The chitin catabolic cascade in the marine bacterium *Vibrio cholerae*: Characterization of a unique chitin oligosaccharide deacetylase

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Received on June 25, 2007; revised on August 31, 2007; accepted on September 1, 2007

Chitin, one of the most abundant organic substances in nature, is consumed by marine bacteria, such as *Vibrio cholerae*, via a multitude of tightly regulated genes (Li and Roseman 2004, Proc Natl Acad Sci USA. 101:627–631). One such gene, cod, is reported here. It encodes a chitin oligosaccharide deacetylase (COD), when cells are induced by chitobiase, (GlcNH₂)₂, or crude crab shells. COD was molecularly cloned (COD-6His), overproduced, and purified to apparent homogeneity. COD is secreted at all stages of growth by induced *V. cholerae*. The gene sequence predicts a 26 N-terminal amino acid signal peptide not found in the isolated protein. COD is very active with chitin oligosaccharides, is virtually inactive with GlcNAc, and slightly active with colloidal [1³H]-N-acetyl-chitin. The oligosaccharides are converted almost quantitatively to products lacking one acetyl group. The latter were characterized by mass spectrometry (ESI-MS), and treatment with nitrous acid. COD catalyzes the following reactions (n = 2–6): (GlcNAc)ₙ→ GlcNAc-GlcNH₂-(GlcNAc)ₙ₋₂ + Ac⁻. That is, COD hydrolyzes the N-acetyl groups attached to the penultimate GlcNAc moiety at the nonreducing terminus of the penultimate GlcNAc moiety at the nonreducing terminus of the oligosaccharide. The COD enzyme (Figure 2).

Results

Molecular cloning, expression, and purification of COD

The *V. cholerae* genomic sequence was used to design primers for subcloning the cod gene. The resulting overexpression vector pET21d:VC1280 encodes eight amino acids more (LEHHHHHH) at the C-terminus than the wild type protein. The construct was introduced into an expression *Escherichia coli* strain, BL21(DE3)pLysS. We found the enzyme not only in the cells, but also in the culture media. With prolonged induction time, the quantity of enzyme in the culture medium increased and accounted for the majority of expressed COD. For example, the extracellular medium contained 83% of the total COD of an overnight culture induced at 30°C. The culture media were therefore, used as the enzyme source. Fractionation of the extracellular fluid with a nickel column followed by a Diethylaminoethyl-Sepharose (DEAE) column, yielded apparently homogeneous enzyme (Figure 2).

The purification procedure gave a 30% recovery and 7.5-fold purification. The apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is 45 kDa.

Keywords: chitin oligosaccharide deacetylase/extracellular/penultimate GlcNAc/Vibrios

Introduction

Chitin, a β,1-4 linked homopolymer of N-acetyl-D-glucosamine (GlcNAc), is produced in astronomical quantities in the marine environment, mostly by copepods. Marine ecosystems would be inundated by this highly insoluble polysaccharide, were it not for chitinivorous bacteria that convert it to biologically useful substances (Zobell and Rittenberg 1937; Poulicek and Jeuniaux 1982). *Vibrio cholerae* is one such organism, and, in fact, a close association with copepods is an important part of its life cycle. It is well recognized that this association plays an important role in *V. cholerae* infections in humans (Nalin 1976; Nalin et al. 1979; Huq et al. 1983; Chakrabarty et al. 1997).

We have reported that there are numerous enzymes and other proteins in the chitin catabolic pathway and some are described in the following references (Keyhani and Roseman 1999; Keyhani, Li, and Roseman 2000; Park et al. 2000). Intermediates include both fully and partially N-acetylated oligosaccharides (see Discussion). In work related to the detection of a novel His-Asp two component sensor (Li and Roseman 2004; Meibom et al. 2004), we found that the disaccharide chitobiase, (GlcNH₂)₂, induced a disaccharide Phosphoenolpyruvate:glycose phosphotransferase system (PTS) transport operon containing six genes (Figure 1), but this operon was apparently linked to another gene transcribed in the opposite direction. We investigated the latter, and the gene product, and found it to be a chitin oligosaccharide deacetylase (COD). The acronym COD is suggested for the protein, and cod for the gene. COD specifically hydrolyzes only the penultimate GlcNAc moiety at the nonreducing terminus of chitin oligosaccharides.
V. cholerae cells grown in 50% ASW-HEPES minimal medium were induced with 1 mM (GlcNH$_2$)$_2$, and the cultures separated into the following fractions: extracellular fluid (culture media), periplasmic, inner membrane, outer membrane, and cytoplasmic. Each fraction was concentrated and the quantity of COD present in each was determined by immunoelectrophoresis.

COD was detected only in the extracellular fluid (growth medium), and accounted for about 10% of the total protein in the extracellular space.

To confirm and extend this result, V. cholerae cells were grown to different stages with and without (GlcNH$_2$)$_2$ as inducer, and cell fractions isolated as indicated above. Again COD was found only in the extracellular medium, and only from induced cells. It was not detectable at the early log stage, around 3795 molecules/cell at mid log stage, 3930 molecules/cell at late log stage and 710 molecules/cell at stationary stage. These results indicate that the enzyme is actively secreted and is not being released into the medium by some other mechanism such as cell lysis. The decrease in enzyme in the stationary phase probably reflects proteolysis. A decreased secretion was expected as the inducer, (GlcNH$_2$)$_2$, was depleted from the medium, but this could not account for the change from 3795 to 710 molecules/cell, since such a change would require a fivefold increase in cell number, and this would have been detected.

Sequence analysis of COD

The cod gene, annotated as VC1280 from the Institute for Genomic Research (TIGR) microbial V. cholerae genome database, contains 431 amino acids before processing. A 26 amino acids signal peptide was predicted by program Signal P 3.0 server (Technical University of Denmark) giving a 405 amino acid mature protein. This is exactly the same as determined by N-terminal protein sequencing shown in Table I for the recombinant protein. With our 6 His-tag protein, the enzyme contains 413 amino acids and the calculated molecular weight is 46,086 Da, in good agreement with the apparent molecular weight on SDS-PAGE. This value was further demonstrated by mass spectrometry of pure recombinant COD.

Subcellular distribution of COD in V. cholerae VCXB21

The enzyme expressed in E. coli was found both in the cells and the extracellular media. Therefore, it became important to determine where the enzyme was located in the V. cholerae cells.
enzyme from *E. coli* (46,025 Da). The results indicated that the secreted enzyme was modified only by cleavage of the signal peptide. The sequence of COD enzyme was also used to search against NCBI Conserved Domain Database (CDD v2.08, 2004) and two types of conserved domains were found, an N-terminal polysaccharide deacetylase domain and a C-terminal type 3 chitin binding domain (ChtBD3). By searching the NCBI database, the protein shows a high degree of similarity (full length) to other Vibrio proteins, such as *V. alginolyticus* H-8 (79.9% identity/85.9% similarity), *V. vulnificus* YJ016 VV2902 (85% identity/91% similarity), *V. vulnificus* CMCP6 VV11481 (85% identity/91% similarity), *V. parahaemolyticus* RIMD2210633 VP2638 (80% identity/88% similarity), *Photorhizobium profundum* SS9 PBPR-A0494 (65% identity/79% similarity). Considering this degree of conservation, it was surprising to find that COD showed only low similarity to the *Nod B* gene of *Rhizobium* SP NGR234 (17% identity/24.3% similarity), which encodes a deacetylase for chitin oligosaccharides that hydrolyzes the acetamido group at the nonreducing end of the chain, rather than the penultimate GlcNAc residue as does COD.

Characterization of enzymatic products

When individual N-acetylcchitooligosaccharides (dp 2–6) were treated with the deacetylase, a single product was isolated from each (Figure 3B).

The reactions catalyzed by COD are summarized in the scheme shown in Figure 3A, that is, the enzyme cleaves one acetyl group from each of the oligosaccharides. The data for this conclusion were obtained by mass spectrometry (ESI-MS), which revealed that the mass of each product was 42 daltons less than the corresponding starting material (Figure 3B, panels A–E).

To determine which N-acetyl group was removed, i.e., where the single free amino group was located in each product, the mono-deacetylated oligosaccharides were treated with nitrous acid and the resulting products analyzed by ESI-MS. It is known that treatment of a 2-amino-glucoside results in specific cleavage of the O-glycosidic bond via deamination and rearrangement, that treatment of a 2-amino-glucoside results in specific cleavage of the O-glycosidic bond via deamination and rearrangement, which releases the glycon portion with a 2,5-anhydro-D-mannose unit at the reducing end, together with the corresponding aglycon fragment (Sashiwa et al. 1993; Tommeraas et al. 2001). Mass spectroscopic analysis of the reaction mixtures revealed that HNO$_2$ treatment of the mono-deacetylated chitooligosaccharides released the disaccharide derivative 4-O-[(N-acetyl-glucosaminyl)-2,5-anhydro-D-mannose (GM) as the sole anhydro-mannose containing unit from each oligosaccharide, together with the corresponding aglycon fragment (Figure 3B and Table II). For example, treatment of the mono-deacetylated disaccharide with HNO$_2$ gave GM as the sole product (Figure 3B, panel F); treatment of the trisaccharide derivative with HNO$_2$ gave the GM disaccharide and GlcNAc (G) (Figure 3B, panel G) (GlcNAc not shown); and treatment of the tetra-, penta-, and hexasaccharides with HNO$_2$ resulted in the formation of GM, together with the N-acetylchitooligosaccharides (GlcNAc)$_2$, (GlcNAc)$_3$, and (GlcNAc)$_4$ respectively (Figure 3B, panels H to J). The results, summarized in Table II, suggest that, regardless of the length of the N-acetylchitooligosaccharides, enzymatic de-N-acetylation by COD occurs specifically at the second GlcNAc unit from the nonreducing end of the chain.

Kinetic properties of COD

Some kinetic properties of COD are illustrated in Figure 4. The optimum pH for the enzyme was between pH 7 and 7.5, depending on the buffer (Figure 4A). It exhibits more than 90% of maximal activity at pH 8.0, the approximate pH of the marine environment. The optimum temperature in terms of activity was 45°C, and it was 75% as active at 37°C (Figure 4B). COD is thermostable up to 50°C while it loses almost all activity when incubated at 60°C for 30 min. Ionic strength experiments showed that COD was maximally active at low ionic strength, and the activity gradually decreased as the ionic strength was increased; it was 57% as active as the control at 0.5 M NaCl or 0.5 M KCl. There was little effect on activity in the presence of 1 mM DTT and 5 mM EDTA. On the other hand, the activity was sensitive to metal ions, e.g., inhibition by 2 mM Ag$^{+}$, Hg$^{2+}$, Al$^{3+}$, Co$^{2+}$, Cu$^{2+}$, and Ni$^{2+}$, but this inhibitory effect may result from the 6-His-tag which caused visible precipitation of COD at higher concentrations of the protein.

Enzyme specificity

COD exhibited no detectable activity with the monosaccharide, GlcNAc, under the assay conditions described above, and a barely detectable activity when GlcNAc was incubated for prolonged periods with large amounts of the enzyme. A slight activity with colloidal $[^3]$H-N-acetyl labeled chitin was detected, but it was too low to obtain reliable kinetic constants. The COD activity with the chitin oligosaccharides, (GlcNAc)$_n$, n = 2–6, is shown in Table III and Figure 4C–G. The isotopic assay was used for these experiments, and CurveExpert 1.3 was used to obtain the kinetic constants. The $K_m$ increases while the $V_{max}$ decreases with increasing chain length. These values are reflected in the turnover numbers of the enzyme, $k_{cat}$, and especially by the catalytic efficiency of the enzyme for these substrates, $k_{cat}/K_m$. The disaccharide, (GlcNAc)$_2$, is the most active substrate, and the activity drops off substantially as chain length increases. The disaccharide is threefold more active than the trisaccharide, and 44-fold more active than the hexasaccharide.

Discussion

We have reported that the chitin degradation system in *V. cholerae* is very complex, involving many genes and enzymes (reviewed in (Keyhani and Roseman 1999; Li and Roseman 2004)). The known pathway includes the following steps: (i) Binding of the cells to chitin by what we have designated (Yu et al. 1987, 1991; Li and Roseman 2004) a nutrient sensor. (ii) Secretion of chitinases. (iii) Partial hydrolysis of the chitin to chitooligosaccharides (see reviews (Keyhani and Roseman 1999)). (iv) Diffusion of the oligosaccharides through the outer

**Table I.** N-terminal amino acid sequences of chitooligosaccharide deacetylase (COD)

<table>
<thead>
<tr>
<th>Deduced from gene sequence.</th>
<th>Recombinant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MDTKMKLKKLALFTAISLAISGHGFASTPKGKITIFDD</td>
</tr>
<tr>
<td>5</td>
<td>NSTPKGTI</td>
</tr>
</tbody>
</table>

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Fig. 3. Evidence for reactions catalyzed by COD—(A). Schematic for general reaction catalyzed by COD. One N-acetamido group is hydrolyzed in each of the oligosaccharides. (Continued)
Chitin oligosaccharide deacetylase (COD)

Fig. 3. (B) A–E, the ESI-MS profiles of the products obtained from the enzymatic deacetylation of the di-, tri-, tetra-, penta-, and hexasaccharides respectively. In these spectra, the major peaks, M, signify the masses of each of the enzymatic products. To determine the location of the free amino groups in the products of the COD reactions, each oligosaccharide was treated with nitrous acid as described in the text. This reaction results in conversion of each glucosamine residue with the free amino group to 2,5-anhydro-D-mannose with simultaneous chain cleavage. Panels F–J show that each oligosaccharide yielded the same product, designated GM. This product exhibits the mass of GlcNAc-2,5-anhydro-mannose. Some oligosaccharides gave an additional fragment in the nitrous acid reaction as follows: Panel F, disaccharide gave only GM; Panel G, trisaccharide yielded GM + GlcNAc (not shown); Panel H, tetrasaccharide yielded GM + G2; Panel I, pentasaccharide gave GM + G3; Panel J, hexasaccharide produced GM + G4, G2, G3, and G4 exhibit the masses corresponding to (GlcNAc)2, (GlcNAc)3, and (GlcNAc)4 respectively. (C) Schematic view of structures of each COD product and how these compounds reacted with nitrous acid. The abbreviation GlcN denotes glucosamine (or GlcNH2).

membrane into the periplasmic space via a chitooligosaccharide specific porin (Keyhani, Li, Roseman 2000). (v) Hydrolysis of the oligosaccharides to GlcNAc and (GlcNAc)2 by a combination of two specific N-acetyl-glucosaminidases located in the periplasmic space. The endoenzyme, a chitodextrinase (Keyhani and Roseman 1996a) cleaves the oligosaccharides to (GlcNAc)2 and (GlcNAc)3. The second, an exoenzyme, hydrolyzes the GlcNAc linkage at the nonreducing termini in the tri- and higher oligosaccharides, but is virtually inactive with (GlcNAc)2 at the pH of sea water or the growth medium (Keyhani and Roseman

Table II. Analysis of enzyme products

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme product</th>
<th>Theoretical mass (Da)</th>
<th>Found ESI-MS (Da) m/z (M + H)+</th>
<th>HNO2 treatment yields</th>
<th>(GlcNAc)-2,5-anhydro-Man Plus</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GlcNAc)2</td>
<td>(GlcNAc)-(GlcNH2)</td>
<td>382.16</td>
<td>383.22 (M + H)+</td>
<td>Yes</td>
<td>none</td>
</tr>
<tr>
<td>(GlcNAc)3</td>
<td>(GlcNAc)-(GlcNH2)-(GlcNAc)</td>
<td>585.24</td>
<td>586.32 (M + H)+</td>
<td>Yes</td>
<td>(GlcNAc)2</td>
</tr>
<tr>
<td>(GlcNAc)4</td>
<td>(GlcNAc)-(GlcNH2)-(GlcNAc)2</td>
<td>788.32</td>
<td>789.52 (M + H)+</td>
<td>Yes</td>
<td>(GlcNAc)3</td>
</tr>
<tr>
<td>(GlcNAc)5</td>
<td>(GlcNAc)-(GlcNH2)-(GlcNAc)3</td>
<td>991.40</td>
<td>992.69 (M + H)+</td>
<td>Yes</td>
<td>(GlcNAc)4</td>
</tr>
<tr>
<td>(GlcNAc)6</td>
<td>(GlcNAc)-(GlcNH2)-(GlcNAc)4</td>
<td>1194.48</td>
<td>1195.88 (M + H)+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

GlcNAc-GlcNH2: ESI-MS: Calculated for C14H26N2O10, M (exact mass) = 382.16; found, 383.22 (M + H)+.
GlcNAc-GlcNH2-(GlcNAc): ESI-MS: Calculated for C22H39N3O15, M (exact mass) = 585.24; found, 586.32 (M + H)+.
GlcNAc-GlcNH2-(GlcNAc)2: ESI-MS: Calculated for C30H52N4O20, M (exact mass) = 788.32; found (m/z), 789.52 (M + H)+.
GlcNAc-GlcNH2-(GlcNAc)3: ESI-MS: Calculated for C38H65N5O25, M (exact mass) = 991.40; found (m/z), 992.69 (M + H)+.
GlcNAc-GlcNH2-(GlcNAc)4: ESI-MS: Calculated for C46H78N6O30, M (exact mass) = 1194.48; found (m/z), 1195.88 (M + H)+.
1996b). (vi) (GlcNAc)₂ is a particularly important product because it is the key signal to a two component signaling system that regulates expression of a host of genes (Li and Roseman 2004; Meibom et al. 2004). (vii) The mono- and disaccharides are then translocated into the cytoplasm by specific transporters (Bouma and Roseman 1996; Keyhani et al. 1996) where they are catabolized (Roseman 1957; Comb and Roseman 1958; Bassler et al. 1991; Yu et al. 1993; Park et al. 2000, 2002) via several enzymes and converted to fructose-6-P, NH₄⁺, and acetate.

### Table III. Kinetic constants of COD

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_m) (µM)</th>
<th>(V_{max}) (µmol/min/mg)</th>
<th>(k_{cat}) (s⁻¹)</th>
<th>(k_{cat}/K_m) (s⁻¹ M⁻¹ × 10⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(GlcNAc)₂</td>
<td>10.0</td>
<td>36.6</td>
<td>28.1</td>
<td>281.2</td>
</tr>
<tr>
<td>(GlcNAc)₃</td>
<td>21.1</td>
<td>26.6</td>
<td>20.4</td>
<td>96.7</td>
</tr>
<tr>
<td>(GlcNAc)₄</td>
<td>38.9</td>
<td>9.4</td>
<td>7.2</td>
<td>18.5</td>
</tr>
<tr>
<td>(GlcNAc)₅</td>
<td>62.7</td>
<td>7.9</td>
<td>6.1</td>
<td>9.7</td>
</tr>
<tr>
<td>(GlcNAc)₆</td>
<td>94.6</td>
<td>8.0</td>
<td>6.1</td>
<td>6.4</td>
</tr>
</tbody>
</table>

*The data in Figure 4 were used to determine the best fit kinetic constants by the software program CurveExpert 1.3.*

There was no detectable activity with GlcNAc under these assay conditions.

The data in Figure 4 were used to determine the best fit kinetic constants by the software program CurveExpert 1.3.

An alternate pathway for chitin catabolism has recently been described that depends on the action of two enzymes molecularly cloned from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. (i) A deacetylase that hydrolyzes chitin oligosaccharide N-acetyl groups located at the non-reducing termini. These products are identical (except for chain length) to those of Nod B (see Figure 5) (ii) An exo β-glucosaminidase that cleaves the GlcNH₂ residue from GlcNH₂-(GlcNAc)ₙ oligosaccharides products of the deacetylase. The authors propose that the sequential action of the two enzymes gives the final products, GlcNH₂ and GlcNAc (Tanaka et al. 2003, 2004).

Here, we describe a COD that specifically removes the N-acetyl group from the penultimate GlcNAc residue of the chitooligosaccharides (GlcNAc)ₙ, \(n = 2–6\). The data banks reveal a high degree of sequence similarity between COD and a deacetylase isolated from *V. alginolyticus* (Ohishi et al. 1997, 2000). Despite this similarity (79.9% identity), the two enzymes exhibit significantly different substrate specificities. The *V. alginolyticus* enzyme is specific for \(N,N'\)-diacetylchitobiose giving the disaccharide (GlcNAc-GlcNH₂), while the *V. cholerae* enzyme hydrolyzes a broader spectrum of chitooligosaccharides, yielding the products (GlcNAc-GlcNH₂-(GlcNAc)ₙ=₂–₆), although (GlcNAc)₂ is kinetically the most active substrate.

The chitin catabolic enzymes are highly conserved in *Vibrio* species. Thus, it is likely that the following genes encode COD enzymes: VV2902 from *V. vulnificus* CMCP6, VV11481 from *V. cholerae* O395, and the PTS transporter operon. This operon is similar to the *E. coli* (GlcNAc)₂ PTS transporter system, but transcription of *cod* and the PTS transporter genes are in opposite directions. In Figure 1, the annotations are those currently in the *V. cholerae* gene bank, that is, the older annotations for the disaccharide transporter proteins, CelA, CelB, CelC, and CelF. This nomenclature was used when it was believed to be a cryptic, cellobiose specific transporter that is expressed when the cells mutated. In fact, it has been clearly shown that these genes are neither cryptic, nor normally function in the catabolism of cellobiose. They are involved in the catabolism of (GlcNAc)₂ (Keyhani and Roseman 1997; Keyhani, Rodgers, Demeler, Hansen, and Roseman 2000; Park et al. 2000, 2002) via several enzymes and converted to fructose-6-P, NH₄⁺, and acetate.

**Fig. 4.** Some kinetic properties of COD—Unless otherwise indicated, all assays were performed at 37°C, and where product formation was proportional to quantity of COD and time of incubation. (A) Effects of pH on COD activity. Assayed by the colorimetric method (see text) with the following buffers: (O), sodium citrate; (□), PIPES; (●), potassium phosphate; (△), McIlwaine broad range; (■), imidazole HCl; (○), Hepes; (▲), sodium borate; (▼), glycine–NaOH. (B) Effect of temperature during assay on COD activity (colorimetric assay). (C)-(G) Effect of substrate concentration on activity of COD. Substrates were assayed by the isotopic method at the indicated concentrations, and where less than 5% of the substrate was utilized.

**Fig. 5.** Relationship between COD and Nod B—As explained in the text (see Discussion), Nod factors are signaling molecules essential for the development of the symbiotic relationships between nitrogen fixing bacteria, such as Rhizobia, and their hosts, such as soybean plants. The Nod factors determine the specificity of this interaction, and Nod B is a key enzyme in this process. The figure shows that COD and Nod B catalyze equivalent reactions, removing one acetyl group from the chitooligosaccharides. The enzymes differ in that Nod B most generally acts on the tetra- and pentasaccharides, and sometimes on the trisaccharide.
can be strongly induced by chitobiose, (GlcNH₂)₂, and by crab Vibrios and Photobacteria. The bottom two lines of Figure 1. The microbial genome sequence annotations and abbreviations for the genes are shown on the Roseman 2000; Keyhani, Bacia, Roseman 2000). The corrected 2000; Keyhani, Boudker, Roseman 2000; Keyhani, Wang, Lee, Roseman 2000; Keyhani, Bacia, Roseman 2000). The corrected annotations and abbreviations for the genes are shown on the bottom two lines of Figure 1. The microbial genome sequence database (www.tigr.org) shows that the same gene cluster and gene order are well conserved among γ-proteobacteria including Vibrios and Photobacteria.

From microarray data, we know that VC1280 to VC1286 can be strongly induced by chitobiose, (GlcNH₂)₂, and by crab shells, but not by (GlcNAc)₂ (Meibom et al. 2004). Considering that COD was coinduced with the PTS transporter system by chitobiose, it seems reasonable to suggest that the products of COD, the oligosaccharides GlcNAc-GlcNH₂-(GlcNAc)ₙ (n = 0–4), are the true substrates for this PTS transporter system, although this hypothesis remains to be tested.

For the COD products to reach the cytoplasm intact requires that they are not hydrolyzed during the steps outlined above, but most especially by a very active outer membrane β-N-acetylhexosaminidase (Jannatipour et al. 1987; Soto-Gil and Zyskind 1989), and furthermore, they must be recognized by the membrane transporter encoded by the genes in Figure 1. These possibilities can be tested experimentally with mutants constructed for these purposes.

Our final speculation may be the most interesting and potentially important. The COD products closely resemble essential intermediates in the biosynthesis of Nod factors (Figure 5). These factors play a critical role in the development of symbiotic relationships between nitrogen fixing bacteria and plants. To show their importance in plant/microbe relationships, it may be noted that there are many thousands of references on nod factors in the literature (around 10,000 in Chem Abstracts and close to 150,000 according to Google). The factors are derivatives of chitin tri-, tetra-, and pentasaccharides. These oligosaccharides are partially hydrolyzed by nod B as shown in Figure 5. The free amino groups are first coupled to fatty acids to provide membrane anchors, and the precursors are then derivatized (fucose, mannose, arabinose, acetate, sulfate, etc.) to form the nod factors that confer specificity on the symbionts.

Thus, there is a great deal of similarity between nod B and COD. Both act on chitin oligosaccharides, and both remove one acetyl group. They differ primarily in the location of the acetyl group that is hydrolyzed from the chain of GlcNAc residues. Despite these similarities, there is little sequence similarity between nod B and COD (17% identity, 24% similarity). Nevertheless, the structural similarities between the products generated by the two enzymes, and the great importance of the nod factors as signaling molecules for such a complex process makes it reasonable, we think, to suggest that the oligosaccharides produced by COD may be precursors of important signaling events yet to be determined.

Materials and methods

The following chemicals, reagents, and materials were purchased from the indicated sources. Chitin, and N-acetylglucosamine (GlcNAc) were from Sigma (St. Louis, MO). Chitin oligosaccharides (GlcNAc)ₙ (n = 2–6) and the corresponding chitosan oligomers (GlcNH₂)ₙ (n = 2–6) were from Seikagaku America, Inc. (Rockville, MD). Reagents for bacterial growth media were from BD Biosciences (Palo Alto, CA) and J.T. Baker (Phillipsburg, NJ). Reagents for molecular biology were obtained from New England Biolabs (Ipswich, MA), Promega (Madison, WI), Stratagene (La Jolla, CA), and Invitrogen (Carlsbad, CA). [³H]-N-Acetyl labeled chitooligosaccharides were synthesized here by quantitative N-acetylation (Roseman and Ludowieg 1954; Roseman and Daffner 1956; Horowitz et al. 1957) of glucosamine oligomers with [³H]-acetic anhydride (TRK2-25mCi, 150–370 GBq/mmol) obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Electrophoresis gels were from Cambrex BioScience Rockland, Inc (Rockland, ME). Other buffers and reagents were of the highest purity commercially available.

The N-terminal sequence of the purified enzyme was determined at the Biosynthesis and Sequencing Facility (Department of Biological Chemistry, Johns Hopkins School of Medicine) using an Applied Biosystems (Foster, CA) 475A protein sequencer.

Two methods were used to determine protein concentrations, the dye-binding procedure of Bradford (Bio-Rad, CA) with BSA as a standard, and the total nitrogen concentration by a reported method (Jaenicke 1974) for the enzyme purified to apparent homogeneity.

Bacterial strains

All strains of V. cholerae were derived from V. cholerae EI Tor N16961, the organism used for sequencing the V. cholerae genome (Heidelberg et al. 2000). A mutant designated VCXB21 was used to determine the subcellular location of the enzyme, and was constructed from the parent strain as follows: (i) The lacZ gene (VC2338) was replaced with a kanamycin cartridge from plasmid pNK2859 (Kleckner et al. 1991). In this procedure, the primers used were: Gal-R, 5‘-GGATCCTGGAACGTCTCATCAA-3’, and Gal-R, 5‘-CCCGGGAGATCTTAAAGGTCTCTTT-3’; DR-F, 5‘-AGATC TCCGGGTGCTGATTTGACCCCA-3’, and DR-R, 5‘-AGATCCACATGACGGCCACCAA-3’.

By bridge PCR, the deletion construct will delete a fragment from 160 bp before and 16 bp after lacZ gene. The PCR product was subcloned in the pGEM-T vector. A BamHI fragment from 160 bp before and 16 bp after lacZ gene was subcloned in pGEM-T vector. The PCR fragment was subcloned in pGEM-T vector, the NruI fragment removed and the resulting construct religated. This treatment resulted in the deletion of 664 bp in the middle of the sucABC gene (from 308 bp to 971 bp). The deletion construct was then transferred to the BamHI site of
conditional suicide vector, pMAKSACB. The resulting mutant was selected by its inability to ferment sucrose.

V. cholerae strains were grown in either Luria Broth (LB) for maintenance or in 50% artificial sea water (50% ASW-HEPES buffered, pH 7.5) supplemented with 0.005% K₂HPO₄, 0.1% NH₄Cl, 0.5% D,L-lactate as carbon source, and the indicated sugar for induction (Yu et al. 1993; Keyhani, Li, Roseman 2000).

E. coli strain BL21 (DE3) pLysS Novagen (La Jolla, CA) was used as the expression strain. Typically, E. coli strains harboring designated plasmid constructs were grown overnight with vigorous shaking in LB medium containing appropriate antibiotics.

Enzyme assay
Two techniques were employed to measure enzyme activity. The first was a colorimetric method using 3-methyl-2-benzothiazolinone hydrazone hydrochloride reagent (MBTH; Sigma Chemical Co.) for quantization of amino groups in benzothiazolinone hydrazone hydrochloride reagent (MBTH; Sigma Chemical Co.) for quantization of amino groups in various sugar chromogenic reagents, such as anthrone sulfates the hexosamine residue, but not the N-acetyl blocked hexosamine residues, yielding the corresponding anhydro sugar product was treated with nitrous acid, which specifically deamidates the hexosamine residue. The correlation coefficient at 650 nm for each of the enzymatic products, i.e., (GlcNAc)ₜ, lacking one acetyl group, the colorimetric procedure was carried out by the MBTH method exactly as described (Tsuji et al. 1969a).

Method 1: Colorimetric The incubation mixture of substrate and product was treated with nitrous acid, which specifically deaminates the hexosamine residue, but not the N-acetyl blocked hexosamine residues, yielding the corresponding anhydro sugar product. The method consisted of using [³H]-CH₃CO-labeled (GlcNAc)ₙ as substrates, and determining the quantity of [³H]- CH₃COOH released by the enzyme.

Method 2: [³H]-Labeled Oligosaccharides Reactions were performed in 200 µL volumes using 20 mM HEPES buffer, pH 7.0, 200 ng COD enzyme. After incubating at 37°C for 15 min, reactions were terminated by boiling for 4 min and the products assayed by the MBTH method exactly as described (Tsuji et al. 1969a).

Product formation was proportional to the quantity of COD in the incubation mixture, and to the time of incubation in all kinetic assays.

Construction of cod overexpression vector
The gene cod (VC1280) was molecularly cloned from V. cholerae El Tor N16961 genomic DNA by PCR with primers (VC1280F- 5′-AACCATGGACAGTACCCCTAA GGGCA-3′; VC1280R-5′-GGGGCTCG AGTAAAGCTGTAATAAGGTG-3′). The PCR product was subcloned into the pGEM-T vector (Promega) and designated as pGEM-VC1280. The 1.3 kb NcoI-XhoI fragment from pGEM-VC1280 was inserted into the corresponding site of pET21d (+) (Novagen) giving the overexpression construct pET21d:VC1280, which now had a His tag at the C-terminus of the cod gene.

Overexpression and purification of COD
E. coli strain BL21 (DE3) pLysS harboring pET21d:VC1280 was grown in LB medium overnight with 75 µg/mL ampicillin. Fresh medium was inoculated with cells from the overnight culture at a 1:50 dilution and the 2000 mL culture grown to mid-exponential phase at 37°C with vigorous shaking. Isopropyl β-D-thiogalactopyranoside was then added to 0.5 mM final concentration and the incubation continued at 30°C for 4–12 h. The culture was first centrifuged at 3000 × g and the supernatant was filtered through a 0.22 µm membrane (Nalgene) to remove any remaining cells. Macromolecules in the filtrate were concentrated by ultrafiltration using a Centricon Plus-80 (Millipore) membrane with a 10,000 Da cut off. After dialyzing against Buffer A (20 mM potassium phosphate buffer, pH 8.0, 0.2 M NaCl, 0.02% NaN₃), the concentrated enzyme solution (170 mL) was applied to a 20 mL Ni²⁺ charged affinity column (Sigma). The column was washed with five volumes of Buffer A followed by three volumes of Buffer A containing 20 mM imidazole. The enzyme was then eluted with an imidazole gradient (20 mM–120 mM) in 400 mL Buffer A. Active fractions with different degrees of purity (38 mL, 92 mL, and 113 mL) were collected and dialyzed against Buffer B (20 mM potassium phosphate buffer, pH 8.0). COD was further purified either by chromatography on a DEAE-Sepharose 4B column (50 mL), or by repeating the Ni²⁺ charged affinity column (20 mL). For the DEAE column, the enzyme was eluted with 0–0.3 M NaCl gradients in buffer B. Activity appeared at about 80 mM NaCl.

The purified enzyme fractions were collected, concentrated, dialyzed, and stored as 50 µL aliquots containing 2.53 mg/mL enzyme solution in buffer B at −80°C. The enzyme was stable for months under these conditions.

Antibody preparation and immunophoresis assay
COD preparations that appeared to be homogeneous on SDS-PAGE were used to generate rabbit antibodies by Covance Research Products Inc (Denver, PA). A highly sensitive, and pre-
cise rocket immunophoresis assay (Laurell 1972) was employed to quantify COD by comparing rocket heights and areas with varying amounts of standard purified enzyme.

**Kinetics properties of COD**

Unless otherwise specified, the standard assay conditions described above were used with COD preparations that appeared homogenous by SDS-PAGE. The colorimetric and/or the isotopic methods were used for the following determinations. Assays were conducted under conditions where product formation was proportional to incubation times and to the quantity of COD. The following parameters were studied:

**Effects of pH and Buffer Type** The following buffers were tested at 20 mM concentration: McIlvaine's sodium phosphate–citric acid broad-range buffer from pH 2.6–7.6, sodium citrate buffer 3.0–6.0, potassium phosphate buffer 6.0–8.0, imidazole–HCl buffer 6.2–7.8, Pipes buffer 6.1–7.5, HEPES buffer 7.0–8.0, glycine–NaOH buffer 8.6–10.6, borate buffer 8.1–10.5.

**Effects of Ionic Strength, Temperature, and Metal Ions** The effect of ionic strength on purified COD enzyme activity was determined with 0 to 1 M NaCl or KCl in the reaction mixtures. The optimum temperature for the assay was determined by incubating replicate reaction mixtures over the range 4°C to 80°C for 15 min. Thermal stability of COD was measured by incubating the enzyme in the assay buffer without substrate at the indicated temperatures for 30 min, stored on ice for 5 min, warmed to 37°C, and residual enzyme activity determined by adding 2 mM \(N,N'\)-diacetylchitobiose to the reaction mixture.

The effects of metal ions, generally as the chloride salts, were tested at 2 mM concentrations in the standard assay. Also, EDTA at 5 mM concentrations, DTT at 1 mM, and KAc from 0 to 0.2 M were tested as possible inhibitors.

**Preparation of enzymatic reaction products**

Chitin oligosaccharides (GlcNAc)_n \((n = 1–6)\), 5 mg each, were incubated at 37°C for 4 h with 62 \(\mu\)g pure COD enzyme in 20 mM pyridine–acetic acid buffer, pH 7.0, total volumes of 6 mL. Chromatography of the reaction mixtures by TLC showed a virtual complete conversion of substrates to products. The developing solvent was 1-butanol: methanol: NH_4OH: H_2O (5:4:2:1) v/v, which separates the fully acetylated and mono-deacetylated chitooligosaccharides). The reaction mixtures were passed through a Centricon filtration device (Millipore) with a 10,000 Da cut off membrane, and the COD content in each was measured by rocket immunophoresis, with isolated COD as standard.

**SDS-PAGE**

Protein samples were heated at 80°C for 5 min in Laemmli buffer (65 mM Tris/HCl, pH 6.8, 0.3% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.1 mg/mL bromphenol blue), followed by electrophoresis in a 4–20% gradient gel. Gels were stained with Coomassie Blue G-250.

**Mass spectrometry**

The mass measurements of pure recombinant COD enzyme from E. coli was performed using a Voyager DE-STR MALDI-TOF in the Mass Spectrometry/Proteomics Facility at Johns Hopkins University, School of Medicine (www.hopkinsmedicine.org/msf/) with support from a National Center for Research Resources shared instrumentation grant 1S 10-RR 14702, the Johns Hopkins Fund for Medical Discovery and the Johns Hopkins Institute for Cell Engineering.

**Electron spray ionization mass spectrometry (ESI-MS) analysis**

ESI-MS was performed on a micromass ZQ-4000 single quadruple mass spectrometer at the University of Maryland Medical School.

**Nitrous acid treatment of mono-deacetylated chitooligosaccharides**

The mono-deacetylated chitooligosaccharides (3 mg) were dissolved in 1 M acetic acid (300 \(\mu\)L) containing 1 mg sodium nitrite. Aqueous HCl (0.1 M, 30 \(\mu\)L) was added to the solution, maintained at 4°C for 10 h, and passed through a Dowex 50W-X8 column (H⁺ form). The column was washed with an equal volume (300 \(\mu\)L) of distilled water, and the eluants combined and subjected to ESI-MS analysis.
Funding
National Institutes of Health (NIH) (GM51215).

Acknowledgements
We wish to thank Dr. Donald Comb who generously made available a grant from New England Biolabs and Ms. Haijing Song for technical support.

Conflict of interest statement
None declared.

Abbreviations
COD, Chitin oligosaccharide deacetylase; DEAE, Diethylaminoethyl-Septarose; GM, 4-O-(N-acetyl-glucosaminy1)-2,5-anhydro-D-mannose; ESI-MS, Electron spray ionization mass spectrometry; GlcNAc, N-acetyl-D-glucosamine; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria Broth; MBTH, 3-methyl-2-benzothiazolinone hydrazone hydrochloride reagent; PMS, Phosphoenolpyruvate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

References


