Chitin Catabolism in the Marine Bacterium Vibrio furnissii
IDENTIFICATION AND MOLECULAR CLONING OF A CHITOPORIN*

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Chitin catabolism by the marine bacterium Vibrio furnissii involves many genes and proteins, including two unique periplasmic hydrolases, a chitodextrinase and a β-N-acetylglucosaminidase (Keyhani, N. O., and Roseman, S. (1996) J. Biol. Chem. 271, 33414–33424 and 33425–33432). A specific chitoporin in the outer membrane may be required for these glycosidases to be accessible to extracellular chitooligosaccharides, (GlcNAc)_n, that are produced by chitinases. We report here the identification and molecular cloning of such a porin. An outer membrane protein, OMP (apparent molecular mass 40 kDa) was expressed when V. furnissii was induced by (GlcNAc)_n, n = 2–6, but not by GlcNAc or other sugars. Based on the N-terminal sequence of OMP, oligonucleotides were synthesized and used to clone the gene, chiP. The deduced amino acid sequence of ChiP is similar to several bacterial porins; OMP is a processed form of ChiP. In Escherichia coli, two recombinant proteins were observed, corresponding to processed and unprocessed forms of ChiP. A null mutant of chiP was constructed in V. furnissii. In contrast to the parental strain, the mutant did not grow on (GlcNAc)_2 and transported a nonmetabolizable analogue of (GlcNAc)_2 at a reduced rate. These results imply that ChiP is a specific chitoporin.

We have previously reported that the chitin catabolic cascade and signal transduction systems expressed by the marine bacterium Vibrio furnissii comprise a large number of genes and proteins, only some of which have been identified (6–15). Two of these proteins are unique periplasmic glycosidases. One is a chitodextrinase (11), and the other is a β-N-acetylglucosaminidase (12). The concerted action of these two enzymes yields GlcNAc and (GlcNAc)_2 from the higher oligosaccharides, which are then taken up by specific cytoplasmic membrane transporters (9, 10, 13).

But how do the higher oligosaccharides penetrate the outer membrane/cell wall complex so that they can be hydrolyzed in the periplasm? There is an extensive literature on outer membrane proteins or porins that are thought to mediate this process (16–18). The nonspecific, constitutive porins of Escherichia coli permit the diffusion of solutes that range up to about 600 Da (19), depending on shape, or roughly the size of a trisaccharide. A few sugar-specific porins, or glycoporins, have been reported. These include, most notably, the LamB protein or λ phage receptor protein, whose crystal structure has been resolved (20) and which permits the diffusion of malto-oligosaccharides (21, 22), and ScrY, involved in sucrose transport (23). The RafY protein is involved in raffinose uptake (24) and may not have a specific binding site for the trisaccharide but functions because it is wider than the constitutive E. coli porins (25).

The present report presents evidence for another glycoporin designated chitoporin. The porin is expressed by V. furnissii in its outer membrane and is induced by chitin oligosaccharides but not by GlcNAc nor by other sugars. The structural gene for the porin, chiP, has been cloned, and its sequence was determined and expressed in E. coli. The physiological behavior of a null mutant of chiP in V. furnissii provided additional evidence that ChiP is a specific chitoporin.

EXPERIMENTAL PROCEDURES

Materials

Buffers, reagents, and cell culture media were purchased from commercial sources and were of the highest purity available. The following reagents were purchased from designated sources: HEPES (Research Organics Inc., Cleveland, OH), GEP glass microfiber filters (Whatman), N-lauryl sarcosine (Sigma), [3H]N-acetylglucosamine (CFA-485, 60 mCi/mmol; Amersham Pharmacia Biotech), [α-32P]dATP (6000 Ci/ mmol; Amersham Pharmacia Biotech). Chitin oligosaccharides (GlcNAc)_n, n = 2–6,2 were prepared by a previously published method (26) or obtained from Seikagaku America, Inc. (Rockville, MD). Methyl β-N- [3H]diacyethylthiochitobiose ([3H]Me-TCB or Me-TCB) was prepared (13, 27) as described. Oligonucleotide primers were synthesized and purchased from Genemed (San Francisco, CA). Purified phosphoenolpyruvate-glycose transferase (PTS) general proteins, Enzyme I and HPr were kind gifts from Dr. Norman Meadow.

Growth and Maintenance of Strains

The strains and plasmids used are given in Table I. E. coli strains were grown in LB or on Luria Agar plates supplemented with 50–75 µg/ml ampicillin where appropriate for selection of recombinants. Cell
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**Table I**

| Strains and plasmids | Description | Source |
|-----------------------|-------------|--------|
| **Strains** | | |
| *V. furnissii* 1514 | Wild type | Ref. 15 |
| *V. furnissii* X1401 | chiP null mutant | This study |
| *V. furnissii* XC1 | X1401 + pSF-chiP | This study |
| E. coli BL21(DE3) | Overexpression host | Novagen |
| E. coli XL-1 | Cloning host | Stratagene |
| E. coli S17–1 | Transconjugation strain | Ref. 37 |
| **Plasmids** | | |
| pBluescriptII KS (+) | Cloning vector | Stratagene |
| pKS-X1 | 90-base pair N terminus of chiP in pBluescript | This study |
| pL7 and pL8 | Cosmid cloning containing chiP | This study |
| pNQ705 | Suicide vector | Refs. 36 and 38 |
| pNQ-X300 | 300-base pair N terminus of chiP in pNQ705 | This study |
| pET21a | Overexpression vector | Novagen |
| pHI148 | Overexpression vector | NEB |
| pVita129 | Cloned *V. furnissii* methylase gene | A. F. |
| pSF4 | Mobilization vector | Ref. 43 |
| pET-chiP, pH-chiP, pSF-chiP | Cloned chiP gene | This study |

* A. Fomenkov, unpublished results.

Cultures were grown at 37 °C, with aeration and turbidity measured at 600 nm. At this wavelength, 1.0 optical density unit corresponds to 0.5 mg of cell protein/ml. *V. furnissii* strains were grown either in high salt LB (LMB, Luria broth supplemented with a 10 g/liter NaCl) or in minimal media containing HEPES (50 mM, pH 7.5), 50% artificial sea water (ASW), 0.1% NH₄Cl, 0.001% K₂HPO₄, and 0.5% mL-lactate (lactate-ASW) (8). The minimal medium was supplemented with carbon sources as indicated. Cell cultures were grown at 30 °C with aeration and growth measured by absorbance at 540 nm; 1.0 optical density unit corresponds to 0.5 mg of cell protein/ml. Growth curves were obtained by growing primary inocula overnight in LMB and diluting 1:100 in the indicated media.

**DNA Manipulations**

DNA preparation and analysis, restriction enzyme digests, ligation, and transformations were performed using standard techniques (28). A cosmid library was constructed using bacterial genomic DNA from *V. furnissii* 1514 as described (28). Library construction, including conditions for partial genomic DNA restriction (using Sau3AI) and ligation into the cosmid vector SuperCos1, was performed as recommended (Stratagene). The ligation mixture was packaged into λ phage using GigaPack Gold III packaging extract (Stratagene). Transfections into various *E. coli* strains were performed according to the supplier’s recommendations. Double-stranded DNA was prepared from recombinant clones and sequenced by the dyeodeoxy method using U.S. Biochemical Sequenase version 2.0 sequencing kit or alternately by the Genetics Company Sequencing Facility (Department of Biological Chemistry, UCLA) was used. The N-terminal sequence of the protein was determined at the Biosynthesis and Sequencing Facility (Department of Biological Chemistry, UCLA) was used. The N-terminal sequence of the protein was determined at the Biosynthesis and Sequencing Facility (Department of Biological Chemistry, UCLA) was used.

**Isolation of Outer Membranes**

Unless otherwise indicated, the following procedures were conducted between 0 and 4 °C.

**Method 1—Outer membrane fractions were prepared from mid-exponential phase cells (29, 30). Typically, 5–10-ml cultures were harvested (7500 × g, 5 min), washed once with the same volume of buffer (50 mM Tris-HCl buffer, pH 7.5, 50 mM EDTA, 15% sucrose), and then resuspended in 0.5 ml of buffer containing 0.3 mg/ml lysozyme.** Samples were maintained on ice for 30 min in a microcentrifuge tube and centrifuged at maximum speed for 15 min. The resulting pellet was resuspended in 1 ml of ice-cold 1% N-lauryl sarcosine to solubilize the inner membrane fraction. The sample was passed through a 22-gauge needle to a 5–10 times and centrifuged for 15 min, and the supernatant fluid was discarded. The pellet was washed either with 1% lauryl sarcosine or with H₂O. SDS-PAGE loading buffer (20–100 μl) was added to the pellet, and samples were boiled for 5 min before being subjected to SDS-PAGE (28).

**Method 2—Outer membrane enriched fractions were prepared from mid-exponential phase cells that were first treated by sonication in 50 mM Tris-HCl buffer, pH 7.5. N-Lauryl sarcosine was then added to a final concentration of 0.5%, and the mixture was incubated at 25 °C for 30 min (31). Samples were centrifuged at 6000 × g for 2 min; the supernatant fraction was then centrifuged in a Beckman Airfuge at 150,000 × g for 30 min. The pellet was resuspended in SDS-PAGE loading buffer and processed as described above for Method 1.**

**Method 3—Inner and outer membranes from *V. furnissii* were prepared essentially as described (32). Briefly, mid-exponential cells, grown in lactate-ASW medium with and without 0.6 mM (GlcNAc)₂, were harvested at 12,000 × g, 10 min, washed with 50 mM Tris-HCl buffer, pH 7.5, containing 50 mM EDTA and 15% sucrose, and resuspended in the same buffer containing 0.3 mg/ml lysozyme. Samples were maintained on ice for 30 min, then slowly poured into four volumes of ice-cold sterile distilled H₂O with rapid stirring, and stirred for an additional 10 min. Unlysed cells were removed by centrifugation at 1200 × g for 15 min. The supernatant was centrifuged at 360,000 × g for 2 h, and the pellet was resuspended in the same volume of buffer without lysozyme and passed through a 22-gauge needle 5–7 times. The membranes were harvested at 360,000 × g, resuspended in 25% sucrose containing 5 mM EDTA, pH 7.5, layered on top of a 30–55% sucrose gradient, and centrifuged at 180,000 × g for 16 h. Fractions were collected by piercing the bottom of the centrifuge tubes and allowing the liquid to flow out. The buoyant density of each fraction was determined from measurements of the refractive index.

**β-N-Acetylexosaminidase Assay**

β-GlcNAcIdase activity was measured either discontinuously or continuously using PNP-GlcNAc (Sigma) as described (8).

**Lipopolysaccharide Estimation (KDO Assay)**

Gradient fractions from the inner/outer-membrane isolation procedure were assayed for 3-deoxyoctulosonic acid (KDO) as described (32). Gradient fractions (100 μl) were precipitated with 1 ml of ice-cold 10% trichloroacetic acid. The pellet was washed twice with 1 ml of distilled H₂O, resuspended in 0.1 ml of 0.02 % H₂SO₄, and hydrolyzed for 20 min at 100 °C. The hydroxylates were assayed for KDO using the thiobarbituric acid method.

**Enzyme II⁺neg Assay**

Fractions from bacterial inner and outer membranes were assayed for phosphoenolpyruvate-dependent GlcNAc phosphorylation catalyzed by the PTS system (33, 34). Each assay mixture contained the following components in 0.2 ml: 0.1 ml of the membrane fraction to be assayed, 2–5 units of homogeneous Enzyme I, 3–5 μM HPr, 125 μg of bovine serum albumin, 50 mM Tris-HCl buffer, pH 8.0, 10 mM potassium-phosphoenolpyruvate, 5 mM MgCl₂, 1 mM dithiothreitol, 10 mMKF, and 2 mM [³⁵C]GlcNAc (315 dpm/nmol). The assay mixtures were incubated for 15 or 30 min at 37 °C and heated for 5 min at 100 °C. GlcNAc-6-P was determined by anion exchange chromatography (35).

**N-terminal Sequence of ChiP**

The protein band observed on SDS gels was electroblotted to an Immobilon-P polyvinylidene difluoride membrane. After transfer, the blot was lightly stained with Coomassie Blue, and the band was cut out. The N-terminal sequence of the protein was determined at the Biosynthesis and Sequencing Facility (Department of Biological Chemistry, UCLA).
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Johns Hopkins School of Medicine) using an Applied Biosystems 47A protein sequencer.

Cloning of chiP

The putative chitoporin gene, chiP, was cloned using a two-step strategy. First, primers were designed based on the N-terminal amino acid sequence and used to clone the 90 base pairs corresponding to the nucleotide sequence of the N terminus. Then the N-terminal nucleotide fragment was used as a probe for screening a recombinant V. furnissii cosmid library in E. coli.

Three degenerate oligonucleotide probes were synthesized based on the N-terminal amino acid sequence of ChiP: (a) primer O1, GGCG-GAATTCAARGARGACNGGCT, which is derived from amino acids 1–5, and an EcoRI site inserted for use in cloning is underlined; (b) Primer O2, GHTTSTAYGNGTSGCGCSATG (amino acids 12–19); and (c) primer O3, TTRTGNCCTTTRCTAGGGGG (the sequence in bold is derived from amino acids 25–29, i.e. direction of primer is opposite to O1, and a BamHI cloning site is underlined. Primers O1 and O3 were used to amplify a 90-base pair N-terminal fragment of chiP by polymerase chain reaction from V. furnissii genomic DNA. The polymerase chain reaction generated fragments were subcloned into the EcoRI-BamHI sites of vector pBluescript II KS (+), and colonies were screened for those containing the correct insert by hybridization to primer O2. Three such isolates designated pKS-X1, pKS-X2, and pKS-X3 were picked, and their inserts were sequenced. All three contained the DNA sequence that encoded the desired amino acid sequence. The N-terminal nucleotide fragment (designated N90f) was cut from pKS-X1 (using EcoRI and BamHI) and random primer-labeled with [α-32P]dATP according to the manufacturer’s recommendations (Amersham Pharmacia Biotech). The radiolabeled probe was used to screen a recombinant V. furnissii cosmid library and two positive clones, designated as pL7 and pL8, containing 25–50-kb inserts were isolated by colony hybridization from approximately 3000 recombinant clones. Restriction analysis showed that both isolates shared many similar restriction fragments and a 4.2-kb EcoRI-HindIII fragment hybridized to N90f in both isolates as detected using a Southern blot. Attempts to subclone this fragment yielded a variety of deletions, and the intact 4.2-kb piece could not be cloned. The putative porin gene was, therefore, sequenced by subcloning fragments of the 4.2-kb piece using a combination of various restriction endonucleases and polymerase chain reaction. Based on the sequences of the fragments, it was possible to derive the nucleotide sequence of the complete gene. This deduced sequence was confirmed by designing the appropriate primers and resequencing the entire 4.2-kb fragment using the original template (pL7). The chiP open reading frame was subsequently subcloned into the expression vectors pET21a and pLIH1145 using appropriate primers based on the nucleotide sequence.

Construction of chiP Deletion Mutant

A knock-out or null mutant of chiP was constructed in V. furnissii by homologous recombination between a cloned fragment of chiP (constructed in the suicide vector pNQ705) and the V. furnissii genome (36). Briefly, this method involves conjugal transfer of plasmids from an E. coli mobilizing donor (strain S17-1) to V. furnissii. A fragment of the target gene is subcloned into the vector pNQ705, which contains an antibiotic resistance marker and is capable of propagating only in the mobilizing donor (strain S17-1) to V. furnissii. The suicide vector pNQ705 (which can propagate in this strain) and a compatible plasmid bearing the cloned V. furnissii DNA-methylation gene. The resultant double transformant was used in conjugation experiments with V. furnissii. Briefly, E. coli and V. furnissii were inoculated and grown separately under appropriate conditions. V. furnissii was grown in 50 ml of LB at 30 °C to an A600 = 0.8; E. coli S17-1 cells harboring pNQ-X300 and pVfu129 (the methylation gene) were grown in 50 ml of LB at 37 °C supplemented with 30 μg/ml chloramphenicol and 50 μg/ml kanamycin to an A600 = 0.8. Aliquots containing 2 × 10⁶ cells of each species were pipetted onto LMB plates (no antibiotics) and allowed to grow for 24 h at room temperature. The cocultured E. coli and V. furnissii cells were resuspended in 1.5–2.0 ml of LMB, and aliquots (0.1 ml) were transferred to LMB plates containing 30 μg/ml ampicillin and 30 μg/ml chloramphenicol. The desired V. furnissii recombinants are ampicillin- and chloramphenicol-resistant, individual colonies were picked, and a single colony was purified.

Complementation of V. furnissii chiP Null Mutant

The V. furnissii chiP gene was cloned into the mobilizable vector pSF4 (kind gift of Dr. V. N. Iyer, Carleton University, Ottawa, Canada) in a two step procedure. Primers were first constructed to clone chiP into the NdeI and BamHI sites of pH1148 immediately following the Ptc promoter present on the plasmid. The fragment containing chiP fused to the Ptc promoter was then cloned into the pSt1 site of pSF4 using polymerase chain reaction and two newly designed primers. The primers used for the cloning were as follows: (a) for cloning into pHI1148: 5′-CGGCGGGGCGCATATGGAAACTCTAGAC-3′ and 5′-GGCCGAATTCCAGCTTACCTCTAGAC-3′ and (b) for cloning into pSF4: 5′-GCGCGGATCCCTGGAGACGTTCG-3′ and 5′-ATGGATCGAATTTCGATAGGTCG-3′. The sequences in bold refer to NdeI, BamHI, NsiI and NsiI sites on the given primers, respectively. It should be noted that NsiI and pSt1 result in compatible ends allowing for ligation into the pSt1 site of pSF4 giving pSF-chiP. pSF-chiP was transferred into V. furnissii X1401 (chiP null mutant) by transconjugation as described above.

Transport Assay

The rate of [3H]Me-TCB uptake by V. furnissii was measured essentially as described (33, 35, 39). Briefly, V. furnissii was grown overnight in LMB media supplemented with 0.6 mM (GlcNAc)₂ (induced) or as indicated. Cells were grown at 30 °C with aeration to an A600 of 0.8–1.2, washed three times at 4 °C with an equal volume of buffered 50% ASW salts, and resuspended in buffered 50% ASW or in 0.4 M sucrose containing 50 mM KCl, using 5% to 5% to 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 of the cells. Uptake was initiated by the addition of an equal volume of cell suspension to [3H]Me-TCB dissolved in the same buffer as the cell suspension. Substrate concentrations ranged from 0.5 to 100 μM. The cell suspension was rapidly mixed at room temperature, and aliquots (0.1 ml) were taken at various times, mixed 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.

RESULTS

Induction of an Outer Membrane Protein by Chitin Oligosaccharides—Three different procedures were employed for the isolation of outer membrane enriched fractions from V. furnissii cells grown in lactate-50% ASW with and without 0.6 mM (GlcNAc)₂. Fig. 1 illustrates the protein pattern from membranes prepared by Method 1. After induction by growth on (GlcNAc)₂, a major new band was observed with an apparent molecular mass of 40 kDa, and representing 10–20% of the total Coomassie-stained protein. Chitin oligosaccharides, (GlcNAc)₃, n = 3–6, also acted as inducers, whereas the following

4 A. Fomenkov, unpublished results.
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**Fig. 1.** SDS-PAGE of outer membrane fractions from wild type *V. furnissii*, X1401 (chiP* null mutant), and XC1 (chiP* complemented null mutant): effect of induction by chitin oligosaccharides. The outer membranes of uninduced and induced *V. furnissii* cells grown in lactate-ASW were prepared as described under “Experimental Procedures.” Sugars tested as inducers were used at 0.6 mM concentrations. Lane A, standards. Wild type *V. furnissii*; lane B, no inducer; lane C, GlcNAc; lane D, (GlcNAc)₂; lane E, (GlcNAc)₃; lane F, (GlcNAc)₅; lane G, (GlcNAc)₆; lane H, (GlcNAc)₇. Lanes I and J, *V. furnissii* X1401 (chiP* null mutant); lane I, no inducer; lane J, (GlcNAc)₂. Lanes K and L, *V. furnissii* XC1 transformant (X1401 complemented with chiP* plasmid): lane K, no inducer; lane L, (GlcNAc)₂. The arrow denotes ChiP.

Sugars did not: GlcNAc, glucose, glycerol, maltose, melibiose, sucrose, trehalose, cellulose, and glucosamine oligosaccharides, (GlcNH₂)₃n, n = 1–3. Although GlcNAc did not act as an inducer when tested as described above, growth on GlcNAc as the sole carbon source (0.5%) did induce a band corresponding to ChiP, although at a greatly reduced level (<10% of that observed using 0.6 mM (GlcNAc)₂ as inducer). It should be noted, however, that most porins migrate at about the same molecular mass on SDS-PAGE, 30–45 kDa (16–18), so that the bands observed after growth on GlcNAc and after induction by (GlcNAc)₂ may or may not be the same proteins. Membrane proteins prepared by an alternate method (Method 2 under “Experimental Procedures”) yielded essentially the same results as described above (data not shown).

To determine the location of the induced protein, and to establish that Methods 1 and 2 did indeed yield outer membranes, membranes were prepared from cells grown in lactate medium with and without 0.6 mM (GlcNAc)₂, n = 2–6, as inducer and lysed by osmotic shock, and inner and outer membranes were isolated by fractionation on sucrose gradients (Method 3). Gradient fractions were analyzed for KDO (outer membranes were isolated by fractionation on sucrose gradients). Gradient fractions were analyzed for KDO (outer membranes were isolated by fractionation on sucrose gradients). The results obtained with (GlcNAc)₂-induced cells are shown in Fig. 2. Two KDO peaks are visible: a major peak at a sucrose density of about 1.220–1.190 and a smaller peak at a density of 1.140–1.110. Aliquots of each fraction were analyzed by SDS-PAGE, and a 40-kDa band was observed in fractions between densities of 1.180 and 1.190, corresponding to a density of 1.220–1.190, with a small quantity detectable in fractions between densities of 1.180 and 1.160. All membrane preparations gave essentially the same profiles after isopycnic sedimentation, but the 40-kDa protein was detected only in the KDO enriched fractions of induced cultures, i.e. cells grown in lactate + 0.6 mM (GlcNAc)₂, n = 2–6. From these results, we concluded that the (GlcNAc)₂-induced protein is localized in the outer membrane of *V. furnissii*.

Expression of ChiP by *V. furnissii*— Cultures were grown to mid-exponential phase in the presence of different concentrations of (GlcNAc)₂, and the quantity of chitoporin or ChiP produced was measured as described under “Experimental Procedures.” The optimal inducer concentration was 0.5–1.0 mM (GlcNAc)₂ (data not shown). Experiments on the time course of induction in the presence of 0.6 mM (GlcNAc)₂ showed that protein expression can be detected after 15 min of exposure to the inducer, with maximal expression occurring at approximately 2.5–3 h (Fig. 3). Cells washed into minimal media, without any carbon source after 3 h of induction, retained ChiP in the outer membrane for as long as 24 h after removal of the inducer.

N-terminal Determination of ChiP—The N-terminal amino acid sequence of a major *V. furnissii* outer membrane protein specifically induced by chitin oligosaccharides was sequenced by electroblotting the protein onto polyvinylidene difluoride membranes as described under “Experimental Procedures.” The 32-amino acid residues determined by sequencing are given in Table II. The sequencing data confirmed that the band observed under (GlcNAc)₂-induced conditions was a single polypeptide, >95% pure.

Cloning and Sequencing of chiP—The N-terminal amino acid sequence was used to construct a series of (degenerate) primers. These were used in turn to clone the gene from a *V. furnissii* library constructed in the cosmid vector SuperCos1, in a stepwise manner as described under “Experimental Procedures.” Two cosmids clones were thus isolated with restriction analysis and hybridization, showing that they contained similar *V. furnissii* genomic inserts.

Initial attempts at subcloning the porin gene from the cosmid clone proved difficult, and although not fully confirmed, it appeared as if the gene immediately following the porin structural gene encodes a toxic protein. (When this segment of the DNA was subcloned from the cosmid into *E. coli* and expressed, the cells lysed. We presume that the cosmid was stably maintained because of low copy number and level of expression.) The sequence derived from these fragments was confirmed by restecquencing the gene using the original cosmid clone as the template and is presented in Fig. 4. The deduced amino acid sequence reveals that there are two potential start sites (methionine residues) separated by two amino acid residues.

Similarity of *V. furnissii* Chitoporin to Other Proteins—A search of the EMBL Protein Data Bank (version nrdb95) identified several proteins with significant similarity to the translated open reading frame of the chitoporin gene. In the following list, the proteins are identified by their data bank accession...
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**Fig. 2.** Separation of V. furnissii inner and outer membranes. V. furnissii cells were grown to an absorbance at 540 nm = 0.5 in lactate-ASW medium containing 0.6 mM (GlcNAc)2. Inner and outer membranes were separated using isopycnic sucrose gradient centrifugation as described under “Experimental Procedures.” A total of 25 fractions (1.0 ml/fraction) were isolated. Aliquots (0.1 ml) of each fraction were analyzed for KDO, II\textsuperscript{Nac} activity, and for protein (+) as described. •, outer membrane marker; ○, inner membrane marker. The II\textsuperscript{Nac} activity is expressed as nmol [14C]GlcNAc-6-P formed per min per 0.1 ml of fraction.

**Fig. 3.** Effect of time of induction on expression of chitoporin in V. furnissii. V. furnissii cultures were grown in lactate-ASW medium with 0.6 mM (GlcNAc)2 added at different times. The values on the abcissa represent the time the culture was grown in the presence of inducer. For example, for the 180-min time point, the inducer was added 180 min prior to harvesting, whereas for the 30-min time point inducer was added 30 min before harvesting. Outer membranes were subjected to SDS-PAGE, and ChiP and total protein quantitated by scanning densitometry of the gel. Induction: minutes in (GlcNAc)2

**FRACTION NUMBER**

numbers, and the numerical values that follow give the percentage of identity/percentage of similarity over the full lengths of the genes: a porin from *Vibrio cholera*\textsuperscript{5} (49% identical, 60% similar); *Salmonella typhimurium* phoE porin, Swissnew P30705 (22/33); and *E. coli* phoE porin, Trembl O87911 (25/37); and *Klebsiella pneumoniae* phoE fragment of the gene, Swissnew P30704 (22/35).

ChiP Expression in *E. coli*—The primary sequence of the chitoporin gene determined as described above was used to subclone the chitoporin gene into the overexpression vector, pET21a under the control of the T7 promoter (“Experimental Procedures”). Outer membrane proteins were analyzed by SDS-PAGE using Method 1 (Fig. 5). In Fig. 5, the position of migration of V. furnissii ChiP is marked by arrow 2; two recombinant proteins are expressed in *E. coli*, marked by arrows 1 and 2. The more rapidly migrating band (arrow 2), displays the same mobility as the *V. furnissii* ChiP. The two bands expressed in *E. coli* were electroblotted onto polyvinylidene difluoride membranes, and their N-terminal sequences were determined. The sequences of ChiP from *V. furnissii* and the *E. coli* recombinant proteins are compared with that predicted from the gene sequence (Table II).

In *V. furnissii*, the first 23 amino acids are processed, presumably as the protein enters the periplasmic space before insertion into the outer membrane. *E. coli* expresses both unprocessed (Fig. 5, arrow 1) and processed forms (Fig. 5, arrow 2) of the ChiP. Thus, the *V. furnissii* ChiP is recognized by the appropriate *E. coli* secretory pathway. Incomplete processing may have resulted from overexpression of the gene, which exceeded the capacity of the secretion/processing system of the *E. coli* cells. Indeed, if cells are grown at a slightly lower rate by decreasing growth temperature from 37 to 30 °C, the processed form of ChiP increases from approximately 10–20 to 50–60% of the total ChiP expressed. Furthermore, it is possible that the outer membrane preparations are contaminated with inclusion bodies containing the unprocessed protein.

**Construction of chiP Null Mutant in V. furnissii**—The nucleotide sequence of the chiP gene was used to construct a null mutant in wild type *V. furnissii* as described under “Experimental Procedures.” Briefly, a 0.3-kb fragment of the chiP gene was subcloned into the suicide vector pNQ705 containing a chloramphenicol resistance selection marker. The resulting plasmid was transconjugated from the recombinant *E. coli*

\textsuperscript{5} Preliminary sequence data were obtained from The Institute for Genomic Research. The *V. cholera* sequence displayed the highest homology (49% identity/60% similarity) to the *V. furnissii* chitoporin. We believe that the *V. cholera* protein is a chitoporin homologue.

**TABLE II**

| Genomic Research. The | V. cholera | mology (49% identity/60% similarity) to the | V. furnissii | detella pertussis gene for porin protein, only the N-terminal half showed similarity, Swiss Q04064 (25/40); a Neisseria flavescens porin, DNA segment, Sptrembl P72072 (29/39); the Neisseria meningitidis porA, Sptrembl O87911 (25/37); and Klebsiella pneumoniae phoE fragment of the gene, Swissnew P30704 (22/35). ChiP Expression in *E. coli*—The primary sequence of the chitoporin gene determined as described above was used to subclone the chitoporin gene into the overexpression vector, pET21a under the control of the T7 promoter (“Experimental Procedures”). Outer membrane proteins were analyzed by SDS-PAGE using Method 1 (Fig. 5). In Fig. 5, the position of migration of V. furnissii ChiP is marked by arrow 2; two recombinant proteins are expressed in *E. coli*, marked by arrows 1 and 2. The more rapidly migrating band (arrow 2), displays the same mobility as the V. furnissii ChiP. The two bands expressed in E. coli were electroblotted onto polyvinylidene difluoride membranes, and their N-terminal sequences were determined. The sequences of ChiP from V. furnissii and the E. coli recombinant proteins are compared with that predicted from the gene sequence (Table II).

In V. furnissii, the first 23 amino acids are processed, presumably as the protein enters the periplasmic space before insertion into the outer membrane. E. coli expresses both unprocessed (Fig. 5, arrow 1) and processed forms (Fig. 5, arrow 2) of the ChiP. Thus, the V. furnissii ChiP signal sequence can be recognized by the appropriate E. coli secretory pathway. Incomplete processing may have resulted from overexpression of the gene, which exceeded the capacity of the secretion/processing system of the E. coli cells. Indeed, if cells are grown at a slightly lower rate by decreasing growth temperature from 37 to 30 °C, the processed form of ChiP increases from approximately 10–20 to 50–60% of the total ChiP expressed. Furthermore, it is possible that the outer membrane preparations are contaminated with inclusion bodies containing the unprocessed protein.

**Construction of chiP Null Mutant in V. furnissii**—The nucleotide sequence of the chiP gene was used to construct a null mutant in wild type V. furnissii as described under “Experimental Procedures.” Briefly, a 0.3-kb fragment of the chiP gene was subcloned into the suicide vector pNQ705 containing a chloramphenicol resistance selection marker. The resulting plasmid was transconjugated from the recombinant E. coli
strain S17-1 in which it can replicate and where it was appropriately methylated, so that it was protected against the \textit{V. furnissii} restriction system. Selection on chloramphenicol gave clones containing a knock-out of the \textit{chiP} gene by homologous recombination. A single insertion in the \textit{V. furnissii} genome corresponding to the region containing appropriate restriction sites for \textit{chiP} was confirmed by Southern hybridization (data not shown).

No protein band corresponding to ChiP was observed in the null mutant under any conditions tested. The null mutant strain was complemented by a construct containing the chitoporin gene fused to a Ptac promoter (pSF-\textit{chiP}) as described under "Experimental Procedures." Expression of the chitoporin in the complemented strain, designated \textit{V. furnissii} XC1, was constitutive.

Growth of \textit{V. furnissii} Strains on Chitin Oligosaccharides—Wild type \textit{V. furnissii}, strain X1401 (\textit{chiP} mutant), and strain XC1 (\textit{chiP} complemented mutant) were grown at 30 °C in minimal media containing one of the following carbon sources: 40 mM lactate, 20 mM GlcNAc, 10 mM (GlcNAc)\textsubscript{2}, or 6.7 mM (GlcNAc)\textsubscript{3}. The different sugar concentrations yield about the same number of GlcNAc equivalents in each mixture. The growth curves are presented in Fig. 6. The data show that the three cell types grow at the same rates on lactate, GlcNAc, and (GlcNAc)\textsubscript{2}. By sharp contrast, the wild type and XC1 strains readily grow on the trisaccharide, (GlcNAc)\textsubscript{3}, whereas the \textit{chiP} mutant grows very slowly, if at all, on this oligosaccharide over the time course of the experiment.

Transport of Me-TCB by Wild Type and Mutant Strains—The \textit{V. furnissii} (GlcNAc)\textsubscript{2} transport system has been characterized using a radioactive nonmetabolizable analogue [\textsuperscript{3}H]Me-TCB (13). The effect of the \textit{chiP} null mutation on the initial rate of Me-TCB uptake was measured as a function of the external concentration of the substrate (Fig. 7 A). Initial rates were calculated from transport experiments conducted between 7 and 21 s in buffered 50% ASW. Wild type \textit{V. furnissii} exhibits a 6-fold greater rate of uptake than the mutant at low substrate concentrations. At high substrate concentrations, the two rates were equal.
Regulation of chiP Expression—Cultures were grown on lactate or on various sugars including GlcNAc, galactose, glycerol, mannose, mannitol, maltose, sucrose, fructose, and glucose (all at 0.5%), with and without 0.6 mM (GlcNAc)2 as inducer. ChiP expression as well as β-GlcNAcidase activities were quantified as described under “Experimental Procedures,” and the data are presented in Table III. β-GlcNAcidase activity was measured in the detergent solubilized fraction; this value represents the sum of all the cellular β-GlcNAcidase activities, including both cytoplasmic and periplasmic enzymes (8, 11, 12, 14). The genes encoding these enzymes do not appear to be in a single operon, and they are probably differentially regulated.

All the sugars tested, except perhaps galactose and glycerol, repressed both ChiP and β-GlcNAcidase induction. Glc seemed to be the most potent inhibitor, and it is interesting to note that the monosaccharide, GlcNAc, also inhibits induction of the “disaccharide” pathway.

To determine whether glucose repression of ChiP and β-GlcNAcidase expression was mediated via reduction in cAMP levels, cultures were grown in lactate or glucose medium with and without the inducer 0.6 mM (GlcNAc)2 and with and without 10 mM cAMP. Table III shows that 10 mM cAMP alleviated rates for uptake between the wild type and mutant strains are identical. Thus, the porin has a significant effect on the uptake rate of the disaccharide only at low concentrations (see “Discussion”).

FIG. 6. Growth of V. furnissii strains on lactate, GlcNAc, (GlcNAc)2, and (GlcNAc)3. Wild type, X1401 (the chiP null mutant), and XCl (the chiP complemented mutant) were grown at 30 °C in minimal ASW media containing one of the following carbon sources: 40 mM lactate, 20 mM GlcNAc, 10 mM (GlcNAc)2, or 6.7 mM (GlcNAc)3. The area between the dashed lines includes all of the growth curves except the V. furnissii chiP mutant grown on (GlcNAc)3 (•).

DISCUSSION

The problem posed in the Introduction to this paper is how chitin oligosaccharides penetrate the cell envelope of V. furnissii so that they can be hydrolyzed by the specific periplasmic β-N-acetylglucosaminidases to the mono- and disaccharides. We speculated that a specific porin, or chitoporin, might be required, and in this paper we present evidence for such a porin, ChiP, encoded by the gene chiP.

Generally, constitutive or nonspecific porins are trimeric outer membrane proteins with pore sizes that permit entry of di- and sometimes trisaccharides, depending on the shapes of the molecules. There are a few sugar-specific porins, or glycoporins, the best characterized being LamB (20, 22, 40–42), which permits the entry of high molecular weight glucose oligosaccharides. E. coli also expresses the specific ScrY porin (23), induced by sucrose.

RafY is induced by the trisaccharide raffinose (24), and Ulmke et al. make the following points: (a) Although the trisaccharide raffinose can diffuse through the constitutive, nonspecific E. coli porins (PhoE, OmpC, and OmpF), the diffusion rate is too slow to permit growth. The Km for raffinose uptake is 2 mM in the absence of RafY and 0.13 mM when it is expressed.
but it is not known whether the GlcNAc-induced protein is the same as in membranes from cells grown on GlcNAc as the sole carbon source, (GlcNAc)\(^2\). Glucosamine, its oligosaccharides, and the maltose permease. In other words, under these conditions, the rate of hydrolysis of GlcNAc, and (GlcNAc)\(^2\). There was, however, a marked difference in the kinetics exhibited by the wild type and chiP\(^-\) mutant cells (Fig. 7), about 6-fold in the rate. As the concentration of the solute is increased >2 \(\mu\)M, the slopes of the lines (wild type and mutant) become equivalent, and at “saturation” of the transporter (>50 \(\mu\)M) the rates are equal. In our earlier work on this permease (13), we discussed the difficulties in obtaining valid kinetic data. With wild type *V. furnissii* cells, we obtained the following kinetic constants: apparent \(K_m\) 1 \(\mu\)M (more accurately, \(\leq 1 \mu\)M); apparent \(V_{\text{max}}\) 2.1 nmol/uptake/min/mg cell protein (more accurately, \(\geq 2.1 \text{ nmol/min/mg}\)). Considering the technical problems, the present results are consistent with the earlier data (Fig. 7). In other words, as the level of the solute is increased, the permease becomes dominant in the uptake process, and it is only at low substrate concentrations that the effect of the porin is apparent. These results are therefore in complete accord with those reported for RafY discussed above. (d) Growth experiments (Fig. 6) were conducted with 20 \(\mu\)M GlcNAc, and equivalent (in terms of carbon content) concentrations of the di- and trisaccharide, as well as lactate. The wild type, chiP\(^-\) mutant, and the transformant (complemented chiP\(^+\)) cells grow at essentially the same rates on lactate, GlcNAc, and (GlcNAc)\(^2\). There was, however, a marked difference in their growth properties on 6.7 \(\mu\)M (GlcNAc)3. Whereas the wild type cells and the complemented mutant grew on the same results as (GlcNAc)\(^2\). Glucosamine, its oligosaccharides, and cellulbiose did not induce expression of chiP.

A protein band that migrated at the same rate as ChiP was observed in membranes from cells grown on GlcNAc as the sole carbon source, but it is not known whether the GlcNAc-induced protein is the same as ChiP.

### RafY

RafY has a relatively broad specificity, which is explained by the fact that the reconstituted porin showed no specific binding of the trisaccharide (25), and the aqueous channel was wider than the constitutive *E. coli* porins. (b) The uptake rates of disaccharides such as maltose and sucrose are dependent on their concentrations. At 5 mM, the diffusion rates through the general porins are so rapid that they do not affect the rates of uptake (which are determined by the respective permeases); at these concentrations, there is no increase in uptake rate when the cells express glycoporins. By contrast, at low substrate concentrations, uptake rates of the disaccharides are increased when glycoporins are expressed. (c) Finally, increased uptake rates of the disaccharides are only observed when the porins are coupled to high affinity permeases (\(K_m\), 1–10 \(\mu\)M) such as the maltose permease. In other words, under these conditions, diffusion through the porins is the rate-limiting step in uptake.

ChiP is believed to be a *V. furnissii* chitin oligosaccharide porin for the following reasons: (a) The protein is induced only by chitin oligosaccharides and is found in the outer membranes of *V. furnissii*. Induction is catabolite repressed and can be alleviated by the addition of cAMP. In addition, the recombinant protein is detectable in the outer membranes of *E. coli*. (b) The gene, chiP, that encodes the protein was cloned and expressed in *E. coli*. Although bacterial porins generally show little or no amino acid sequence similarity, the deduced amino acid sequence of ChiP shared homology with bacterial porins from several *Neisseria* species, *B. pertussis*, and with the *Sal-

### TABLE III

| Carbon source for growth\(^a\) | (GlcNAc)\(^2\) (0.6 min) | cAMP (10 min) | ChiP (%\(^b\)) | \(\beta\)-GlcNAcIdase activity\(^c\) |
|-------------------------------|--------------------------|---------------|----------------|----------------------------------|
| Lactate                       | ND                       | ND            | ND             | ND                               |
| +                             | 14                       | 20.4          |                |                                  |
| Glycerol                      | ND                       | ND            | ND             | ND                               |
| +                             | 8.0                      | 10.2          |                |                                  |
| Galactose                     | ND                       | ND            | ND             | ND                               |
| +                             | 6.5                      | 21            |                |                                  |
| Maltose                       | ND                       | ND            | ND             | ND                               |
| +                             | 2.5                      | 10.5          |                |                                  |
| GlcNAc                        | 2.5\(^b\)                | 1.0           |                |                                  |
| +                             | 3.5                      | 6.0           |                |                                  |
| Glucose                       | ND                       | ND            | ND             | ND                               |
| +                             | 2.0                      | 4.0           |                |                                  |
| +                             | 11                       | 17            |                |                                  |
| Mannitol                      | ND                       | ND            | ND             | ND                               |
| +                             | 2.3                      | 6.0           |                |                                  |
| Sucrose                       | ND                       | ND            | ND             | ND                               |
| +                             | 3.0                      | 6.0           |                |                                  |
| Fructose                      | ND                       | ND            | ND             | ND                               |
| +                             | 3.5                      | 5.0           |                |                                  |

\(\text{a The minimal medium was buffered 50% ASW supplemented with the indicated carbon source at 10 mM concentration. The compounds above the space are not substrates of the PTS in this organism, whereas those below the space are substrates. PTS substrates, such as glucose, are strong catabolite repressors in *E. coli* and other bacteria.}\)

\(\text{b ChiP was determined as described in the text (Coomassie-stained SDS-PAGE gels) and is presented as the percentage of total outer membrane protein. ND, not detected, less than 1% of the total protein in the lane.}\)

\(\text{c \(\beta\)-GlcNAcIdase activity in crude extracts was determined by measuring the rate of hydrolysis of \(p\)-nitrophenyl \(\beta\)-N-acetyl-D-glucosamin-}

\(\text{id; activity} = \text{nmmol/min/\mu g protein at 25 °C. It should be emphasized that the extracts contain several enzymes that hydrolyze this substrate.}\)

\(\text{d Higher chitin oligosaccharides, (GlcNAc)\(_n\), } n = 3–6, \text{ gave essentially the same results as (GlcNAc)\(_2\). Glucosamine, its oligosaccharides, and cellulbiose did not induce expression of chiP.}\)

\(\text{e A protein band that migrated at the same rate as ChiP was observed in membranes from cells grown on GlcNAc as the sole carbon source, but it is not known whether the GlcNAc-induced protein is the same as ChiP.}\)

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\(\text{f These cells eventually grow on the trisaccharide after overnight incubation, possibly by release of the periplasmic \(\beta\)-GlcNAcIdase for which the trisaccharide is an excellent substrate.}\)
Identification and Cloning of Chitoporin from V. furnissii