straw (APWS) is essentially devoid of nitrogen. Combined nitrogen must be exogenously supplied if APWS is to be considered a suitable feedstuff for ruminants.

Recently, Waterbury et al. (14) isolated a cellulolytic nitrogen-fixing bacterium from the gland of Deshayes in six species of marine shipworms. The isolates are morphologically similar and probably represent a single species. Furthermore, Waterbury et al. believe that the bacterium is the primary source of cellulolytic enzymes for the shipworms and that nitrogen fixation by the bacterium enables the shipworms to meet their nitrogen requirements when growing on a nitrogen-poor diet. Because the shipworm bacterium uniquely possesses two properties, cellulose digestion and nitrogen fixation, we considered it to be a viable candidate for production of single-cell protein from nitrogen-deficient cellulosic substrates such as APWS. In this paper we report the growth characteristics of the shipworm bacterium on APWS, as well as on other cellulosic substrates.

MATERIALS AND METHODS

Stock cultures. Bacterial cultures (not yet officially classified) isolated from shipworm (*Psiloteredo healdi*) gland of Deshayes were generously supplied by John Waterbury (Woods Hole Oceanographic Institution). Stock cultures were maintained on a colloidal cellulose-agar medium in aluminum seal-type culture tubes (Bellco Glass, Inc.) fitted with gray butyl rubber stoppers (Wheaton Industries). These stoppers allowed limited, aseptic exchange with the atmosphere. The stock culture medium consisted of basal salt medium supplemented with 0.5% Sigmacell 100 and 0.2% agar. Cultures grew as a visible band just below the surface of the medium.

Experimental cultures. Unless otherwise stated, basal medium contained the following, per liter of deionized H_2O: $NaCl_2$, 18.8 g; KCl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 1.9 g; $MgCl_2 \cdot 6H_2O$, 1.5 g; $CaCl_2 \cdot 2H_2O$, 0.4 g; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 4.9 g; solution A, 10 ml; trace metal solution, 1 ml. Solution A consisted of the following (per liter of H_2O): $K_2HPO_4 \cdot 3H_2O$, 2.0 g; Na_2CO_3, 1.0 g; sodium citrate, 0.4 g; $Fe_2(SO_4)_3$, 0.3 g; EDTA, 0.05 g. Trace metal solution consisted of the following (per liter of H_2O): H_3BO_3, 2.9 g; $MnCl_2 \cdot 4H_2O$, 1.8 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g; $Na_2MoO_4 \cdot 2H_2O$, 0.04 g; $CoSO_4 \cdot 7H_2O$, 0.05 g; $CuSO_4 \cdot 5H_2O$, 0.08 g. The pH of the basal medium was adjusted to 8.0 with NaOH. Carbon supplements were 0.5% Sigmacell 100, 0.5% APWS (3), or 0.1% cellodextrins. Combined nitrogen was added as NH_4Cl, 0.3 g/liter. The standard growth temperature was 30°C.

Aerated growth was conducted with 25-ml cultures in 125-ml Erlenmeyer flasks shaken at 125 rpm. Microaerophilic growth on cellulose as a carbon source was conducted with static 25-ml cultures in 25-ml Erlenmeyer flasks. Growth on cellodextrins as a carbon source was conducted with 15-ml cultures in the above-described stock culture tubes. Measuring optical density was unsuitable for evaluation of growth because the bacterial cells tended to form clumps and to adhere to the side of the tube. Therefore, all growth was measured by protein content as determined by the method of Lowry et al. (11). Protein was assayed in quadruplicate, and cultures were first sonicated (Branson model S125 probe Sonifier) to ensure a more representative sample. After addition of the Lowry et al. (11) alkaline-copper solution, samples were incubated at 80°C for 30 min to aid cell disruption. Residual cellulose content was calculated from aqueous glucose concentrations (determined by high-pressure liquid chromatography) after digestion by

* Corresponding author.

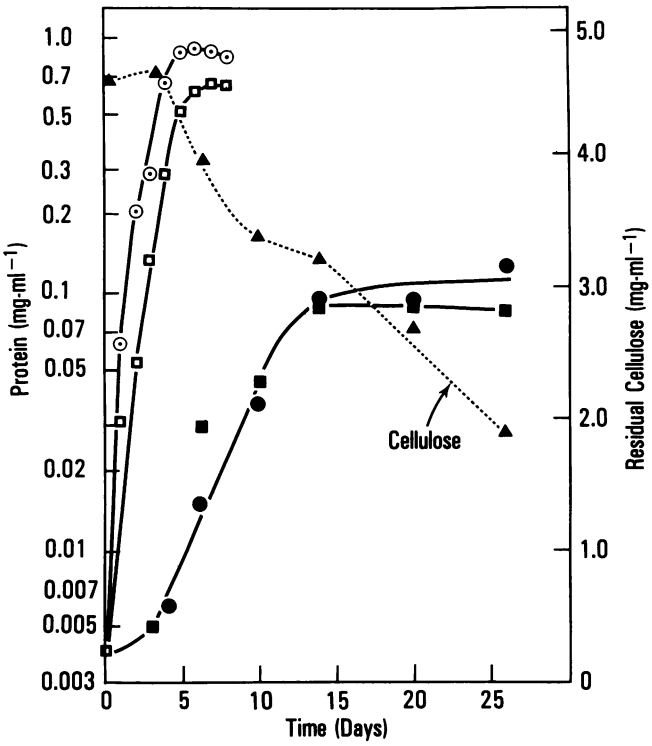

FIG. 1. Growth of the shipworm bacterium at 30°C on Sigmacell 100 or APWS with and without added combined nitrogen. Protein concentration symbols: (■) unshaken culture supplemented with Sigmacell 100; (●) unshaken culture supplemented with APWS; (□) shaken culture supplemented with Sigmacell 100 and NH_4Cl; (⊙) shaken culture supplemented with APWS and NH_4Cl. (▲), represents residual cellulose content in the unshaken culture supplemented with APWS. All points represent the average of triplicate cultures.

Trichoderma reesei cellulase, as described elsewhere (3). Evolution of $^{14}CO_2$ from [^{14}C]lignin was determined by the method of Kirk et al. (10).

Analysis of culture media. Organic acids were determined with a Dionex model 2010i ion chromatography system equipped with an HPIC-AS1 separator column and an ISC suppressor column. The eluent was 1 mM HCl. Peaks detected by conductivity were processed by digital integration (Spectra-Physics model SP 4270 integrator) and identified by comparison to retention times of authentic standards. Succinic acid concentrations were also determined enzymatically by the method of Beutler (1).

Cellodextrins were separated by high-pressure liquid chromatography with a Bio-Rad HPX-87P column heated to 85°C after samples were initially desalted with a mixed-bed ion-exchange resin (Bio-Rad AG 501-X8D). The eluent was H_2O. Peaks detected by changes in refractive index (Spectra-Physics model SP 6040 refractometer) were processed with a Hewlett-Packard model 3393A integrator and identified by comparison to retention times of authentic standards.

Sources of chemicals. APWS was a gift of J. Michael Gould. [^{14}C]lignin was a gift of T. Kent Kirk. Sigmacell 100 and Folin-Ciocalteu phenol reagent were from Sigma Chemical Co. Cellodextrins were prepared by the method of Freer and Detroy (2). *T. reesei* cellulase was from Miles Laboratories, Inc. Succinyl-coenzyme A synthetase, pyruvate kinase/lactate dehydrogenase, coenzyme A, phosphoenolpyruvate, ITP, NADH, and glycylglycine were from Boehringer Manneheim Biochemicals. All other chemicals were reagent grade.

RESULTS

The growth of the shipworm bacterium at 30°C on APWS or Sigmacell 100 as a sole carbon source is shown in Fig. 1. Under conditions conducive to nitrogen fixation (static flasks, no added combined nitrogen), growth on APWS and Sigmacell 100 was essentially identical. Typically, after inoculation a lag phase of about 2 days was observed, which was not significantly shortened by reinoculating fresh growth medium with actively growing cultures. Lag phase was followed by a growth phase that exhibited a doubling time of about 2 days. Maximum growth was attained between 12 and 16 days. Generally, the cultures produced a maximum of about 0.1 mg of protein ml^{-1}, although cultures with protein content approaching 0.4 mg ml^{-1} have been observed. No growth was observed in vigorously shaken cultures lacking combined nitrogen (not shown), probably due to oxygen poisoning of the nitrogenase. However, if NH_4Cl was added to vigorously shaken cultures, growth was rapid (doubling times, 8 to 15 h) and extensive (protein content approaching 1.0 mg ml^{-1}). APWS appeared to support better growth than did Sigmacell 100 under these latter conditions. In agreement with previous conclusions (14), the data indicate that the bacterium fixes molecular nitrogen under microaerophilic conditions, but requires a source of combined nitrogen if aerated.

The remainder of this paper presents only data obtained under nitrogen-fixing conditions since a prime purpose of the work was to explore the feasibility of utilizing the shipworm bacterium to introduce single-cell protein into nitrogen-deficient cellulosic material without adding combined nitrogen. The residual cellulose content in nitrogen-fixing cultures grown on APWS is also presented in Fig. 1 (dotted line). After 12 days of growth, 1.5 mg of cellulose ml^{-1} was degraded. The corresponding protein content at that time was 0.1 mg ml^{-1}, representing a yield of 7.5 g of protein 100 g of $cellulose^{-1}$. Lignin accounts for about 10% of the dry weight of APWS (6), and it is possible that the bacterium may have derived some additional metabolic energy from lignin degradation. However, no $^{14}CO_2$ evolution was observed when [^{14}C]lignin was added to actively growing cultures (not shown), indicating that the bacterium could not significantly metabolize lignin.

The effect of temperature and salinity upon growth of the shipworm bacterium is shown in Fig. 2. Maximum growth was achieved between 30 and 35°C, while growth was absent at 40°C. When the concentration of NaCl was varied, growth was observed between 0.1 and 0.6 M NaCl. The optimal concentration of NaCl for growth was about 0.3 M.

The effect of culture medium pH on bacterial growth is shown in Table 1. Growth was observed for a rather wide range of pH values (10.5 to 6.0), but optimum growth occurred at initial medium pH values of about 8.5. Culture growth was accompanied by a lowering of medium pH. In the most actively growing cultures, the pH decrease was dramatic (>2 pH units), suggesting that considerable amounts of organic acid were excreted by the bacterium.

A Dionex ion chromatography system specific for organic acids was used to analyze the culture medium because the system was found to be highly sensitive and exhibited negligible interference from high concentrations of salt. The major acid produced during bacterial growth was succinic

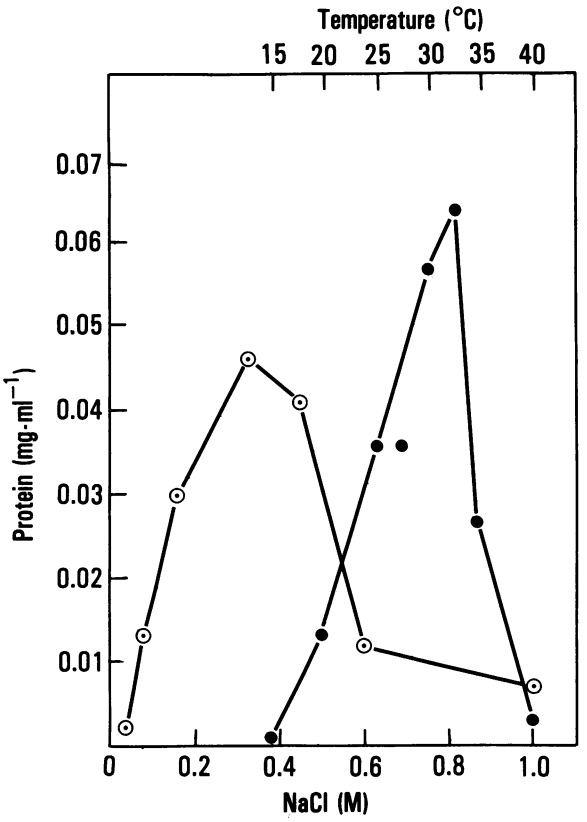

FIG. 2. Effect of variable temperature (●) or NaCl concentration (⊙) on growth. Points represent the average of triplicate cultures grown for 11 days on APWS.

acid (Table 2). This was confirmed enzymatically by the method of Beutler (1). Lesser amounts of acetic and formic acids were also present in the culture medium. The production of succinic and acetic acids appears to coincide with primary growth (Fig. 1 also), while most of the formic acid appears to be produced during stationary growth phase. Adding the concentration of each organic acid measured from 12-day-old cultures (Table 2) to uninoculated culture medium lowered the medium pH from 8.0 to 6.4. This is in reasonable agreement with the measured pH decrease (8.0 to 6.2) found in 12-day-old cultures (Table 1). Ion chromatography also indicated that another minor unidentified organic

TABLE 1. Effect of medium pH on bacterial growth[a]

Initial pH	Protein concn (mg ml^{-1})	Final pH
10.5	0.027	9.5
10.0	0.043	8.5
9.5	0.052	8.4
9.0	0.066	7.8
8.5	0.069	6.4
8.0	0.059	6.2
7.5	0.046	5.9
7.1	0.039	5.8
6.5	0.022	5.6
5.5	0.010	5.4

[a] The buffer system used was 5 mM HEPES, 5 mM MES (morpholine ethanesulfonic acid), 5 mM TAPS [tris(hydroxymethyl)methylaminopropanesulfonic acid], and 5 mM CHES (2-*N*-cyclohexylaminoethanesulfonic acid). Data represent the average of triplicate cultures grown for 12 days on APWS.

TABLE 2. Production of organic acids by the shipworm bacterium

Culture age (days)	Concn of organic acids in growth medium (%, $\times 10^3$)[a]		
	Succinate	Acetate	Formate
1	0.1 (0)[b]	0.0	0.1
3	3.0 (2)	0.1	0.8
6	22.5 (15)	10.3	1.1
12	26.8 (23)	6.4	2.6
18	18.3 (18)	5.0	6.9
28	18.7 (23)	8.3	12.7

[a] The carbon source in the growth medium was APWS.
[b] Parenthetical numerals represent succinate concentrations determined enzymatically by the method of Beutler (1).

acid was present in the growth medium, which could account for the slight discrepancy above.

Figure 3 illustrates the ability of the shipworm bacterium to grow on cellodextrins of various chain lengths. In contrast to the strains examined by Waterbury et al. (14), the strain from *P. healdi* showed little or no growth with glucose as the sole carbon source. Cellobiose supported variable weak growth. Cellodextrin chain lengths greater than two (cellotriose through cellohexaose) supported extensive growth.

Growth media from the above cultures were analyzed for residual cellodextrins, and results are presented in Tables 3 and 4. The concentrations of longer-chain cellodextrins (≥cellotetraose) are presented as a pool because they were not resolved by the high-pressure liquid chromatography system. As expected, 4-day-old cultures catabolized little original glucose or cellobiose, while cellotriose and longer-chain cellodextrins were readily degraded (Table 3). Furthermore, this degradation appeared to be accompanied by the formation of cellodextrins containing one less glucose unit. For instance, cellobiose was the major carbohydrate de-

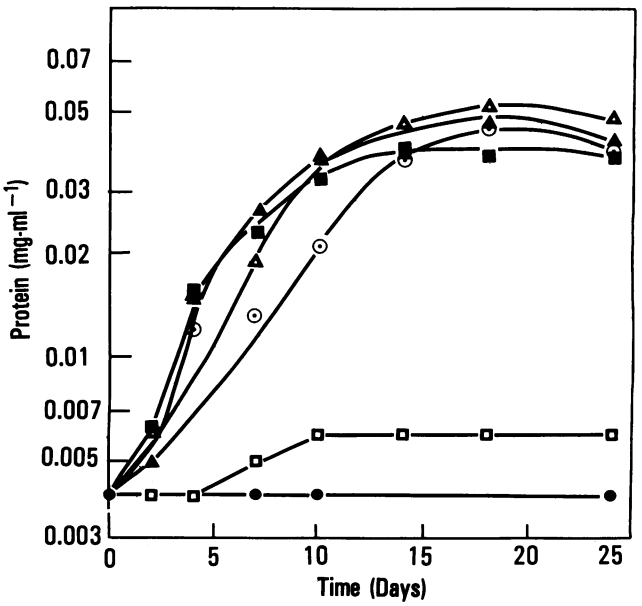

FIG. 3. Bacterial growth on cellodextrins of various chain lengths. Symbols for carbon source: (●) glucose; (□) cellobiose; (⊙) cellotriose; (△) cellotetraose; (■) cellopentaose; (▲) cellohexaose. Points represent the average of two cultures.

TABLE 3. Residual cellodextrins in 4-day-old growth media with cellodextrins of different chain lengths as carbon source

Original carbon source (0.1%)	Concn of cellodextrins in media (%)			
	G_1	G_2	G_3	$G_{\geq 4}$
G_1	0.091	ND[a]	ND	ND
G_2	0.010	0.083	ND	ND
G_3	ND	0.045	0.043	ND
G_4	ND	0.011	0.040	0.031
G_5	ND	0.012	0.019	0.050
G_6	ND	0.007	0.013	0.081

[a] ND, Not detectable (<0.005%).

tected in the growth medium for the original cellotriose substrate. Similarly, cellotriose was the major carbohydrate detected in the medium from the original cellotetriose growth substrate (Table 3). The data in Table 4 support this observation. Cultures grown on cellotetraose first accumulated cellotriose. Then, as they matured, cellobiose accumulated. Glucose did not appear to accumulate significantly, which was somewhat unexpected.

DISCUSSION

Our findings confirm the report of Waterbury et al. (14) that the shipworm bacterium is capable of both nitrogen fixation and cellulose digestion. As we demonstrate (Fig. 1), this unique combination of properties allows for the conversion of cellulosic material into substantial amounts of protein without the necessity of adding combined nitrogen. Several observed growth characteristics bear upon potential commercial utilization of this organism to produce single-cell protein. First, the microaerophilic nature of the shipworm bacterium simplifies growth procedures since oxygenation is unnecessary. Second, significant growth requires at least 0.1 M NaCl (Fig. 2), which would have to be removed for the product to be a suitable feedstuff. Third, under nitrogen-fixing conditions the rate and extent of growth are relatively low (Fig. 1), which might be expected since significant metabolic energy must be devoted to the task of reducing nitrogen gas (7). Currently, efforts are under way to increase bacterial production with APWS as a carbon source, as well as to reduce the salinity requirements for growth.

A somewhat unexpected result was that the bacterium favors larger cellodextrins (degree of polymerization > 2) for growth (Fig. 3). Analysis of the residual cellodextrins in the growth media suggested that the bacterium predominantly removed one glucose moiety from the end of the chain (Tables 3 and 4), indicative of exogluconase activity. Since little growth was observed with glucose as the sole carbon source (Fig. 3) and glucose did not significantly accumulate in growth media containing longer cellodextrins as the sole carbon source (Table 3), it seems feasible that the cleavage reaction is coupled to transport of an excised glucose unit, possibly through a phosphorylation mechanism. Phosphorolytic cleavage of cellodextrins has previously been described in *Clostridium thermocellum* (12, 13). However, additional work is necessary to determine if the shipworm bacterium metabolizes cellodextrins in a similar manner to *C. thermocellum*.

TABLE 4. Residual cellodextrins in growth medium as a function of culture age based on 0.1% G_4 as the original carbon source

Culture age (days)	Concn of cellodextrins in medium (%)			
	G_1	G_2	G_3	$G_{\geq 4}$
4	ND[a]	0.011	0.040	0.031
7	ND	0.035	0.051	0.010
10	0.012	0.031	0.012	0.015
14	0.007	0.016	0.010	ND

[a] ND, Not detectable (<0.005%).

Analysis of growth media from cultures grown on 0.5% APWS revealed little accumulation of cellodextrins (<0.005%) (not shown), supporting the premise that the predominant bacterial activity is exoglucolytic. These cultures did, however, accumulate significant quantities of organic acid (Table 2), of which succinic acid was the most prevalent. This is interesting because succinate is an intermediate of the tricarboxylic acid cycle and could be a ready source of energy for the host shipworm. Also, in a truly symbiotic relationship at a biochemical level, the bacterium might depend on the removal of organic acids by the shipworm since their accumulation could lower the pH in the gland of Deshayes to inhibitory levels (Table 1). An analogous situation occurs in ruminant animals where certain cellulolytic bacteria excrete volatile fatty acids, which are in turn easily absorbed by the rumen (8).

The preliminary work presented here on the shipworm bacterium indicates a necessity for further research. The ability of the bacterium to produce protein from cellulosic agricultural residues, such as APWS, needs to be optimized for possible commercial application. This task should be simplified by characterization of the nitrogenase and cellulase systems, as well as the bioenergetic interrelationships which allow these two systems to coexist uniquely in this organism. Lastly, the production of organic acids could be of commercial value by itself or as an intermediate metabolite in a coculture. Investigations designed to determine the feasibility of such application are under way in our laboratory.

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