The Chitin Disaccharide, \( \text{N}, \text{N}' \)-diacetylchitobiose, Is Catabolized by Escherichia coli and Is Transported/Phosphorylated by the Phosphoenolpyruvate:Glycose Phosphotransferase System

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We have previously reported that wild type strains of Escherichia coli grow on the chitin disaccharide \( \text{N}, \text{N}' \)-diacetylchitobiose, \((\text{GlcNAc})_2\), as the sole source of carbon (Keyhani, N. O., and Roseman, S. (1997) Proc. Natl. Acad. Sci., U. S. A. 94, 14367–14371). A nonhydrolyzable analogue of \((\text{GlcNAc})_2\), methyl \( \beta-N', \text{N}'-\text{[H]} \text{diacetylthiochitobiose} \) was used to characterize the disaccharide transport process, which was found to be mediated by the phosphoenolpyruvate:glycose phosphotransferase system (PTS). Here and in the accompanying papers (Keyhani, N. O., Bouddker, O., and Roseman, S. (2000) J. Biol. Chem. 275, 33091–33101; Keyhani, N. O., Bacia, K., and Roseman, S. (2000) J. Biol. Chem. 275, 33102–33109; Keyhani, N. O., Rodgers, M., Demeler, B., Hansen, J., and Roseman, S. (2000) J. Biol. Chem. 275, 33110–33115), we report that transport of \([\text{H}]\text{Me-TCB}\) and \((\text{GlcNAc})_2\) involves a specific PTS Enzyme II complex, requires Enzyme I and HPr of the PTS, and results in the accumulation of the sugar derivative as a phosphate ester. The phosphoryl group is linked to the C-6 position of the GlcNAc residue at the nonreducing end of the disaccharide. The \([\text{H}]\text{Me-TCB}\) uptake system was induced only by \((\text{GlcNAc})_2\), \(n = 2\) or 3. The apparent \(K_m\) of transport was \(50–100 \mu\text{M}\), and effective inhibitors of uptake included \((\text{GlcNAc})_2\), \(n = 2\) or 3, cellobiose, and other PTS sugars, i.e. glucose and GlcNAc. Presumably the PTS sugars inhibit by competing for PTS components. Kinetic properties of the transport system are described.

We have reported that wild type Escherichia coli and a mutant unable to transport GlcNAc can utilize the chitin disaccharide, \(\text{N}, \text{N}' \)-diacetylchitobiose or \((\text{GlcNAc})_2\), as the sole source of carbon for growth (6).\(^2\) An E. coli transposon mutant was selected that was unable to grow on \((\text{GlcNAc})_2\), but behaved normally on GlcNAc, and the mutant was used to clone the \((\text{GlcNAc})_2\) catabolic operon. Sequence analysis of the genes in the operon compared with the sequence of the complete E. coli genome (7) showed that the \((\text{GlcNAc})_2\) operon corresponded to the previously described "cryptic" cellobiose operon (8, 9). In the preliminary report (6) we demonstrated that the wild type allele of this operon encoded for \((\text{GlcNAc})_2\) but not cellobiose utilization; the cel genes were therefore renamed and constitutive the chb \((\text{N}, \text{N}' -\text{diacetylchito-biose})\) operon.

In earlier work (10), a \((\text{GlcNAc})_2\) permease was described in the Gram-negative, chitinolytic marine bacterium Vibrio furnissii. For these experiments, we employed a nonhydrolyzable \((\text{GlcNAc})_2\) analogue, methyl \( \beta-N', \text{N}'-\text{[H]} \text{diacetylthiochitobiose} \) \(([\text{H}]\text{Me-TCB})\). In V. furnissii, the transport product was characterized as unmodified \([\text{H}]\text{Me-TCB}\), and its apparent \(K_m\) for uptake was \(<1 \mu\text{M}\).

In the present studies, we show that transport of \((\text{GlcNAc})_2\) \(([\text{H}]\text{Me-TCB})\) in E. coli is mediated via the phosphoenolpyruvate:glycose phosphotransferase system (PTS), with the sugar accumulated as its phosphorylated derivative. The phosphoryl group is linked to the C-6 position of the nonreducing GlcNAc. In E. coli, the apparent \(K_m\) for \(([\text{H}]\text{Me-TCB})\) uptake is 50–100 \(\mu\text{M}\). Thus, the chitin disaccharide, \((\text{GlcNAc})_2\), is transported by different mechanisms in these two closely related organisms. This is in sharp contrast to other sugars, as discussed below.

EXPERIMENTAL PROCEDURES

Materials—Reagents for bacterial media were obtained from Difco Laboratories (Detroit, MI), J. T. Baker (Phillipsburg, N.J.), and BBL Microbiology Systems (Cockeysville, MD). Molecular biology reagents were obtained from New England Biolabs (Beverly, MA), U.S. Biochemical Corp. (Cleveland, OH), and Stratagene (La Jolla, CA). Radioisotopes were purchased from Life Science Products. Whatman GF/F glass microfiber filters, and thin layer chromatography plates (Silica-Gel 60) were purchased from EM Science (Cherry Hill, NJ). (GlcNAc)_2 was prepared as described (11) or by modifications to be described elsewhere. Other buffers and reagents were of the highest purity commercially available. PTS proteins, such as homogenous Enzyme I, HPr, and IIA^{chb} were kind gifts from Drs. Norman Meadow, Regina Svatchova, and Roshan Mattoo.

Bacterial Strains—E. coli strain XL1-Blue MR (ΔmerC;hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lacI^q) was purchased from Stratagene. E. coli strains were grown at 37°C in LB or on Luria Agar plates supplemented with ampicillin (50 \(\mu\text{g/ml}\)) and/or tetracycline (15 \(\mu\text{g/ml}\)) where appropriate for selection of recombinant E. coli cells. Alternately, cells were grown on minimal media (M9 salt) supplemented with 0.5 mM thiamine, 0.01% casamino acid.

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1 The abbreviations used are: (GlcNAc)_2, \(\beta-N', \text{N}'-\text{[H]} \text{diacetylthiochitobiose}, \text{Me-TCB}, \text{PTS}, \text{PEP}, \text{HPr}
2 The subject matter of the accompanying manuscripts is as follows: characterization of IIA^{chb} from E. coli (1); characterization of phospho-IIB^{chb} and of a potential transition state analogue in the phosphotrans-
acids, a carbon source, typically 0.25% lactate (lactate-minimal media) and further supplemented as indicated. Fermentation was assayed by using Difco MacConkey Agar Base supplemented with the indicated carbon source, typically at a concentration of 10 mM. Plates were incubated at 37 °C for 15–20 h before individual colonies were scored as positive (*) or negative (white).

Transport Experiments—E. coli was grown overnight in LB and diluted 50-fold in lactate-M9 salts minimal media supplemented with 0.6 mM (GlcNAC)₂ (for induction) or as indicated. Cells were grown at 37 °C with aeration to an absorbance at 600 nm of 0.8–1.2, washed three times at 4 °C with equal volumes of M9 salt medium, and finally resuspended in M9 salt using 1/3 to 1/2 volume of fresh medium. The suspension was stored on ice and maintained at room temperature for 15 min prior to use. Transport experiments were conducted no later than 2 h after harvesting and washing of the cells. Uptake was initiated by adding an equal volume of cell suspension to [³H]-Me-TCB or other substrate (1–5,000 cpm/nmol) dissolved in M9 salts and aerated by shaking (150 rpm, 37 °C). Aliquots (0.1 ml) were taken at the indicated times, rapidly mixed with 10 ml of wash buffer (M9 salts) at room temperature and filtered through Whatman GF/F glass microfiber filters. After washing with an additional 10 ml of buffer, the cells on the filter were solubilized with Packard Soluene-350 and counted in a Packard Liquid Scintillation Spectrometer. Although aliquots from a given cell suspension gave excellent replicates, the cell preparations varied as much as 6-fold with respect to nmol [³H]-Me-TCB taken up per mg protein per min. Conceivably this variability resulted from different rates of catabolism of the inducer, (GlcNAC)₂, from one cell culture to another.

Assay for Intracellular Products of Transport—Transport experiments were performed as described above with the following modifications. At each time point, the glass microfiber filters, containing the harvested, washed cells, were immediately removed from the filtration apparatus and immersed in 2.0 ml of 70% EtOH and boiled for 3 min. The resulting cell extract was centrifuged (15,000 × g, 10 min) to remove cell and filter debris, and the quantity of sugar phosphate determined by anion exchange chromatography (12). Briefly, samples (0.5 ml) were transferred to columns containing Dowex-1 AG-X8 resin (1 ml), chloride form, the columns were washed thrice with 1 ml of water each, and sugar phosphate was eluted with 0.6-ml aliquots (three times) of 1.0 M NaCl. Typically, both the flow through/wash fractions and the salt eluate were collected in scintillation vials containing 3.0 ml of HIONIC fluor (Packard), and the samples were counted using a Packard Liquid Scintillation Spectrometer.

Toluidinedi-ziedi—E. coli was grown overnight in LB and diluted 50-fold in lactate-M9 salts media supplemented with 0.6 mM (GlcNAC)₂ (for induction) or as indicated. Cells were grown at 37 °C with aeration to an A₆₀₀ = 0.8–1.0, washed three times at 4 °C with equal volumes of toluenization buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 0.5% NaCl), and resuspended using 1.0 ml of toluenization buffer/0.2 gm of cells (wet weight). Toluenization was performed as described (13). Briefly, samples (0.5 ml) were transferred to columns containing Dowex-1 AG-X8 resin (1 ml), chloride form, and sugar phosphate was eluted with 0.6-ml aliquots (three times) of 1.0 M NaCl. Typically, both the flow through/wash fractions and the salt eluate were collected in scintillation vials containing 3.0 ml of HIONIC fluor (Packard), and the samples were counted using a Packard Liquid Scintillation Spectrometer.

RESULTS

(GlcNAC)₂ is actively catabolized by both V. furnissii and E. coli and cannot be used to obtain accurate kinetic results in transport experiments. To circumvent this problem, nonmetabolizable analogues were employed to characterize a (GlcNAC)₂ transport system in the marine bacterium V. furnissii (10). Similar experiments were conducted in the present studies on the E. coli transporter. The substrates contained [¹⁴C]N-acetyl groups, and were methyl β-N,N-diacyltelitobiose Me-TCB and methyl α-N,N,N-triacyltelitoctosiode (Me-TCT).

Three E. coli strains (6) were employed in these studies: (a) wild type E. coli strain XL1-Blue MR, (b) strain Xn1.4, a transposon insertion mutant of XL1-Blue incapable of utilizing (GlcNAC)₂, and (c) Xn1.4:pES1, the mutant strain harboring a plasmid containing a 7.3-kilobase E. coli genomic fragment that spans the entire chb operon. The transposon was found to be inserted in the chbC gene (the membrane protein) by polymerase chain reaction analysis (data not shown).

In Vitro PTS Assay—The PTS was reconstituted in vitro with purified components: Enzyme I, HPr, IIAChb and IIBChb (see accompanying paper (1)), and high speed membrane fractions derived from the indicated strains. Membranes were prepared as follows. E. coli was grown overnight in LB and diluted 50-fold in lactate-M9 salts media supplemented with 0.6 mM (GlcNAC)₂ (induced) or as indicated. Cells were grown at 37 °C with aeration to an A₆₀₀ = 0.8–1.0, washed three times at 4 °C with 50% v/v lactate-phosphate buffer, pH 7.5, containing 50 mM NaCl, and resuspended using 1.0 ml buffer/4 ml/g wet cells (wet weight). Cells were lysed by passage through a French pressure cell (two or three times), and unlysed cells and cell debris were removed by low speed centrifugation (10,000 × g, 10 min). The membrane fraction was isolated by high speed centrifugation (150,000 × g, 1 h). The supernatant was poured off, and the remaining liquid was removed using a cotton tip applicator. A membrane suspension was obtained by resuspending the pellet in 1% of the original culture volume; the solution contained 50 mM sodium phosphate buffer, pH 7.5, and 50 mM NaCl. Membranes were not washed unless otherwise indicated.

PTS assay mixtures (50 µl) contained 5 mM PEP (or ATP as a control), 50 µM Tris-HCl buffer, pH 8.0, 5 mM MgCl₂, 10 mM KF, 0.5 mM dithiothreitol, 1 mM indicated radioactive substrate (specific activity, 10⁵ cpm/mmol), 3–5 units (1–2 µg) of Enzyme I, 5–10 µg of HPr, and 0.5–2.5 µg of IIAChb. The membranes contained endogenous IIBChb, but in some experiments (e.g. Fig. 5), 5 µg of homogeneous IIBChb were added to increase the rate of sugar phosphorylation. Reactions were initiated by the addition of membranes (4–9 µl), 20–40 µg of protein (as determined by the Lowry method (14) or by the Bradford Coomassie dye binding assay (15)). Preparation of Phospho-Me-TCB—The PTS assay reaction mixture described above was increased 100-fold (5 ml of total reaction volume) using the same final concentrations of reactants and with 5 µg [³H]-Me-TCB as substrate (specific activity, 10⁵ cpm/mg). The reaction was initiated by adding membranes and incubated at 37 °C for 12 h, after which 5 ml of 95% EtOH was added and the reaction was stopped by boiling for 5 min. Precipitated material was removed by centrifugation; the supernatant contained >95% of the ³H-counts/min that was transferred into a Dowex AGX18-H⁺ resin column (25 ml, bicarbonate form), the column was washed with water, and the sugar phosphate was purified by eluting with a 0–1 M gradient of triethylammonium bicarbonate (500 mM), pH 7.6. The fractions containing the labeled material were pooled, concentrated repeatedly with water to remove the buffer, and applied to a Sephadex G-15 column (45 × 1.2 cm). The final product exhibited a single band on TLC. Approximately 60% of the starting material sugar was converted to the sugar phosphate. Phospho-(GlcNAC)₂ was isolated by the same procedure, starting with (GlcNAC)₃.

NMR Analyses—¹H and ³¹P NMR spectra were recorded at 25 °C with a Bruker AMX-300 spectrometer.

Mercuric Acetate Hydrolysis—The thioglycosidic bond of (phospho)Me-TCB was hydrolyzed using mercuric acetate (16) at room temperature. Mixtures contained 10 nmol of thiglycoside and 25 nmol of mercuric acetate at 0.02 M acetic acid. Mercuric ion was removed by Dowex 50 AGX8 (H⁻ form), and the product was measured by high performance anion exchange chromatography on an system consisting of a Bio-LC (Dionex Corp., Sunnyvale, CA), and a Dionex CarboPac PA-1 column (4 × 250 mm) (17).
NAc, maltose, lactose, melibiose, trehalose, cellobiose, salicin, and arbutin. Only (GlcNAc)$_2$, (GlcNAc)$_3$, and Me-TCB were inducers of the transport system, whereas higher chitin oligosaccharides, $(\text{GlcNAC})_n$, $n = 5–6$, did not act as inducers.

The effect of concentration of (GlcNAc)$_2$ on induction of the transporter is shown in Fig. 1B. It is interesting to note, although probably coincidental, that about 0.6 mM is the optimal concentration of disaccharide for inducing the transport systems in both $E. coli$ and $V. furnissii$, although these systems function by different mechanisms. With 0.6 mM (GlcNAc)$_2$ as inducer, $E. coli$ cells expressed the transport system after 30 min of incubation at 37 °C. The effect of concentration on induction by (GlcNAc)$_3$ and by Me-TCB were not tested, but these compounds were about as effective as (GlcNAc)$_2$ between 0.5 and 1 mM.

The mutant strain Xm1.4 is incapable of transporting either analogue under any conditions tested, whereas the complemented strain, Xm1.4:pCBU7.3, displayed the inducible transport system. The rate of transport in the complemented mutant was greater than wild type, both for Me-TCB and Me-TCT, which probably reflects the multiple copy number of the plasmid when compared with the single copy of the gene in the wild type cell.

**Kinetics of Me-TCB and Me-TCT Transport**—Kinetic studies on the transport of the labeled analogues were conducted with two $E. coli$ strains, XL1-Blue (wild type) and Xm1.4:pCBU7.3. Initial uptake rates were determined as described under “Experimental Procedures,” with the first time point taken at 6 s after mixing the labeled analogue with the cell suspension. The effects of substrate concentrations on the uptake rates are shown in Fig. 2. The data were analyzed by Woolf-Augustinsson-Hofstee plots ($v$ versus $v/S$; data not shown) to obtain the corresponding $K_m$ and $V_{max}$ values. A
summary of the calculated apparent kinetic constants of transport is given in Table I. The $K_m$ for the disaccharide in both the wild type and Xm1.4:pES1 strains were similar (50–100 μM), whereas the $V_{max}$ observed for Xm1.4:pES1 was 3-fold higher than that of wild type (see above). Similarly, the $K_m$ for the trisaccharide was determined to be between 300 and 400 μM, with an approximately 8–10-fold higher rate of transport observed in Xm1.4:pES1 than the wild type strain.

Competition Experiments—The specificity of the transporter was analyzed by competition experiments with potential substrates or inhibitors of the permease. These were performed by determining the initial rates of uptake of $[^3H]$Me-TCB (6–60 s) in the presence and absence of various sugars. The potential inhibitors were tested at 4–6 concentrations, whereas the substrate concentration was 10 μM.

Lactose and maltose were inactive, requiring >500 μM for 50% inhibition. Apparent competitors of Me-TCB uptake were (GlcNAc)$_2$, (GlcNAc)$_3$, cellobiose, GlcNac, and glucose. The concentrations of inhibitors that gave 50% inhibition of Me-TCB uptake were 5–10 μM (GlcNAc)$_2$, 50–100 μM (GlcNAc)$_3$, 50–100 μM cellobiose, and 50–100 μM GlcNac and glucose. One possible explanation for the inhibition by GlcNac and glucose is that they compete with the analogue for components of the PTS, which they share, such as PEP, Enzyme I, etc. (lactose and maltose are not PTS sugars in E. coli).

Me-TCB Accumulates as an Anionic Derivative in E. coli XL1-Blue MR—The $[^3H]$Me-TCB accumulated by the cells was analyzed by fractionation on Dowex AG1X2 chloride form columns. Me-TCB is a neutral sugar and does not bind to the resin.

Transport experiments were conducted as described under “Experimental Procedures” over 60 min (Fig. 3). At each time point, the cells were filtered and quickly washed once with buffer, and the filter was immediately placed in 1 ml of 70% EtOH and boiled for 3 min. A 0.5-ml aliquot of the supernatant was diluted with 3.0 ml of H$_2$O and transferred to a Dowex AG1X2 chloride form column. The column was washed with H$_2$O and eluted with 1 M NaCl as described under “Experimental Procedures.” The counts in the water wash and in the 1 M NaCl eluates are shown in Fig. 3; at each time point, more than 90% of the total radioactivity placed on the column appeared in the 1 M NaCl eluate.

The mutant strain Xm1.4 did not transport Me-TCB, whereas results similar to those shown in Fig. 3 were obtained with the mutant strain complemented by the cloned $cbb$ operon (Xm1.4:pES1). A parallel experiment was performed with V. furnissii cells; 85–95% of the cpm transferred to the column appeared in the flow through and the water wash fractions (data not shown), and less than 1% was eluted with 1 M NaCl. These results are consistent with the previous observation (10) that Me-TCB is transported in unmodified form (or is dephosphorylated faster than can be detected in the assay) in V. furnissii. These results are discussed below, but they indicate that Me-TCB, and presumably the native substrate (GlcNAc)$_2$, are transported by different mechanisms in E. coli and V. furnissii.

PPE-dependent Phosphorylation of Me-TCB by Toluenized XL1 Blue Cells—It appeared likely that the Me-TCB anionic derivative was a phosphate ester. Toluenized cells were therefore used (“Experimental Procedures”) to test this idea, with PEP and ATP as potential phosphoryl donors. Fig. 4 shows that Me-TCB was phosphorylated in the presence of PEP. A very low level of ATP-dependent phosphorylation of Me-TCB was also observed, possibly owing to the generation of small amounts of PEP over this time course. There was no detectable phosphorylation of Me-TCB by toluenized cells of the mutant strain Xm1.4 under any of the conditions tested, whereas similar preparations of Xm1.4:pCBU7.3 phosphorylated Me-TCB at 2–3-fold the rate observed with the wild type cells.

Because it appeared that the PTS was catalyzing the phosphorylation of Me-TCB, a mutant strain of XL1-Blue was generated using P1 transduction from strain BL21.E1, which contains a kanamycin resistance marker that disrupts Enzyme I of the PTS. This Enzyme I-deficient strain could not ferment the disaccharide, transport $[^3H]$Me-TCB, or phosphorylate the substrate in the toluenized cell assay in the presence of either PEP or ATP (data not shown).

In Vitro PTS-dependent Phosphorylation of Me-TCB—The PTS system can be reconstituted in vitro by adding homogeneous Enzyme I and HPr to a membrane fraction containing the permease. Membranes were prepared from induced wild type, mutant, and Xm1.4:pES1 E. coli cells (as well as from uninduced cells of each strain). As expected, Xm1.4:pES1 membranes gave the highest activity and were used in the experiments described below. Similar results were obtained using membranes from wild type cells that had been induced with (GlcNAc)$_2$.

No phosphorylation of Me-TCB was detected in the initial
nents of the transport system designated IIAChb and IIBChb, which suggested that it encoded two additional soluble components. This was in accord with an analysis of the nrous IIBChb increased the rate of phosphorylation about 2-fold.

**Fig. 4.** PEP-dependent phosphorylation of Me-TCB by toluenized E. coli XL1-Blue MR cells. Cells were grown to mid-exponential phase in M9 minimal media containing 0.5% lactate, 0.5 mM thiamine, and 0.05% casamino acids, supplemented with 0.6 mg of (GlcNAc)2. The cells were harvested, washed, toluenized, and assayed for sugar phosphorylation as described under “Experimental Procedures” with no exogenous phosphoryl donor (■), with ATP (●), or with PEP (○).

experiments with membranes supplemented with Enzyme I, HPr, PEP, and Mg2+. When the protein IIAChb was added to the mixture, the same result was obtained. It therefore appeared likely that the missing components were (GlcNAc)2-specific proteins. This was in accord with an analysis of the chb operon, which suggested that it encoded two additional soluble components of the transport system designated IIAChb and IIBChb, respectively (the membrane protein is IICChb). Me-TCB phosphorylation was detected only when the components of the reaction mixture listed above were supplemented with an aliquot of high speed supernatant (cell extract). To definitively determine whether the putative IIA and/or IIB protein was required for Me-TCB phosphorylation, the individual proteins were subcloned into an overexpression vector and purified to homogeneity (see accompanying papers (1–3)). A complete reaction mixture containing purified Enzyme I, HPr, IIAChb, Xm1.4:pCBU membranes, and PEP resulted in Me-TCB phosphorylation (Fig. 5). Presumably, the membranes contained sufficient bound IIBChb to serve its required catalytic role because exogenous IIBChb was not required. When added, exogenous IIBChb increased the rate of phosphorylation about 2-fold. No phosphorylation of Me-TCB was detected in the following controls: omission of any of the components listed above, substitution of IIAChb for IIAChb, or membranes from the transport mutant, Xm1.4. Uninduced cells yielded membranes that exhibited less than 5% of the activity observed with the membranes from induced cells; the addition of purified IIBChb to the membranes from uninduced cells had no effect.

**Characterization of Phosphorylated Me-TCB and Phosphorylated (GlcNAc)2.** To isolate and characterize phospho-Me-TCB and phosphorylated (GlcNAc)2, the cells were harvested, washed, toluenized, and assayed for sugar phosphorylation as described under “Experimental Procedures.” Phospho-Me-TCB was obtained in a yield of about 50%. Essentially the same result was obtained. It therefore appeared likely that the missing components were (GlcNAc)2-specific proteins. This was in accord with an analysis of the chb operon, which suggested that it encoded two additional soluble components of the transport system designated IIAChb and IIBChb, respectively. The membrane protein is IICChb. Me-TCB phosphorylation was detected only when the components of the reaction mixture were supplemented with an aliquot of high speed supernatant (cell extract). To definitively determine whether the putative IIA and/or IIB protein was required for Me-TCB phosphorylation, the individual proteins were subcloned into an overexpression vector and purified to homogeneity (see accompanying papers (1–3)). A complete reaction mixture containing purified Enzyme I, HPr, IIAChb, Xm1.4:pCBU membranes, and PEP resulted in Me-TCB phosphorylation (Fig. 5). Presumably, the membranes contained sufficient bound IIBChb to serve its required catalytic role because exogenous IIBChb was not required. When added, exogenous IIBChb increased the rate of phosphorylation about 2-fold. No phosphorylation of Me-TCB was detected in the following controls: omission of any of the components listed above, substitution of IIAChb for IIAChb, or membranes from the transport mutant, Xm1.4. Uninduced cells yielded membranes that exhibited less than 5% of the activity observed with the membranes from induced cells; the addition of purified IIBChb to the membranes from uninduced cells had no effect.

Characterization of Phosphorylated Me-TCB and Phosphorylated (GlcNAc)2. To isolate and characterize phospho-Me-TCB, the reaction mixture described above was scaled up to 5 mg of Me-TCB (see “Experimental Procedures”). The phospho-Me-TCB was separated from unreacted Me-TCB, PEP, and P, by ion exchange chromatography, followed by Sephadex G-15 chromatography (“Experimental Procedures”). The final product was obtained in a yield of about 50%. Essentially the same procedure was used for preparing phospho-(GlcNAc)2. The structures of the phospho-glycosides were determined using mass spectroscopy, NMR, and chemical cleavage of the thiogly-}

**Fig. 5. In vitro phosphorylation of Me-TCB.** The PTS system was reconstituted in vitro, and sugar phosphorylation was measured as described under “Experimental Procedures.” ○, complete system; ●, lacking exogenous IIBChb; □, lacking Enzyme I; +, lacking HPr or membranes.

**Mass Spectroscopy—**Negative mode Laser Secondary Ion Mass Spectroscopy analyses were kindly conducted by Drs. Igor A. Kaltashov and Robert Cotter of the Middle Atlantic Mass Spectroscopy Laboratory (Johns Hopkins University). The method was used to determine purity and to obtain the molecular weights of the phosphorylated products from Me-TCB and from (GlcNAc)2. A single peak was observed in each case (data not shown); the ions should be deficient in one mass unit (proton) relative to the starting mass. The following theoretical values are for the anions. The molecular mass of starting material, Me-TCB, was found to be 454 (theoretical, 453.4), whereas the purified product from the reaction mixture was 533 (theoretical, 533.4). The molecular mass of (GlcNAc)2 was found to be 424 (theoretical, 423.4), and that of the purified phosphorylated product was 503 (theoretical, 503.4).

**NMR—**Phospho-Me-TCB was isolated as the triethylammonium salt. 31P NMR (D2O, 300 MHz): δ 4.706 (d, 1H, J1,2 = 10.4 Hz, H-1’), 4.409 (d, 1H, J1,2 = 7.8 Hz, H-1), 4.115 (dd, 1H, J5,6a = 1.5 Hz, J6a,5b = 4.5, J6a,6b = 12.2 Hz, H-6’a), 4.039 (dd, 1H, J6b,5b = 1.8 Hz, J6b,6a = 12 Hz, H-6’a), 3.950 (m, 1H, H-6’b), 3.850 (dd, 1H, J5,6b = 5.0 Hz, J6b,6a = 12 Hz, H-6’b), 3.744–3.600 (m, 5H, H-2, 2’, 3, 4’, 5), 3.575–3.490 (m, 2H, H-3, 3’), 3.449 (s, 3H, OCH3), 3.149 (q, 10H, J = 10.4 Hz, CH2 from triethylamine), 3.149 (q, 10H, J = 10.4 Hz, CH2 from triethylamine), 3.185 (t, 1H, J3,4 = 12 Hz, J4,5 = 10.4 Hz, H-4), 1.989 (s, 6H, 2 acetyl), 1.225 (s, 15H, J = 7.3 Hz, 5 CH3 from triethylamine). The NMR data therefore suggest that the phosphoryl group is linked to the C-6 position of one of the hexosamine residues.

**Mercuric Acetate Cleavage of the Thioglycoside—**The disaccharide phosphate contains two potential sites of linkage (C-6) of the phosphoryl residue. To determine which GlcNAc residue contained the phosphoryl group, the thioglycosidic bond was specifically hydrolyzed using mercuric acetate as described under “Experimental Procedures.” After passage of the reaction mixture over a Dowex-50 AGX8 column, only the sugar from the nonreducing terminus is recovered (16). Phospho-Me-TCB and Me-TCB were subjected to the cleavage reaction, and after removal of the cationic reaction products (which includes the
GlcNAc permease (10), and some of its properties actively taken up and catabolized by the cells independent of participant in many of these phenomena. For example, it is the

cleavage reaction and age of Me-TCB and phospho-Me-TCB.

duress" and in Ref. 32. After removal of the Hg2+-thiosugar bound to Hg2+), the monosaccharides generated from the nonreducing termini were analyzed by anion exchange chromatography (Fig. 6). The product from the control, Me-TCB, was GlcNAc as expected. However, the product derived from phospho-Me-TCB was eluted at the same position as standard GlcNAc-6-P. These data indicate that the product of Me-TCB transport by E. coli is phospho-Me-TCB, with the phosphate linked to the C-6 position of the nonreducing terminal sugar.

DISCUSSION

The marine bacterium V. furnissii grows on chitin as the sole source of carbon and nitrogen; this catabolic cascade involves at least four sophisticated signal transducing systems and a large source of carbon and nitrogen; this catabolic cascade involves at least four sophisticated signal transducing systems and a large

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The chitin disaccharide, N,N’-diacetylchitobiose or (GlcNAc)2, is a key participant in many of these phenomena. For example, it is the major product of chitin hydrolysis by the secreted chitinase, is actively taken up and catabolized by the cells independent of GlcNAc uptake, and is a required inducer or derepressor of many of the genes in the cascade. We have described the V. furnissii (GlcNAc)2 permease (10), and some of its properties are reviewed below. For those studies, we used the nonmetabolizable disaccharide analogue, Me-TCB, and the trisaccharide analogue, Me-TCT.

In attempts to clone the permease, we discovered that E. coli can utilize (GlcNAc)2, and identified the catabolic operon as the previously described so-called cryptic cellodextrin or cel operon (6). The operon is not cryptic and is induced by (GlcNAc)2 and not by cellodextrin, and the cells do not utilize cellodextrin until some of the genes of the operon are mutared (which is why it originally was designated cryptic). We report here some of the properties of the E. coli (GlcNAc)2 permease, which is encoded by three genes, chbA, chbB, and chbC.

The nonmetabolizable analogue Me-TCB was again used to study the kinetics of the permease. As was found with V.

furnissii, Me-TCB is physiologically active in E. coli in that it induces expression of the transporter. The results with E. coli can be summarized as follows: (a) Uptake of Me-TCB is driven by the PTS. For example, in whole cells, uptake requires Enzyme I of the PTS. (b) The uptake product by whole cells is phospho-Me-TCB, and the same product is formed by toluenized cells when they are supplemented with Me-TCB and PEP (but not ATP). (c) Phospho-Me-TCB and phospho-(GlcNAc)2 are formed in vitro when the disaccharides are treated with PEP, Mg2+; homogenous Enzyme I, HPr, IIAChb, and membranes containing IICChb and (presumably) residual IIBChb. The glucose-specific protein, IIAChb could not replace II-AChb. (d) In phospho-Me-TCB, the phosphoryl group was shown to be linked to the C-6 position of the nonreducing terminal GlcNAc residue. (e) Phospho-(GlcNAc)2 is hydrolyzed by the product of the chbF gene (which does not cleave (GlcNAc)2).

For wild type cells, there is a 3–6-fold increase in $K_m$ and a 3-fold decrease in $V_{max}$ for the trisaccharide analogue, Me-TCT, compared with the disaccharide analogue, Me-TCB. These results therefore suggest why (GlcNAc)2 per se is utilized so poorly by E. coli, if it is catabolized at all.

No attempt was made to directly measure the kinetics with (GlcNAc)2 because it is metabolized, but it competed efficiently with Me-TCB, and from these results, it is likely that the $K_m$ for uptake of the natural disaccharide is similar to that of the analogue. (GlcNAc)2, but not higher oligosaccharides, was also effective as an inhibitor/competitor of Me-TCB uptake, whereas much higher concentrations of cellodextrin, Glc, and GlcNAc were required to obtain this effect. We assume that Glc and GlcNAc compete indirectly because they are PTS sugars and would compete for components of the PTS, such as phospho-HPr. We do not know whether cellodextrin is transported by or only binds to the permease.

Organisms that degrade cellodextrin express cellodextrin permeases. For example, a cellodextrin phosphotransferase system has been isolated from Bacillus stearothermophilus (21). Sequence comparisons between it and the system described in this paper revealed considerable identity and similarity. In the following comparisons, cel genes are from B. stearothermophilus, and chb genes are the corresponding genes from E. coli. Identity:similarity are as follows: celA (Bacillus):chbB (E. coli), 43%-59%; celB:chbC, 33%-60%; celD:chbA, 42%-67%. We emphasize, however, that at least for the B. stearothermophilus celA and celC genes, high degrees of identity:similarity were observed to other unrelated PTS systems such as the L. lactis and S. aureus lactose operons.

Why does E. coli express a PTS-driven transporter for (GlcNAc)2, whereas the V. furnissii permease functions by a different mechanism? The two organisms are very similar. In both, the following sugars are taken up and phosphorylated by the PTS: Glc, GlcNAc, Man, Fru, mannitol, trehalose, and sucrose. Similarly, both organisms have the same array of non-PTS sugars: Gal, Mal, glycerol, Rib, and Glc-6-P (20). Furthermore, the structural genes of three of the sugar-specific Enzyme II (Glc, GlcNAc, Man) complexes of V. furnissii have been cloned and sequenced and show 30–67% identity to the corresponding genes of E. coli (22, 23). These V. furnissii proteins efficiently substitute for the corresponding E. coli proteins in vivo. Given these similarities in the transporters and catabolic pathways of the sugars, it was surprising to find that (GlcNAc)2 is transported by different mechanisms in the two organisms.

3 N. Keyhani and J. Thompson, unpublished results.

4 V. furnissii does not grow on the following carbohydrates (13, 20): t- and l-fucose, d-arabinose, raffinose, sorbitol, l-sorbose, d-xyllose, lactose, cellodextrin, or melibiose. Many of these compounds are used by E. coli.
One can speculate on the teleological reasons for these differences. The $K_m$ for (GlcNAc)$_2$ uptake is estimated to be $<1$ μM in $V. furnissii$, and 50–100 μM in $E. coli$. The high affinity uptake system of $V. furnissii$ is precisely what is required, because extracellular bacterial chitinases yield primarily the disaccharide from chitin, and capturing (GlcNAc)$_2$ from marine waters as it diffuses and is diluted cannot be an easy task. But why does $E. coli$, which does not express a chitinase, utilize (GlcNAc)$_2$ at all? This again, we believe, is an adaptive response to its environment. Chitinases have been reported in vertebrates including man (24–31). Intestinal flora may contain chitinase-producing bacteria, and thus concentrations of (GlcNAc)$_2$ that reach the lower part of the intestine are likely to be much higher than those that occur in the ocean waters. Additionally, many or most intestinal $E. coli$ eventually become incorporated into terrestrial ecosystems, which are rich in chitin-producing organisms such as insects and in soil micro-organisms that hydrolyze chitin primarily to (GlcNAc)$_2$; conceivably, $E. coli$ should thrive in mixed cultures under such conditions.

It is possible that either the $E. coli$ or $V. furnissii$ transporter may have evolved from the other, or alternatively, that their functions may have arisen through convergent evolution. Isolation and sequencing of the $V. furnissii$ genes may reveal such a relationship.

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