The glucomannokinase of *Prevotella bryantii* B4 and its potential role in regulating β-glucanase expression

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*Prevotella bryantii* B4 has a transport system for glucose and mannose, but β-glucanase expression is only catabolite-repressed by glucose. *P. bryantii* B4 cell extracts had ATP-dependent gluco- and mannokinase activities, and significant phosphoenolpyruvate- or GTP-dependent hexose phosphorylation was not observed. Mannose inhibited glucose phosphorylation (and vice versa), and activity gels indicated that a single protein was responsible for both activities. Glucose was phosphorylated at a faster rate than was mannose \([V_{\text{max}} 280 \text{ nmol hexose (mg protein)}^{-1} \text{ min}^{-1} \text{ versus } 60 \text{ nmol hexose (mg protein)}^{-1} \text{ min}^{-1}, \text{ respectively}]\) and glucose was a better substrate for the kinase \([K_m 0.12 \text{ mM versus } 1.2 \text{ mM, respectively}]\). The purified glucomannokinase (1250-fold) had a molecular mass of 68 kDa, but SDS-PAGE gels indicated that it was a dimer (monomer 34 kDa). The N-terminus (25 residues) had an 8 amino acid segment that was homologous to other bacterial glucokinases. The glucomannokinase was competitively inhibited by the nonmetabolizable glucose analogue 2-deoxyglucose (2DG), and cells grown with glucose and 2DG had lower rates of glucose consumption than did cells given only glucose. When the ratio of 2DG to glucose was increased, the glucose consumption rate decreased and the β-glucanase activity increased. The glucose consumption rate and the glucomannokinase activity of cells treated with 2DG were highly correlated \([r^2 = 0.98]\). This result suggested that glucomannokinase activity was either directly or indirectly regulating β-glucanase expression.

**Keywords:** rumen bacteria, sugar transport, regulation, catabolite repression

**INTRODUCTION**

Early work indicated that the bacteria now classified as *Prevotella* species are found at high numbers in the rumen (Bryant et al., 1958; Van Gylswyk, 1990), and molecular probes and 16S rDNA clonal libraries support the idea that these bacteria are important members of the rumen microbial ecosystem (Whitford et al., 1998; Wood et al., 1998). *Prevotella bryantii* B4 utilizes a variety of sugars and polysaccharides, and it appears to occupy a relatively broad niche. The β-glucanase andmannanase activities of *P. bryantii* B4 were found to be repressed by glucose but not mannose, but the nature of this regulation was not defined (Gardner et al., 1995).

*P. bryantii* B4 transports glucose and mannose rapidly, and the carriers appear to operate as facilitated diffusion systems at high substrate concentrations (Fields & Russell, 2000). *P. bryantii* B4 lacks phosphotransferase activity (Martin & Russell, 1986), but enzyme assays indicated that cells had ATP-dependent glucokinase activity (Fields & Russell, 2000). The glucokinase was inhibited by mannose, but it was unclear whether the glucokinase was catalysing mannose phosphorylation. Bacterial glucokinases (EC 2.7.1.2) are often glucose specific, but eukaryotes have hexokinases that can phosphorylate glucose and mannose. The mannokinase (EC 2.7.1.7) of *Streptomyces violaceoruber* has a very

**Abbreviations:** 2-DG, 2-deoxyglucose; 2-ME, 2-mercaptoethanol; PEP, phosphoenolpyruvate.

The SWISS-PROT accession number for the sequence of *P. bryantii* B4 glucomannokinase reported in this paper is P82680.
low affinity for glucose and a separate gluco kinase was needed for rapid glucose utilization (Sabater et al., 1972). The mannokinase of Escherichia coli also phosphorylated glucose, but the affinities were 70-fold different for the two sugars (Sebastian & Asensio, 1967).

The following experiments characterized the gluco kinase and mannokinase activities of P. bryantii B4 and were designed to: (1) compare the glucose and mannose kinase activities to see if rates of hexose phosphorylation could explain growth rate differences, (2) determine if both reactions are being catalysed by the same protein, and (3) evaluate the role of the glucose and mannose kinase(s) as potential regulators of catabolite repression.

**METHODS**

**Growth conditions.** Prevotella bryantii B4 was obtained from M. P. Bryant (University of Illinois, Urbana) and has been maintained at Cornell University for 16 years. Prevotella bryantii, formerly Prevotella ruminicola B4, was recently reclassified (Avgustin et al., 1997), but remains in the Cytophaga/Bacteroides/Flexibacter phylum (Shab & Collins, 1990). Cultures were grown anaerobically at 39°C in a basal medium containing salts, 0.5 mg yeast extract ml⁻¹, 1.0 mg Trypticase ml⁻¹, 0.6 mg cysteine hydrochloride ml⁻¹ and volatile fatty acids, as previously described (Fields et al., 1997). Energy sources (glucose and mannose), 2-deoxyglucose (2DG) and iodoacetate were prepared anoxically and sterilized separately. Growth was monitored by measuring optical density (600 nm, 1 cm path length, Gilford Spectrophotometer).

**β-Glucanase activity.** Cells for enzyme assays were harvested in the late-exponential phase of growth, washed twice in potassium phosphate buffer (50 mM, pH 7.0) and PMSF was added to a final concentration of 1 mM. Cells were sonicated (Branson model 200 sonifier, micro-tip, output 5, 50% duty cycle, 0°C, 10 min with intermittent cooling) and cell debris was removed by centrifugation (10000 g, 5°C, 10 min). Cell extracts were stored at −20°C and assayed for β-glucanase activity as previously described using 2% (w/v) carboxymethylcellulose (Fields et al., 1997). Protein concentrations were determined by the Lowry method, using serum albumin as a standard. All assays were performed in duplicate and the variation was less than 10%.

**Gluco kinase and mannokinase activities.** Cells were harvested (500 ml) at the late-exponential phase of growth (160 μg protein ml⁻¹, 7000 g, 10 min, 4°C) and washed once in 100 mM sodium/potassium phosphate buffer (pH 7.2) containing 5 mM MgCl₂ and 1 mM dithiothreitol. Cell pellets were resuspended in 5 ml of the same buffer containing 1 mM PMSF. Cells were sonicated (Branson model 200 sonifier, micro-tip, output 5, 50% duty cycle, 0°C, 10 min with intermittent cooling), the cell debris was removed by centrifugation twice (15000 g, 4°C, 15 min) and the cell extract was collected and stored on ice. Glucose phosphorylation (gluco kinase) in cell-free extracts was determined aerobically using an NADPH-linked assay. The reaction mixture (300 μl) contained 10 mM ATP, phosphoenolpyruvate (PEP) or GTP, 0.8 mM NADP⁺, 2 U glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and differing concentrations of glucose in the same buffer as described above. The glucose-6-phosphate dehydrogenase assay could not detect mannose phosphorylation but mannose 6-phosphate could be converted to fructose 6-}

phosphate if phosphomannosiosemerase (EC 5.3.1.8) was added and the fructose 6-phosphate could be converted to glucose 6-phosphate if phosphoglucosiosemerase (EC 5.3.1.9) was added. Preliminary experiments indicated phosphomannosiosemerase and phosphoglucosiosemerase were in excess if the assay mixture contained 5 U of each enzyme in the 300 μl volume. All assays were performed in duplicate and the variation was less than 10%.

**ATP measurements.** Intracellular ATP concentrations were measured by a luciferin–luciferase method as previously described (Russell & Strobel, 1990). Neutralized cell extract was diluted 30-fold in a buffer containing 40 mM Tris-SO₄ (pH 7.75), 10 mM MgSO₄·7H₂O, 2 mM EDTA and 0.3 mM PEP. Samples were diluted and the luciferase reaction was prepared according to the supplier’s recommendations (Sigma-Aldrich). The reaction was immediately measured on a luminometer (model 1250, LKB Instruments) with ATP used as a standard. The assay was done in triplicate and the variation was less than 10%.

**Thin-layer chromatography.** The phosphorylation of glucose and mannose (ATP- and PEP-dependent) was assayed by thin-layer chromatography using silica gel plates (Merck Art. 5737 silica gel 60 kieselguhr F₂₅₄ pre-coated, 0.25 mm). Samples and sugar standards (1–3 μg hexose) were separated by an n-propanol/ethyl acetate/water mobile phase (7:1:4, by vol). Dried plates were sprayed with an anisaldehyde-based reagent (27 ml ethanol, 0.3 ml acetic acid, 1.5 ml sulphuric acid, 1.5 ml anisaldehyde) and placed at 110°C for 5 min.

**SDS-PAGE, native activity gels and agarose overlays.** PAGE was performed as described by Laemmli (1970). Protein samples were combined with 5 × SDS loading buffer [SDS, 20 g; Tris (1 M, pH 6.8), 80 ml; glycerol, 10 ml; bromphenol blue, 20 mg; distilled H₂O to 100 ml] to achieve a 1 × solution. Samples were boiled for 5 min in the presence of dithiothreitol (50 mM) and loaded onto 8%, 10%, or 12% SDS-polyacrylamide gels.

Native PAGE was performed as described by Laemmli (1970), except gels did not contain SDS or a reducing agent. Polyacrylamide gels (12%) were loaded with similar amounts of protein for glucose or mannose phosphorylation and electrophoresis was performed (4°C, 100 V, 3–4 h). Glucose and mannose phosphorylation was detected with agarose overlays following the protocol of Martinez-Barajas & Randall (1998). Briefly, activity was developed in the dark at 37°C by overlaying the gel with a solution of 25 mM Tris/HCl (pH 8.0), 50 mM KCl, 1 mM ATP, 3 mM MgCl₂, 0.3 mM NAD, 0.6 mM 2-(p-iophenyl)-3-nitrophenyltetrazolium chloride, 6 μM phenazine methosulphate, 12 U glucose-6-phosphate dehydrogenase ml⁻¹, 2% low-melting agarose and 10 mM glucose or mannose. Mannose phosphorylation was detected with the above solution that included 4 U phosphoglucoisomerase ml⁻¹ and 4 U phosphomannosiosemerase ml⁻¹. Glucose phosphorylation was observed within 25 min and mannose phosphorylation was observed within 90 min.

**Purification of the gluco mannokinase.** Cells were grown in basal medium (4 l) with glucose and harvested at late-exponential phase growth (approx. 0.6 g cell protein l⁻¹). The cells were washed twice in buffer (250 mM potassium phosphate, pH 7.0, 5 mM 2-mercaptoethanol (2-ME), 0.2 mM PMSF) and resuspended in the same buffer with the addition of 0.5 mM EDTA. Cells were passed twice through a French pressure cell-press (Y-1517, Spectronic Instruments) at 1000 p.s.i. (6.9 MPa). Cell debris and unbroken cells were removed by centrifugation (10000 g, 10 min) and the super-
The sample was loaded on an octyl-Sepharose column 20% (NH₄)₂SO₄ saturation (Englard & Seifter, 1990), stirred for 1 h at 0°C, centrifuged (16000 g, 30 min) and the supernatant was collected. The sample was brought to 30% (NH₄)₂SO₄ saturation (Englard & Seifter, 1990), stirred for 1 h at 0°C and centrifuged (16000 g, 30 min). The pellet was resuspended in 45 ml potassium phosphate buffer [25 mM, pH 7–0, 5 mM 2-ME, 20% (NH₄)₂SO₄]. All purification steps were performed aerobically at 0–4 °C unless stated otherwise.

The sample was loaded on an octyl-Sepharose column (3 × 11 cm) equilibrated in buffer B (25 mM potassium phosphate, pH 7–0, 5 mM 2-ME) containing 20% (NH₄)₂SO₄. Protein was eluted from the column using a linear gradient from 20% to 0% (NH₄)₂SO₄ (100 ml). The glucomannokinase eluted at approximately 8% (NH₄)₂SO₄. The active fractions were pooled and dialysed overnight against buffer B. The desalted pooled sample was applied to a diethylaminoethyl cellulose column (3 × 13 cm) equilibrated in buffer B. The glucomannokinase eluted at 0.35 M KCl during a linear gradient (0–6 M KCl). The active fractions were pooled and dialysed overnight against buffer C (15 mM potassium phosphate, pH 7–0, 5 mM 2-ME).

The dialysed fraction was applied to a ceramic hydroxyapatite column (3 × 3 cm) and developed with a linear gradient (50 ml) from 15 mM to 200 mM potassium phosphate. The glucomannokinase eluted at approximately 115 mM potassium phosphate. The pooled fractions were concentrated with a 30K centrifugal concentrator (Pall Gelman Lab.) and the buffer was exchanged with buffer B. The concentrated sample was applied to a phosphocellulose column (1 × 10 cm), and the proteins were eluted with a linear gradient from 25 mM to 200 mM potassium phosphate. The glucomannokinase activity eluted at approximately 30 mM potassium phosphate and the pooled fractions were concentrated by centrifugation (30K centrifugal concentrator). The buffer was exchanged with buffer C containing 25 mM NaCl. The concentrated sample was loaded on a Sephacryl S-200-HR column (1 × 20 cm) equilibrated in buffer C containing 25 mM NaCl, and the proteins were eluted with the same buffer (20 ml). The pooled fractions were dialysed overnight against buffer B.

Purity of the enzyme was checked by native and denaturing SDS-PAGE. The denatured molecular mass was determined using SDS-PAGE at 8%, 12% and 15% polyacrylamide concentrations. The native molecular mass was determined using size exclusion chromatography. The N-terminal sequence of the purified glucomannokinase (two independent preparations) was determined using Edman degradation on a PE/Applied Biosystems Procise 492 protein sequencer by the Cornell Bioresource Center. Samples were electroblotted on a polyvinylidene difluoride membrane prior to analysis. The P. bryantii B₄ glucomannokinase N-terminal sequence reported in this paper will appear in the SWISS-PROT Protein Database under accession number P82680.

Preliminary Porphyromonas gingivalis sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org. The other glucokinase sequences were obtained from protein databases and the accession numbers are as follows: Streptomyces coelicolor [P40184] (Angell et al., 1992), Zymomonas mobilis [D37855] (Barnell et al., 1990), Brucella abortus [Q59171] (Essenberg, 1995), Bacillus subtilis [PS4495], Haemophilus influenzae [AAC21816] and Staphylococcus xylosus [Q56198] (Wagner et al., 1995). Database searches for identity and similarity and sequence comparisons were performed using BLAST (Altschul et al., 1990; Gish & States, 1993).

Reagents. All chemicals were analytical reagent grade. Hexokinase, glucose-6-phosphate dehydrogenase, phosphomannomutase, phosphoglucomutase, streptomycin sulphate, ammonium sulphate, Sephacryl S-200 HR, octyl-Sepharose CL-4B and luciferin–luciferase extract were purchased from Sigma-Aldrich. Ceramic hydroxyapatite (type 1, 20 μM) was purchased from Bio-Rad and diethylaminoethyl cellulose (DE52) and phospho-cellulose (P-11) were purchased from Whatman International. [¹⁴C]Mannose was from American Radiolabeled Chemicals.

RESULTS

Glucose and mannose phosphorylation

When P. bryantii B₄ cell extracts were incubated with glucose or mannose and PEP, phosphorylated intermediates were not detected (Fig. 1). Phosphorylated intermediates were observed if ATP was the phosphoryl donor and very little unphosphorylated sugar remained (Fig. 1). The glucose and mannose phosphorylation rates were 10-fold lower if GTP was the phosphoryl donor (data not shown).

Glucose phosphorylation could also be measured with an enzyme-linked assay that used NADP⁺, ATP and glucose-6-phosphate dehydrogenase. Mannose phosphorylation could not be detected enzymically until

![Fig. 1. Detection of phosphorylated intermediates by thin-layer chromatography of P. bryantii B₄ cell extracts incubated with glucose or mannose and PEP or ATP. Lanes: A, glucose; B, mannose; C, glucose-6-phosphate; D, fructose 1,6-bisphosphate; E, glucose + PEP; F, mannose + PEP; G, glucose + ATP; H, mannose + ATP.](image-url)
phosphoglucoisomerase and phosphomannoisomerase were added, but these enzymes could be added in excess. These latter assays indicated that both sugars were phosphorylated, but glucose was phosphorylated at a faster rate. Lineweaver–Burk plots indicated that the $K_m$ and $V_{max}$ values for glucose and mannose phosphorylation were approximately 0·12 mM and 280 nmol hexose (mg protein)$^{-1}$ min$^{-1}$ and 1·2 mM and 60 nmol hexose (mg protein)$^{-1}$ min$^{-1}$, respectively.

The $^{[14]}$C-glucose phosphorylation was not significantly inhibited by a 10-fold excess of unlabelled glucose 6-phosphate, but was inhibited by unlabelled mannose (Fields & Russell, 2000). $^{[14]}$C-Mannose phosphorylation could be strongly inhibited by unlabelled glucose and a 1:1 ratio of unlabelled glucose to $^{[14]}$C-mannose caused more than a 50% reduction in the mannose phosphorylation rate (Fig. 2). The $^{[14]}$C-mannose phosphorylation rate was nearly undetectable when there was a fourfold excess of unlabelled glucose.

Sugar phosphorylation activity could be detected with agarose overlays that contained NADP$^+$, ATP, glucose-6-phosphate dehydrogenase, phosphoglucoisomerase, phosphomannoisomerase and phenazine methosulphate as a detection system. When the cell extracts were separated using native PAGE the glucose and mannose phosphorylation activities co-migrated and only a single activity band was observed in each case (Fig. 3).

**Purification of the glucomannokinase**

The glucomannokinase of glucose-grown cells was purified 1250-fold by monitoring glucokinase activity (Table 1). The final fraction also had mannokinase activity and the rate of mannose phosphorylation was approximately fourfold lower than the glucose phosphorylation rate. SDS-PAGE indicated that the protein had an apparent molecular mass of 34·5 kDa (Fig. 4), but size-exclusion chromatography indicated that the native protein had an apparent molecular mass of 68·0 kDa (not shown). The first 25 N-terminal amino acids were MNEQSMKPYVGLDLGGSVESVFGIV. The sequence had high identity (56%) and high similarity (74%) with an unannotated *Porphyromonas gingivalis* putative amino acid sequence (Fig. 5a). The same segment of the *P. gingivalis* genome has an open reading frame with significant homology to other known glucokinases (30% identity and 45% similarity), as well as transcriptional regulators (25% identity and 40% similarity) belonging to the ROK protein family. A segment of the *P. bryantii* B4 N-terminal glucomannokinase sequence (approx. 8 amino acids) was also homologous to other glucokinase sequences from bacteria, including *Staphylococcus xylosus*, *Haemophilus influenzae*, *Streptomyces coelicolor*, *Zymomonas mobilis* and *Bacillus subtilis* (Fig. 5b).

**Role of glucomannokinase in catabolite repression**

*P. bryantii* B4 cultures grew faster on glucose than on mannose and the hexose consumption was also greater (Table 2). Mannose-grown cells had high $\beta$-glucanase activity, but glucose repressed $\beta$-glucanase activity. When cells were given glucose and the glycolytic inhibitor iodoacetate, the growth rate and hexose consumption rate were twofold lower, but $\beta$-glucanase expression was still repressed (Table 2). If cells growing on mannose were treated with iodoacetate, $\beta$-glucanase activity was still high. Cells grown with glucose and 2-deoxyglucose, a non-metabolizable glucose analogue,
Table 1. Purification steps for the *P. bryantii* B14 glucomannokinase

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Sp. act.*</th>
<th>Purification factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>65</td>
<td>0.03</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>AS† precipitation</td>
<td>45</td>
<td>0.30</td>
<td>9</td>
<td>63</td>
</tr>
<tr>
<td>Octyl-Sepharose</td>
<td>40</td>
<td>0.96</td>
<td>32</td>
<td>45</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>25</td>
<td>3.92</td>
<td>126</td>
<td>41</td>
</tr>
<tr>
<td>Hydroxypatite</td>
<td>12</td>
<td>22.4</td>
<td>748</td>
<td>40</td>
</tr>
<tr>
<td>Sephacryl H-200-SR</td>
<td>2.7</td>
<td>23.5</td>
<td>757</td>
<td>19</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>1.5</td>
<td>38.7</td>
<td>1249</td>
<td>4.9</td>
</tr>
</tbody>
</table>

* Units are µmol glucose (mg protein)^-1 min^-1.
† Ammonium sulphate (53% pellet).

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had slower rates of growth and glucose consumption and the β-glucanase activity was nearly as high as for mannose-grown cells (Table 2).

The decrease in the glucose consumption rate caused by 2DG was dose-dependent. When the ratio of 2DG to glucose increased, the hexose consumption rate decreased gradually (Fig. 6a), but there was little increase in β-glucanase activity until the glucose consumption rate was less than 275 nmol hexose (mg protein)^-1 h^-1 (Fig. 6b). Once the hexose consumption rate was less than 150 nmol hexose (mg protein)^-1 min^-1, there was little further increase in β-glucanase activity. The glucomannokinase activity was also inhibited by 2DG and the glucose consumption rate and

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Table 2. Growth rate, hexose consumption rate (q^hexose^), β-glucanase activity and intracellular ATP concentration of cells grown with different substrates and inhibitors

<table>
<thead>
<tr>
<th>Growth with</th>
<th>μ (h^-1)</th>
<th>q^hexose^</th>
<th>β-Glucanase</th>
<th>ATP</th>
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<tr>
<td>Glucose</td>
<td>0.70</td>
<td>25</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.45</td>
<td>15</td>
<td>300</td>
<td>13</td>
</tr>
<tr>
<td>Glucose+IAA</td>
<td>0.33</td>
<td>14</td>
<td>45</td>
<td>1.3</td>
</tr>
<tr>
<td>Mannose+IAA</td>
<td>0.31</td>
<td>12</td>
<td>260</td>
<td>1.5</td>
</tr>
<tr>
<td>Glucose+2DG</td>
<td>0.32</td>
<td>13</td>
<td>270</td>
<td>6.4</td>
</tr>
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</table>

Cultures were incubated with 5.5 mM glucose or mannose. Hexose consumption rate was calculated as µmol hexose (mg protein)^-1 h^-1. Some cultures were treated with 2,4-dinitrophenol (2DG; 12.2 mM). β-Glucanase activity is expressed as nmol hexose (mg protein)^-1 min^-1 and ATP concentration as nmol (mg protein)^-1.

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Fig. 4. SDS-PAGE gel showing the purification steps of the *P. bryantii* B14 glucomannokinase. An equal amount of protein was loaded from each fraction: octyl-Sepharose (A), DEAE-cellulose (B), hydroxyapatite (C), Sephacryl S-200-HR (D) and phosphocellulose (E). The protein standards were as follows (kDa): phosphorylase b (97), bovine serum albumin (68), ovalbumin (43), carbonic anhydrase (29), β-lactoglobulin (18) and lysozyme (14).

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Fig. 5. Amino acid sequence comparison of the *P. bryantii* B14 glucomannokinase N-terminus with an unannotated Porphyromonas gingivalis ORF (a) and with other bacterial glucokinases (b) from sequence databases. Identical residues are shown in bold and similar residues are underlined. The accession numbers are as follows: *Porphyromonas gingivalis* W83 DNA sequence 1823057–1825113 (http://www.tigr.org), *Staphylococcus xylosus* [Q56198], *Haemophilus influenzae* [AAC21816], *Streptomyces coelicolor* [P40184], *Bacillus subtilis* [P54184] and *Zymomonas mobilis* [D37855].

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Gram-positive bacteria, but the nature of this regulation causes inducer (lactose) exclusion. The phosphorylation of lactose enzyme IIA, and competition for HPr is preferred, but cAMP is not a transcriptional regulator. (Postma et al., 1997). Glucose and mannose were, however, utilized simultaneously and P. bryantii B4 had a glucose/mannose carrier (Fields & Russell, 2000). P. bryantii B4 also had an alternative glucose carrier that was induced by high concentrations of glucose, but mutants deficient in the alternative glucose carrier still repressed β-glucanase expression when glucose was available (Fields & Russell, 2000). Because P. bryantii B4 does not have phosphotransferase system activity (Martin & Russell, 1986) and has very low concentrations of cAMP (Cotta et al., 1994), it appeared that previously defined catabolite regulatory mechanisms could not explain the regulation of β-glucanase in P. bryantii B4.

P. bryantii B4 cells that were grown on glucose had higher rates of hexose consumption than mannose-grown cells. However, previous work indicated that the glucose/mannose carrier operated as a facilitated diffusion system, and both sugars were transported rapidly if the substrate concentration was high (Fields & Russell, 2000). Glucose- and mannose-grown cells had similar ATP concentrations, and the glycolytic inhibitor iodoacetate decreased the ATP concentration and hexose consumption rate of cells grown on either sugar. However, in the presence of iodoacetate, β-glucanase activity was still expressed by mannose-grown cells and repressed by glucose-grown cells. Based on these results, the β-glucanase regulation of P. bryantii B4 could not be explained by transport activity or ATP availability.

Thin-layer chromatography indicated that P. bryantii B4 cell-free extracts could phosphorylate either glucose or mannose and that these activities were constitutive. The thin-layer assays did not indicate the position of phosphorylation, but enzyme assays indicated that the phosphorylated derivatives could eventually be converted to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. Mannose was not converted to 6-phosphogluconate until phosphoglucoisomerase and phosphomannoisomerase were added. This observation indicated that mannose was phosphorylated in the number 6 position, but cell-free extracts lacked these latter activities. Because the rate of glucose conversion to 6-phosphogluconate was always greater than mannose phosphorylation even if phosphoglucoisomerase and phosphomannoisomerase were in excess, P. bryantii B4 appeared to have greater glucokinase than manno-kinase activity.

The glucose consumption rate of P. bryantii B4 could be decreased by the non-metabolizable glucose analogue, 2DG, and 2DG alleviated the glucose-dependent repression of β-glucanase. Because 2DG was a competitive inhibitor of glucose phosphorylation, it appeared that the kinase reaction was regulating β-glucanase expression. Previous work indicated that mannose could inhibit the ‘glucokinase’ activity of P.

**DISCUSSION**

Enteric proteobacteria grow faster on glucose than lactose, and lactose is not utilized until the glucose is depleted (Magasanik, 1976). When glucose is available, cAMP concentrations are low, the cAMP-catabolite receptor protein cannot bind DNA, and the lactose permease gene is not transcribed (Peterkofsky, 1976). In Gram-positive lactic acid bacteria, glucose is also preferred, but cAMP is not a transcriptional regulator (Postma et al., 1993; Romano et al., 1979). Glucose enzyme IIα of the phosphotransferase system has a higher affinity for phospho-HPr (Postma et al., 1993) than lactose enzyme IIA, and competition for HPr causes inducer (lactose) exclusion. The phosphorylation state of HPr can also regulate transcription in some Gram-positive bacteria, but the nature of this regulation is not completely understood (Jones et al., 1997; Saier et al., 1996).

Previous work indicated that P. bryantii B4 grew faster on glucose than mannose, and only glucose repressed β-glucanase expression. Glucose and mannose were, however, utilized simultaneously and P. bryantii B4 had a glucose/mannose carrier (Fields & Russell, 2000). P. bryantii B4 also had an alternative glucose carrier that was induced by high concentrations of glucose, but mutants deficient in the alternative glucose carrier still repressed β-glucanase expression when glucose was available (Fields & Russell, 2000). Because P. bryantii B4 does not have phosphotransferase system activity (Martin & Russell, 1986) and has very low concentrations of cAMP (Cotta et al., 1994), it appeared that previously defined catabolite regulatory mechanisms could not explain the regulation of β-glucanase in P. bryantii B4.

**Fig. 6.** Relationship between (a) hexose consumption rate and increasing ratios of 2DG to glucose, and (b) β-glucanase activity and hexose consumption rate of P. bryantii B4 cells that were treated with 2DG.
bryantii B4 (Fields & Russell, 2000) and the current study showed that [14C]mannose phosphorylation was inhibited by unlabelled glucose. Activity gels indicated that the gluco- and mannokinase activities from B4 cell extracts co-migrated and the purified glucomannokinase phosphorylated only glucose and mannose. The glucomannokinase of P. bryantii B4 was more active when glucose was the substrate compared to mannose, and this observation supported the idea that sugar phosphorylation was regulating β-glucanase expression.

Staphylococcus xylosus has a glucose-dependent mechanism that represses β-galactosidase activity and six other enzymes (Wagner et al., 1995). S. xylosus has a glucose phosphotransferase system, but genetic studies indicated that repression was dependent upon a regulatory glucokinase (Wagner et al., 1995). When the regulatory glucokinase was inactivated, a redundant glucokinase was employed, but the β-galactosidase and other measured activities were not repressed (Wagner et al., 1995). Studies with Streptomyces coelicolor also indicated that glucokinase mutants still grew on glucose, but these mutants no longer repressed glycerol kinase and agarase activities (Kwakman & Postma, 1994).

Glucokinases generally have a lower affinity for glucose than hexokinases (Cardenas et al., 1969), glucokinases are smaller than hexokinases (24–35 kDa versus 50–100 kDa) (Cardenas et al., 1998), and hexokinases are inhibited by glucose 6-phosphate (Barman, 1969). The purified P. bryantii B4 glucomannokinase had a Km for glucose of 120 μM and it was not inhibited by glucose 6-phosphate. Most glucokinases are not sensitive to oxygen (Barman, 1969), and P. bryantii cell-free extracts that were assayed aerobically had rates of glucose phosphorylation that were similar to the glucose consumption of whole cells [0.28 nmol (mg protein)−1 min−1 versus 0.40 nmol (mg protein)−1 min−1]. SDS-PAGE indicated that the P. bryantii B4 glucomannokinase had an apparent molecular mass of 34.5 kDa, but size-exclusion chromatography indicated that the native protein was a dimer (approx. 68 kDa).

Glucokinases are often specific for glucose but E. coli has a low-affinity ‘glucokinase’ that is able to phosphorylate glucose as well as mannose (Fukuda et al., 1984). E. coli also has a mannokinase, and this enzyme has a 70-fold higher affinity for mannose than glucose (Sebastian & Asensio, 1967). Sabater et al. (1972) indicated that Streptomyces violaceoruber had a ‘mannoglucokinase’, but this enzyme had an 80-fold lower affinity for glucose than mannose and a different glucokinase was necessary for rapid glucose utilization. The P. bryantii B4 glucomannokinase had only a 10-fold higher affinity for glucose than mannose, and activity gels indicated that there was no other enzyme catalysing glucose or mannose phosphorylation. These results suggested that the B4 glucomannokinase was a novel sugar kinase specific for glucose and mannose and was the only mechanism for glucose and mannose catabolism in this micro-organism.

Glucokinases and hexokinases utilize ATP as a phosphoryl donor, but Glass & Sherwood (1994) indicated that the ruminal bacterium Fibrobacter succinogenes had a glucokinase that was coupled to GTP rather than ATP. The P. bryantii B4 glucomannokinase utilized ATP as a phosphoryl donor and significant amounts of phosphorylated derivatives could not be detected when GTP or PEP was added. Hexokinases phosphorylate 2DG as well as glucose and mannose, but most glucokinases cannot phosphorylate 2DG (Romano et al., 1979). The ruminal bacterium Selenomonas ruminantium has a low-molecular-mass glucokinase that was not inhibited by glucose 6-phosphate, but this enzyme had an ATP-dependent 2DG phosphorylation activity. 2DG was a competitive inhibitor of the P. bryantii B4 glucomannokinase but little 2DG phosphorylation was detected.

We have not yet cloned and sequenced the entire P. bryantii B4 glucomannokinase gene, but the N-terminal sequence (25 residues) had homology (44% and 36% similarity, respectively) with the N termini of the Streptomyces coelicolor and Staphylococcus xylosus regulatory glucokinases. The N-terminal sequence of the P. bryantii B4 glucomannokinase also shared a common sequence [D(I/L)GGT] with other glucokinase sequences from bacteria including Bacillus subtilis, Haemophilus influenzae, Escherichia coli and Zymomonas mobilis, and the sequence is most probably the ATP-binding motif (Spath et al., 1997). 16S rDNA sequencing indicates that Porphyromonas gingivalis and Prevotella bryantii B4 are closely related (approx. 20% difference) and the N-terminal sequence of the B4 glucomannokinase had 56% identity and 74% similarity with a Porphyromonas gingivalis putative amino acid sequence. The segment of the P. gingivalis putative amino acid sequence that had homology with the B4 N terminus also contained an open reading frame that had significant homology with the regulatory glucokinases of Streptomyces coelicolor and Staphylococcus xylosus (29% identity, 47% similarity). The P. gingivalis putative glucokinase also had significant homology with transcriptional regulators from a variety of micro-organisms and appeared to belong to the ROK protein family proposed by Titgemeyer et al. (1994).

Previous work indicated that genes can be moved into P. bryantii B4 via a two-step conjugation process (Shoemaker et al., 1991), but this system has not been modified to allow transposon mutagenesis. The study of
β-glucanase regulation in *P. bryantii* B4 is likewise complicated by the observation that there seems to be only one mechanism of glucose and mannose phosphorylation. Further work will be needed to identify the protein(s) involved in catabolite regulation of *P. bryantii* B4, but results indicated that the glucosaminokinase was either directly or indirectly involved. Hexose flux via the glucosaminokinase appeared to be a signal, but how the signal is sensed has yet to be determined. In yeast, glucose and ATP binding causes a conformational change in the hexokinase (Bennett & Steitz, 1978); the B4 glucosaminokinase could undergo a similar change and might function as a co-regulator. A different model would involve a second regulatory protein that might form homo- or heterodimers with the glucosaminokinase or be covalently modified. Preliminary experiments indicate that β-glucanase activity is transcriptionally regulated and that neither fructose 1,6-bisphosphate nor glucose 6-phosphate is a signal.

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