The Chitin Catabolic Cascade in the Marine Bacterium *Vibrio furnissii*

CHARACTERIZATION OF AN N,N'-Diacetyl-chitobiose TRANSPORT SYSTEM*

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Nemat O. Keyhani, Lai-Xi Wang, Yuan C. Lee, and Saul Roseman†

From the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218

The disaccharide N,N'-diacetyl-chitobiose, (GlcNAc)₂, is critical in chitin dissimilation by *Vibrio furnissii* and, as reported here, is taken up by a specific permease. Since (GlcNAc)₂ is rapidly catalyzed by *V. furnissii*, a non-hydrolyzable thioglycoside analogue was used: methyl β-N,N'-[3H]diacetyl-thiochitobioside (Me-[3H]TCB). Me-TCB and TCB substitute for (GlcNAc)₂ as chemoattractants and inducers of β-N-acetylglucosaminidase activity. The [3H]Me-TCB uptake system was induced only by (GlcNAc)₂ and was not affected by Na⁺ or K⁺. Uptake appeared to be unidirectional, and in 0.4 m sucrose (± K⁺) the cells accumulated [3H]Me-TCB until it was depleted from the medium, giving an internal concentration of 0.1 and an internal/external ratio > 1,000. The only effective inhibitors of uptake were: (GlcNAc)₅, n = 2–4 > cellobiose > (GlcNAc)₂.

In 50% artificial sea water (or sucrose/Na⁺), [3H]Me-TCB accumulation attained a constant steady state level because of efflux, a Na⁺-dependent process. The physiological implications of these results are considered.

We have reported (1–4) that chitin catabolism by *V. furnissii* is a complex phenomenon in which the bacteria must find the chitin or chitin producing organism, adhere to it, and degrade it without losing significant quantities of carbon or nitrogen to the environment. The process involves multiple signal transduction systems. One of the important signals and intermediates in this pathway is the disaccharide N,N'-diacetylchitobiose or (GlcNAc)₂.†

V. furnissii and a mutant unable to transport GlcNAc grow on (GlcNAc)₂ as the sole source of carbon (3). This is not surprising, since the disaccharide is the product of hydrolysis of chitin by all reported bacterial chitinases (5). In addition, (GlcNAc)₂ and (GlcNAc)₅ are formed in the periplasmic space of *V. furnissii* by the hydrolysis of higher chitin oligosaccharides. Finally, the disaccharide is the most potent inducer of a number of the proteins involved in chitin catabolism in this organism, one of which is a cytoplasmic "chitobiase" that hydrolyzes the di- to the monosaccharide (3).

These observations suggested that *V. furnissii* expresses a disaccharide transport system, and the present studies provide evidence for such a permease and for a separate export system.

EXPERIMENTAL PROCEDURES

Materials—Buffers, reagents, and cell culture media were purchased from standard commercial sources. Chitin oligosaccharides (GlcNAC)ₙ, n = 2–6, were obtained from Seikagaku America, Inc. (Rockville, MD), HEPES from Research Organisms Inc. (Cleveland, OH), GFF glass microfiber filters from Whatman, and thin layer chromatography plates (Silica-Gel 60) from EM Science (Cherry Hill, NJ). Fluoro EN3HANCE spray (surface autoradiography enhancer) and [³H]acetic anhydride (NET-018A, 50 mCi/mmol) were purchased from DuPont NEN.

Bacterial Strains—*Vibrio furnissii* strain 7225 (1) was grown in either high salturia Luria Broth containing 1% Bacto-tryptone, 0.5% yeast, 2.0% NaCl or in buffered 50% ASW, containing 0.5% Na-lactate as the carbon source supplemented with 0.1% NH₄Cl and 0.001% phosphate (lactate-ASW). Cells were grown at 30 °C with aeration (shaking), and turbidity measured as absorbance at 540 nm. Cell cultures (lactate-ASW) were supplemented with various other carbon sources as indicated (usually at 0.6 mm)

Preparation of [³H]Acetyl-labeled Methyl β-N,N'-Diacetyl-thiochitobiose (Me-TCB)—Me-TCB was prepared as described (6, 7), de-N-acetylated with 85% hydrazine (8), the methyl β-thiochitobiose purified, and the amino groups quaternarily reacylated (9, 10) with [³H]acetic anhydride.

The final reaction mixture contained the following components: 18 mg of de-N-acetylated Me-TCB in 2 ml of 10% methanol, 0.25 mmol of [³H]acetic anhydride dissolved in 0.5 ml of ice water, and 2.5 mmol of sodium bicarbonate. After 1 h on ice, with intermittent stirring, an additional 0.5 mmol of cold acetic anhydride was added and the mixture stored at 4 °C overnight. The reaction mixture was then deionized with a 3-fold excess of mixed bed ion-exchange resin (Dowex AG50W-X8, hydrogen form, and Dowex AG1-X8, birecarbonate form, both 100–200-mesh).

After concentrating the supernatant and water washings of the resin, the product was purified over a Sephadex G-10 column (45 cm × 1 cm). Only a single radioactive component was detected on TLC (see below).

Assays—β-GlcNAcidase activity was measured by the continuous spectrophotometric assay with PNPGlcNAc as the substrate (3).

Transport Experiments—*V. furnissii* was grown overnight in high salturia Broth and diluted 50-fold in lactate-ASW supplemented with 0.6 mm (GlcNAc)₂ (induced) or as indicated. Cells were grown at 30 °C with aeration to an A₅₅₀ = 0.8–1.0, washed three times at 4 °C with either equal volumes of buffered 50% ASW (no lactate, NH₄Cl, or P) or as indicated. Cells were then suspended in 50% ASW using 1/25 the volume of the growth medium. The suspension was stored on ice and transferred to room temperature 15 min prior to use. Transport experiments were conducted no later than 2 h after harvesting and washing the cells. Uptake was initiated by the addition of an equal volume of cell suspension to [³H]Me-TCB dissolved in the same buffer (50% ASW or as indicated) and aerated by shaking (150 rpm, room temperature). Aliquots (0.1 ml) were taken at various times, added to 10 ml of wash buffer at room temperature (50% ASW or as indicated), and filtered. This paper is available on line at http://www-jbc.stanford.edu/jbc/
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 100 and counted in a Packard liquid scintillation spectrometer. Typically, the specific activity in the uptake experiments ranged from 4500 to 5000 cpm/nmol [3H]Me-TCB. Protein concentrations were estimated using a conversion factor of 1 mg of protein/10^9 cells, and cell density was determined by the absorbance of the suspension at 540 nm.

Efflux experiments were conducted with cells that had accumulated [3H]Me-TCB in standard uptake experiments for 45–60 min. The cells were harvested by centrifuging (10,000 g, 3 min, 4 °C), washed once with 10 ml of the indicated buffer, and resuspended in 10 ml of the same buffer. The suspensions were warmed to 25 °C, 1-ml aliquots removed at the indicated times, filtered through a Millipore Millex-GV 0.22-μm filter, and the eluates collected and counted. At the last time point, a separate aliquot was filtered, and radioactivity remaining in the cells was determined by counting the cells on the filter.

Efflux data are presented as the quantity of [3H]Me-TCB that remains in the cells as a function of time at 25 °C. These values are derived as follows: [intracellular [3H]Me-TCB at time t] = [extracellular [3H]Me-TCB at time t] = [intracellular [3H]Me-TCB at time t]

V. furnissii Cell Extracts—Induced V. furnissii cells were allowed to accumulate [3H]Me-TCB, filtered through Whatman glass microfiber filters, and washed as described above. The cells on the filters were extracted with boiling H₂O or 70% ethanol, and the extracts treated either sequentially with Dowex AG1-X8 (bicarbonate form) and AG50W-X8 (hydrogen form) or with a mixed resin of both forms. The deacetylated samples were then analyzed by thin layer chromatography on Silica-gel 60. Treated extracts were analyzed using three different solvent systems: chloroform, methanol, water, 45:53:7, 55:40:5, and 65:25:4. These systems gave markedly different Rₜ values for Me-TCB.

TLC plates were sprayed with Fluoro EN'HANCE prior to placing the film (Kodak X-Omat ARS, 165-1454) for autoradiography. Films were stored at -70 °C for 5–7 days in the dark before being developed.

Chemotaxis Assays—The procedure is a modified capillary assay method (11) and has been described elsewhere (2, 4). In the present studies, cells were grown and labeled with [32P]P in lactate-ASW with and without 0.6 mM GlcNAc₂. The cells were grown to mid-log, harvested, washed at room temperature, and resuspended in sterile Taxis Buffer: 50% ASW, 50 mM HEPES, pH 7.5, 0.1% NH₄Cl, 0.002% K₂HPO₄ (2, 4). Aliquots of the cell suspension (150 μl) were placed in test tubes (6 × 50 mm), and a 5-μl glass capillary (sealed at the top), containing the specified chemotaxant in transit buffer, was placed vertically in the tube. After incubation at 30 °C for 1 h, each capillary was removed, the outside washed with deionized water, wiped dry, cut at the sealed end, and the contents expelled directly into vials containing 4 ml of Hydrosol fluor liquid scintillation fluid (National Diagnostics, Manville, NJ). All experiments were conducted with triplicate capillaries per data point.

RESULTS

Induction of Hexosaminidases with Me-TCB and TCB—In wild-type V. furnissii, (GlcNAc)₂ specifically induces both the cytoplasmic and periplasmic β-GlcNAcidases (3). Since the analogues TCB and Me-TCB are potential gratuitous inducers, they were tested using the same experimental system.

The relative potencies of (GlcNAc)₂, TCB, and Me-TCB as inducers of total β-GlcNAcidase activity are shown in Fig. 1. Both analogs are capable of inducing β-GlcNAcidase activity, and, with experimental error (reproducibility from one culture to another), the analogues are almost as active as (GlcNAc)₂. In the experiment shown in the figure, Me-TCB was about 20% less active than the natural disaccharide, while TCB was about half as active. These results show that the analogues are not only taken up by the cells, but since they induce expression of the hydrolyases, they presumably interact with the transcriptional regulatory DNA-binding proteins that control expression of these enzymes. However, for reasons given below (see “Discussion”), we believe that the analogues are probably not as effective as the natural disaccharide as transcriptional regulators.

Chemotaxis to Me-TCB and TCB—(GlcNAc)₃ is a potent chemotaxant (2), and the analogues were therefore tested in chemotaxis assays. Fig. 2 shows that cells grown in the presence of inducing concentrations (0.6 mM) of (GlcNAc)₃ display chemotaxis toward GlcNAc, (GlcNAc)₂, (GlcNAc)₃, Me-TCB, and TCB. Thus, Me-TCB and TCB are chemotaxants and interact with the chemoreceptors and the chemotaxis machinery induced by (GlcNAc)₂.

Me-TCB Transport in V. furnissii Cells—(GlcNAc)₂-inducible—V. furnissii was grown in 50% ASW containing 0.5% lactate as the carbon source, with and without 0.6 mM (GlcNAc)₂, as inducer. The cells were harvested, and chemotaxis to GlcNAc, (GlcNAc)₂, Me-TCB, and TCB was assayed by the capillary method. The assays were conducted with different concentrations of attractants in the capillaries, and the optimum concentrations (10 mM) are shown.

Me-TCB is not catabolized by V. furnissii—The thioglycosides, Me-TCB and TCB, were used in the present studies because V. furnissii expresses many β-GlcNAcidases that cleave the O-linked oligosaccharides, but presumably not the thioglycosides. However, the cellular accumulation of radioactivity (Fig. 3) may represent metabolic products derived from [3H]acetate rather than [3H]Me-TCB. Several deacetylases for GlcNAc and GlcNAc derivatives have been reported (12–17), and the label in the analogue [3H]Me-TCB is located in the
N-acetyl groups. The radioactive material in the cells was therefore characterized as follows.

V. furnissii cells (induced) were allowed to accumulate [3H]Me-TCB in buffered 50% ASW for 45 min as in a transport assay. The cells were then filtered, washed, and extracted (see "Experimental Procedures"). More than 98% of the radioactivity in the extracts was recovered after treatment with mixed-bed ion exchange resin. The filtrate and resin washings were then combined, concentrated, and subjected to TLC.

Fig. 4 shows an autoradiograph of a TLC plate containing a sample of deionized V. furnissii extract and a standard lane containing pure [3H]Me-TCB. The radioactivity recovered from V. furnissii corresponds to the Me-TCB standard. Since more than 98% of cellular radioactivity was accounted for, we concluded that [3H]Me-TCB is translocated unchanged by V. furnissii.

Kinetics of Me-TCB Transport—The initial rate of Me-TCB transport into V. furnissii was measured as a function of external concentration of substrate. Initial rates were calculated from transport experiments conducted between 6 and 18 s in 50% ASW. The data are depicted in Fig. 5 in a plot of rate of uptake versus external substrate concentration. A reciprocal plot of 1/rate versus 1/substrate concentration yielded an apparent $K_m$ of 1 μM and an apparent $V_{max}$ of Me-TCB entry of 2.1 nmol/min/mg cell protein. However, we emphasize that these values are only rough approximations.

The problem was that the initial rates of uptake were so rapid. More than 30% of the labeled analogue in the medium was taken up by the cells by the first time point, 6 s, and from 50 to 70% of the substrate had been taken up by 18 s. Thus, we were unable to determine accurate initial rates, and we conclude that the kinetic constants are minimum values. That is, the apparent $K_m \leq 1 \mu M$, $V_{max} \geq 2.1$ nmol/min/mg protein.

Competition Experiments—One way to determine the specificity of an uptake system is by competition experiments with other potential substrates of the permease. These were performed by determining initial rates of uptake of [3H]Me-TCB (6–30 s) in the presence and absence of various sugars. The potential inhibitors were tested at 4–6 concentrations, while the substrate was used at its

The following compounds were inactive: GlcNAc, glucose, maltose, melibiose, sucrose, and trehalose. Most of these compounds are transported by V. furnissii via their own transporters (4). The only competitors of Me-TCB uptake tested were chitin oligosaccharides, (GlcNAc)$_n$, $n = 2–5$, and cellobiose. The efficacy of competition decreased with increasing chain length of the chitin oligosaccharides, and the concentrations of inhibitors that gave 50% inhibition were as follows: (GlcNAc)$_n$, $n = 2–4$, 1.0 μM; (GlcNAc)$_5$, 3.5 μM; cellobiose, 1–3 μM.

We do not know whether (GlcNAc)$_n$, $n = 3–5$, are in themselves competitors of [3H]Me-TCB, or act indirectly, i.e. after conversion to the disaccharide in the periplasmic space of V. furnissii. It should be noted that cellobiose does not induce the Me-TCB uptake system.

Effect of Na$^+$ and K$^+$ on Me-TCB Uptake—Fig. 6 shows the progress curve for the uptake of [3H]Me-TCB by V. furnissii cells under "standard" conditions, i.e. in 50% ASW. The initial concentration of the glycoside is 1 mM, and a steady state level was reached in 45–75 min. The steady state internal concentration was about 40 mM, and the cells accumulated 60–70% of the labeled Me-TCB in the medium. The concentration ratio of the solute inside/outside is therefore about 100 under these conditions (i.e. 40/0.4).

Since the cells are grown in 50% ASW, and the transport is conducted in this medium, it seemed possible that the accumulation of labeled Me-TCB resulted from a sodium co-transport (symport) process, similar to the symport of Na$^+$/melibiose in E. coli (18). The ASW in the medium was therefore replaced with buffered sucrose (5 mM Tris, pH 7.5) with or without 0.1 M NaCl or KCl. In the buffer alone, or with the KCl, no steady state level of accumulation was reached under the conditions of the assay. V. furnissii continued to accumulate [3H]Me-TCB until it was virtually depleted from the medium. The initial external concentration of substrate was 1 mM; the observed accumulation therefore corresponds to an internal concentration of approximately 0.1 M Me-TCB. We also emphasize that the accumulation ratio of the substrate at the last point in the experiment must have been very high, exceeding 1000, since there were only traces of [3H]Me-TCB left in the medium at this time.
To our surprise, in the presence of 0.1 M NaCl, uptake reached only about half of the steady state level obtained with 50% ASW (Fig. 6); the steady state level represents about a 20-fold accumulation over the concentration in the medium.

To determine whether the Na\(^+\) effect resulted from a decrease in the rate of uptake, transport experiments were conducted in the media described in Fig. 3, but over 6–30 s to measure initial rates. The data showed that in the presence or absence of either Na\(^+\) or K\(^+\), there was no significant change in the initial rate of Me-TCB uptake (data not shown).

**Efflux of Me-TCB**—The results obtained above were explained by efflux experiments. Induced *V. furnissii* cells were allowed to accumulate \(^{[3]}\)HMe-TCB for 1 h, washed once and resuspended in the indicated buffer as described under "Experimental Procedures." The quantity of \(^{[3]}\)HMe-TCB that effluxed from the cells at room temperature was then measured. Fig. 7 shows that there is little or no efflux of labeled Me-TCB when the cells are washed and resuspended in 0.4 M sucrose + 0.1 M KCl. In 50% ASW approximately half of the accumulated Me-TCB effluxed from the cells over a 1-h time course. In 0.4 M sucrose containing 0.1 M NaCl, however, almost all of the accumulated Me-TCB effluxed from the cells over the same time course.

Thus, the efflux system is dependent on the presence of Na\(^+\). Furthermore, the shapes of the uptake curves are also explained by Fig. 7. That is, in 0.4 M sucrose ± KCl, the cells can take up the \(^{[3]}\)HMe-TCB, but cannot eject it. In the presence of Na\(^+\), however, the efflux system begins to function as the concentration of the solute inside the cell rises. Ultimately, a steady state is reached where the rates are equal for influx and efflux. The 50% ASW and NaCl/sucrose media do not give quantitatively identical efflux results (Fig. 7), thereby explaining the differences in steady state (Fig. 6). It seems likely that components of the 50% ASW affect the efflux system.
DISCUSSION

The enormous turnover of chitin in marine waters is an essential component of the carbon and nitrogen cycles and appears to be catalyzed primarily by bacteria. Since chitinolytic bacteria first hydrolyze chitin to the disaccharide (GlcNAc)_2, the catabolism of the latter compound plays a pivotal role in maintaining marine (and probably terrestrial) ecosystems. V. furnissii catabolizes (GlcNAc)_2 very rapidly (3) and expresses many β-GlcNAcase (24–26). Therefore, a definitive study of (GlcNAc)_2 transport required the use of suitable non-metabolizable analogues of the disaccharide.

Thioglycosides are generally resistant to hydrolysis by glycosidases, and N,N'-diacetyl-thiochitobiose (TCB) and its methyl β-glycoside (Me-TCB) are excellent analogues of (GlcNAc)_2. They act as chemoattractants for the (GlcNAc)_2 chemotaxis system, and as gratuitous inducers of β-GlcNAcidas in V. furnissii. The latter activity suggests that the analogues are transported by these cells and bind to regulatory proteins, such as repressors, that normally recognize (GlcNAc)_2. This paper reports the properties of the transport systems.

The schematic shown in Fig. 8 summarizes our transport results. There are two distinguishable systems in V. furnissii, one for the uptake and the other for the efflux of [3H]Me-TCB.

A (GlcNAc)_2-inducible, specific transport system catalyzes the uptake of the disaccharide analogue, with an apparent K_m ≈ 1 μM, and the analogue accumulates unchanged in the cell. With the exception of celllobiose, the uptake system is inhibited only by chitin oligosaccharides, possibly only by (GlcNAc)_2 itself. However, celllobiose does not induce the transporter. The accumulation gradient (Me-TCB concentration inside/outside) can reach values as high as 1,000. Neither Na^+ nor K^+ affected the initial rate of transport.

While the accumulation ratio of [3H]Me-TCB reaches extraordinary levels, this is true only because the analogue cannot be hydrolyzed by the cytoplasmic β-GlcNAcase. Normally, one would expect to find very low steady state levels of (GlcNAc)_2 in the cell. From this point of view, the results in Fig. 1 may give an erroneous picture of the efficiency of the analogues as inducers compared to the natural inducer, (GlcNAc)_2, since the intracellular steady state level of the latter during induction is probably much lower than that of the analogues.

Bacterial transport systems in common bacteria, such as E. coli, can be classified into four major groups. (a) One is facilitated diffusion, which occurs for glycerol, but apparently not for other solutes. Facilitated diffusion systems are bidirectional and do not require metabolic energy. (b) Another group consists of symport (co-transport) or antiport (counter-transport) systems, where the permease translocates one solute down its electrochemical gradient (e.g. H^+ or Na^+) and utilizes this energy to transport a second solute against its gradient. The lactose, melibiose, and most amino acid permeases are examples of this type. The H^+/Na^+ antiport system functions to pump Na^+ out of the cell. These transporters are generally bidirectional, and metabolic energy is required to maintain the electrochemical gradient of the requisite ion, such as H^+ or Na^+

(c) Group translocation is another transport system, the most common system being the phosphoenolpyruvate-glycose phosphotransferase system. In this case, the sugar is phosphorylated as it is translocated, energy is required (PEP), and since the membrane is impermeable to the sugar-P, it accumulates in the cell. Examples of the PTS in V. furnissii are described in accompanying papers (22, 23). (d) Unidirectional, ATP-dependent permeases appear to be the systems most relevant to the (GlcNAc)_2 permease described here. These transporters generally have low K_m values for their substrates, do not require cotransported ions, and frequently have periplasmic solute binding proteins as components of the process. The binding constants of solutes to their periplasmic binding proteins is generally very high, and it is these complexes that are recognized by the membrane permeases.

The maltose/maltodextrin transport system (19, 20) is an example of the ATP-dependent uniport system. It involves at least five gene products, one of which is the periplasmic maltose binding protein. As is often the case for periplasmic solute binding proteins, the maltose-binding protein serves two roles, one in transport and the other as a chemoreceptor for chemotaxis to the solute. The K_m for maltose uptake in this system is 1 μM.

We know little about the Me-TCB efflux system, except that it is Na^+-dependent. Some possibilities are: (a) antiport or symport through the uptake system, (b) a different transporter involving Na^+ cotransport, or conceivably (c) efflux via a non-specific system, such as the ATP-dependent ABC exporters of bacterial cells that have been implicated in drug resistance (21). Whatever the mechanism, it seems unlikely that (GlcNAc)_2 would be exported from wild type V. furnissii because it would probably be degraded (1–3) before the internal concentration rose to the level required for efflux.

The results reported here describe the general properties of the (GlcNAc)_2 uptake and efflux systems. The molecular details of these transporters await molecular cloning and characterization, and these studies are in progress.