The importance of chitinase and *N*-acetylglucosamine (GlcNAc) uptake in *N,N'*-diacetylchitobiose [(GlcNAc)₂] utilization by *Serratia marcescens* 2170

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N,N'-diacetylchitobiose [(GlcNAc)₂] is the main degradation product from chitin by the action of chitinases of *Serratia marcescens* 2170. Uptake of (GlcNAc)₂ via a (GlcNAc)₂-specific enzyme II permease by this bacterium has been demonstrated previously. Here, we report the contribution of chitinase and *N*-acetylglucosamine (GlcNAc) uptake to the utilization of (GlcNAc)₂. When *S. marcescens* 2170 was cultivated in a medium containing chitin, chitinase activity was detected both inside and outside the cells; intracellular chitinase was more abundant and suggested to be mainly located in the periplasm. Production of chitinase was induced by GlcNAc and more effectively by (GlcNAc)₂. For induction of chitinase, uptake of (GlcNAc)₂ was essential but ChiR, an essential regulator of chitinase induction, was not required. *S. marcescens* 2170 grew well on both GlcNAc and (GlcNAc)₂ but mutants defective in either chitinase or NagE, the GlcNAc-specific enzyme II permease, showed reduced growth on (GlcNAc)₂. These results suggest that, in addition to uptake as (GlcNAc)₂, a proportion of the (GlcNAc)₂ is converted to GlcNAc by chitinase, mainly in the periplasm, and incorporated into the cytoplasm by NagE. The mutant defective in chitinase grew more slowly on (GlcNAc)₂ than on GlcNAc, indicating that (GlcNAc)₂ is less efficiently fermented by *S. marcescens* 2170 in the absence of chitinase. Therefore, uptake as both (GlcNAc)₂ and GlcNAc is important for efficient utilization of (GlcNAc)₂ in *S. marcescens*.

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INTRODUCTION

Chitin is the second most abundant biopolymer in nature and is composed of β -1,4-linked *N*-acetylglucosamine (GlcNAc) residues (Shahidi *et al.*, 1999). *Serratia marcescens* 2170 is one of the most effective bacteria for the degradation of chitin. This bacterium is known as an insect pathogen, and chitinase plays an important role in its virulence, together with protease and lecithinase (Grimont & Grimont, 1978). When *S. marcescens* is cultivated in the presence of chitin, four chitinases (A, B, C1 and C2) and a 21 kDa chitin-binding-protein (CBP21) can be detected in the culture supernatant (Suzuki *et al.*, 1998). *N,N'*-Diacetylchitobiose [(GlcNAc)₂] is the minimal substrate for chitinase and CBP21 induction (Watanabe *et al.*, 1997), and is the major product of chitin hydrolysis by chitinases of this bacterium (Suzuki *et al.*, 2002). Therefore,

(GlcNAc)₂ is considered to be the key molecule in the recognition and utilization of chitin. It has been shown that the uptake of (GlcNAc)₂ is mediated by the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), and a (GlcNAc)₂-specific enzyme II permease is involved in the PTS system in *S. marcescens* 2170 (Uchiyama *et al.*, 2003). The PTS sugars are phosphorylated during translocation across the inner membrane. The generated (GlcNAc)₂-6P in the cytoplasm is presumably hydrolysed by phosphochitinase to GlcNAc-6P and GlcNAc. Disruption of the (GlcNAc)₂-specific enzyme II permease encoded by the *chb* operon drastically reduces the production of chitinases and CBP21, and impairs growth on colloidal chitin (Uchiyama *et al.*, 2003).

S. marcescens has a gene encoding a chitinase of 98.5 kDa and 885 amino acids, including the signal sequence (Tews *et al.*, 1996). Chitinase hydrolyses the β -1,4-glycosidic bond in (GlcNAc)₂ and chito-oligosaccharides. Many chitinolytic bacteria possess β -*N*-acetylglucosaminidases, like the chitinase of *S. marcescens* (Bassler *et al.*, 1991; Tsujibo *et al.*, 2000; Matsuo *et al.*, 1999; Yang *et al.*, 2006).

Abbreviations: GlcNAc, *N*-acetylglucosamine; (GlcNAc)₂, *N,N'*-diacetylchitobiose; 4-MU-GlcNAc, 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide; PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; WS-chitin, water-soluble chitin.

It has been reported that β -*N*-acetylglucosaminidases located in the periplasm of *Vibrio furnissii* hydrolyse chito-oligosaccharides to GlcNAc (Bassler *et al.*, 1991). In addition, GlcNAc produced from chito-oligosaccharides is thought to be taken up by GlcNAc permease (Bassler *et al.*, 1991). However, the importance of chitobiase and GlcNAc uptake for the utilization of (GlcNAc)₂ and chito-oligosaccharides in *S. marcescens* is poorly understood. In this study, to clarify the utilization mechanism of degradation products from chitin by *S. marcescens*, the contribution of chitobiase and GlcNAc uptake to the utilization of (GlcNAc)₂ was examined.

The gene encoding chitobiase was previously designated *chb* (Tews *et al.*, 1996). However, this name may cause confusion with the *chb* operon encoding the genes required for PTS-mediated transport and metabolism of (GlcNAc)₂. Therefore, the gene name *chb* for chitobiase is not used in this paper; instead, the chitobiase gene is tentatively named *ctb*.

METHODS

Bacterial strains, plasmids and culture conditions. *S. marcescens* 2170 and its mutants were grown at 33 °C with shaking in a yeast extract-supplemented minimal (YEM) medium containing various carbon sources (Monreal & Reese, 1969). The yeast extract concentration in this medium was reduced to 0.025%. *Escherichia coli* strains were grown at 30 or 37 °C in Luria–Bertani (LB) medium containing appropriate antibiotics. Growth was monitored by measuring OD₆₆₀ using a mini-photo 518 photometer (Taitec). Antibiotics were used at the following concentrations: ampicillin, 100 µg ml⁻¹; chloramphenicol, 50 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; tetracycline, 25 µg ml⁻¹.

Construction of mutants with disrupted *ctb* and *nagE* genes.

Mutants were constructed by homologous recombination using *pir*-dependent replication plasmid pFS200 (Uchiyama *et al.*, 2003). A 290 bp internal region of the *ctb* gene and a 400 bp internal region of the *nagE* gene were amplified by PCR with primers *chb*1-F (5'-GACGGCAAAGACTGGGTCATCTATTCCAC-3') and *chb*1-R (5'-AACGGCGCCACAACTGATCCAGGTTTTC-3') for the *ctb* mutant, and *nagE*-F (5'-AAGGCGCTGTCGTTCTTCAG-3') and *nagE*-R (5'-GACGCGCGCAGTGATAGATG-3') for the *nagE* mutant, using chromosomal DNA of *S. marcescens* 2170 as a template. PCR products were cloned into pT7Blue T-vector or pUC119 and sequenced. From the plasmids, target regions were excised and cloned into pFS200. The resulting plasmids were first introduced into *E. coli* S17-1(λ *pir*) by electroporation and then transferred to *S. marcescens* 2170 by conjugation, as previously described (Uchiyama *et al.*, 2003). Individual transconjugants were selected on LB agar plates containing chloramphenicol and tetracycline. Disruption of the target genes was confirmed by sequencing the PCR product obtained with primers complementary to the sequence within the chloramphenicol-resistance gene and to the sequence outside of the target region.

Intracellular, extracellular and total chitobiase activities. The culture supernatant (extracellular fraction) was prepared by centrifugation at 8000 g for 5 min. After removing the supernatant, the cell pellet was suspended in 0.1 M sodium phosphate buffer (pH 6.0). To prepare the intracellular fraction, the cell suspension was then lysed by sonication for 30–120 s. To measure the total activities containing

intra- and extracellular activities, the culture was permeabilized by adding chloroform at a ratio of 100 µl ml⁻¹ and vortexing vigorously for 10 s. Chitobiase activity was measured using 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide (4-MU-GlcNAc) (Sigma) as the substrate. A 50 µl sample was mixed with 500 µl of 10 µM 4-MU-GlcNAc in 100 mM sodium phosphate buffer (pH 6.0). After incubation for 5 min at 37 °C, the reaction was stopped by adding 500 µl 0.2 M Na₂CO₃. The fluorescence of the liberated 4-MU was measured with excitation at 360 nm and emission at 450 nm using an RF-5300 spectrofluorophotometer (Shimadzu). One unit of chitobiase activity was defined as the amount of enzyme that liberates 1 nmol 4-MU per min.

SDS-PAGE and activity staining. SDS-PAGE was carried out as described by Laemmli (1970) using 12.5% polyacrylamide gels under reducing conditions. After electrophoresis, the gel was washed three times with 2.5% Triton X-100 for 60–90 min to remove SDS and renature the proteins. The gel was then incubated for 60 min in 0.1 M sodium phosphate buffer (pH 6.0). The polyacrylamide gel was overlaid with an agarose gel containing 0.1 mM 4-MU-GlcNAc in 0.1 M sodium phosphate buffer (pH 6.0) for 10 min. Enzyme activity was detected as fluorescent bands under UV light. Proteins separated by SDS-PAGE were stained with Coomassie brilliant blue R-250.

Chemicals. GlcNAc was purchased from Seikagaku Kogyo Co. Water-soluble chitin (WS-chitin) and *N,N'*-diacetylchitobiose were kindly provided by Yaizu Suisan Chemical Co. The degree of deacetylation of the WS-chitin is 38.8%, and its approximate molecular mass is 200–300 kDa. WS-chitin is soluble in water due to partial deacetylation and the limited size of the chitin chain. Therefore, it is possible to measure cell growth in medium containing WS-chitin by optical density.

RESULTS

Arrangement of genes and construction of mutants

(GlcNAc)₂ is the major product of chitin hydrolysis by chitinases of *S. marcescens* 2170 (Suzuki *et al.*, 2002). Uptake of (GlcNAc)₂ via a (GlcNAc)₂-specific enzyme II permease has been demonstrated previously (Uchiyama *et al.*, 2003). However, the presence of chitobiase in this organism suggests that (GlcNAc)₂ is in part hydrolysed by chitobiase to GlcNAc, and then taken up by GlcNAc permease. *S. marcescens* has a *nag* operon (*nagEBACD*), highly homologous to the *E. coli* *nag* operon, which is involved in GlcNAc uptake and metabolism (Plumbridge, 1989; Postma *et al.*, 1993). The *nagE* gene encodes GlcNAc-specific enzyme II permease (Lengeler *et al.*, 1994). The *nag* operon is positioned near the *ctb* gene on the genome of *S. marcescens*. The *ctb* gene is clustered with genes homologous to *E. coli* *ybfM* and *ybfN*, which are genes with unknown function. *nagE* and *nagBACD* are transcribed in opposite directions. To clarify the contribution of chitobiase and GlcNAc uptake to the utilization of chitin, *ctb* and *nagE* mutants were constructed. Mutants were constructed by homologous recombination using pFS200, a *pir*-dependent replication plasmid containing a chloramphenicol-resistance gene. Briefly, the internal regions of the target genes were cloned into pFS200. The resulting plasmids were transferred from *E. coli* S17-1(λ *pir*) to *S. marcescens* 2170 by conjugation, and

transconjugants were selected based on chloramphenicol resistance. Chloramphenicol-resistant transconjugants should contain the respective plasmids integrated into the target gene on the chromosome by homologous recombination, leading to two incomplete copies of the target gene. Integration of the respective plasmids into the chromosomal DNA of *S. marcescens* 2170 was confirmed by PCR and the desired mutants were obtained.

Chitinase production by *S. marcescens* 2170

When *S. marcescens* QMB1466 is cultivated in a medium containing chitin, (GlcNAc)₂-hydrolysing activity, which is probably due to chitinase, is detected in the culture supernatant (Monreal & Reese, 1969). In contrast, chitinase produced in *E. coli* carrying the *S. marcescens* *ctb* gene is secreted into the periplasm (Tews *et al.*, 1996). Therefore, we first investigated the localization of chitinase in *S. marcescens*. Wild-type 2170 and the *ctb* mutant were grown to mid-exponential phase in YEM medium containing 0.2% WS-chitin. The *ctb* mutant grew more slowly than the wild-type strain. Extra- and intracellular fractions were analysed by SDS-PAGE and activity staining with 4-MU-GlcNAc (Fig. 1). A thin protein band of approximately 96 kDa, which coincided with the predicted molecular mass of chitinase, was detected in the extracellular fraction of the wild-type strain in SDS-PAGE analysis. This band was considered to be chitinase because it was not detected in the *ctb* mutant. In activity-staining analysis, only a single fluorescent band at the position corresponding to the protein band of chitinase was observed in both fractions of the wild-type strain. This fluorescent band was not observed in the *ctb* mutant. Accordingly, chitinase was found to be located both inside

and outside the cell. It is worth noting that chitinases and CBP21 were produced normally in the *ctb* mutant.

We then tried to determine extra- and intracellular chitinase activity. However, attempts to measure intracellular activity quantitatively failed because chitinase was inactivated by sonication to disrupt the cells. Therefore, total activity, representing the sum of extra- and intracellular activity, was measured as described in Methods. Significant activities were detected in the wild-type strain, while activities were barely detected in the *ctb* mutant, indicating that chitinase is the main enzyme that hydrolyses this substrate. In the wild-type strain, total activity (10.1 ± 2.2 U ml⁻¹ per OD₆₆₀ unit) was much higher than extracellular activity (3.5 ± 0.9 U ml⁻¹ per OD₆₆₀ unit). When the extracellular fraction was analysed by SDS-PAGE, weak protein bands presumably derived from cellular proteins were detected, suggesting that some, but not all, extracellular chitinase activity was released from the cells by cell lysis. Chitinase from *S. marcescens* possesses the signal sequence in its precursor form, and is secreted into the periplasm when expressed in *E. coli* (Tews *et al.*, 1996). Therefore, intracellular chitinase is suggested to be mainly located in the periplasm, and is more abundant than extracellular chitinase. The attempt to extract chitinase from the periplasm by the osmotic shock procedure was unsuccessful. However, this was not unexpected because it has been reported that the periplasmic proteins of *S. marcescens* are resistant to release by osmotic shock (Cerny & Teuber, 1972).

Growth of *S. marcescens* strains on various carbon sources

(GlcNAc)₂ is the major product of chitin hydrolysis by chitinases of *S. marcescens* 2170. Because chitinase

Fig. 1. Chitinase and chitinase production in *S. marcescens* 2170 strains. Wild-type 2170 (wt) and *ctb* mutant (cm) were grown to mid-exponential phase in YEM medium containing 0.2% WS-chitin. (a) SDS-PAGE analysis of the extra- and intracellular fractions corresponding to 500 µl culture. M, size marker. The gel portion near 97 kDa extracellular fractions is enlarged below. (b) Activity-staining analysis after SDS-PAGE. Chitinase activity was detected as fluorescent bands using 4-MU-GlcNAc.

hydrolyses (GlcNAc)₂ to GlcNAc, it has been thought to be involved in the utilization of (GlcNAc)₂. To clarify the involvement of chitobiase in the utilization of (GlcNAc)₂, *S. marcescens* 2170 and its mutants were grown in YEM medium containing 0.1 % glycerol, 0.1 % GlcNAc or 0.1 % (GlcNAc)₂ (Fig. 2). The wild-type strain grew well on all carbon sources examined. On the other hand, growth of the *ctb* mutant on (GlcNAc)₂ was significantly retarded compared with that of the wild-type strain, indicating that chitobiase plays an important role in the utilization of (GlcNAc)₂.

As described above, chitobiase is suggested to be localized both in the periplasm and outside the cells. When chitobiase hydrolyses (GlcNAc)₂ in the periplasm and supernatant, GlcNAc permease is predicted to be required partially for the utilization of (GlcNAc)₂ but not that of glycerol. To test the above prediction, a mutant for *nagE*, encoding GlcNAc-specific enzyme II permease, was constructed. The *nagE* mutant grew very slowly on GlcNAc (Fig. 2b), indicating that NagE is the main permease for GlcNAc. Slow growth of the *nagE* mutant on GlcNAc suggests slight uptake of GlcNAc through other minor permeases. On (GlcNAc)₂, the *nagE* mutant initially grew slowly, similar to the *ctb* mutant, and then it showed a greatly reduced growth rate (Fig. 2a). The *nagE* mutant produced chitobiase on (GlcNAc)₂ and total activity at 6 h cultivation was 15.9 U ml⁻¹ per OD₆₆₀ unit. The involvement of NagE in the growth on (GlcNAc)₂ suggests that GlcNAc uptake is important for the utilization of (GlcNAc)₂.

Induction of chitobiase

Wild-type and mutant strains were grown to mid-exponential phase in YEM medium containing 0.1 % glucose, glycerol, GlcNAc, (GlcNAc)₂ or glycerol + (GlcNAc)₂. Total chitobiase activities were measured using 4-MU-GlcNAc as substrate (Fig. 3). Chitobiase activity was

detected on GlcNAc, (GlcNAc)₂ and glycerol + (GlcNAc)₂ in the wild-type strain. Culture on (GlcNAc)₂ showed fivefold higher activity than that on GlcNAc. Therefore, the expression of chitobiase is induced by GlcNAc and (GlcNAc)₂, and (GlcNAc)₂ is a more effective inducer. On the other hand, the *ctb* mutant did not show significant chitobiase activity on GlcNAc and (GlcNAc)₂.

In *S. marcescens* 2170, the LysR-type transcriptional regulator ChiR and (GlcNAc)₂ uptake are essential for the production of all chitinases and CBP21 (Suzuki *et al.*, 2001; Uchiyama *et al.*, 2003). The *chbC* encodes the (GlcNAc)₂-specific enzyme IIC component of the PTS. Disruption of *chbC* drastically reduced the production of chitinases because of a lack of (GlcNAc)₂ uptake, as demonstrated previously (Uchiyama *et al.*, 2003). To examine whether ChiR and (GlcNAc)₂ uptake is required for the induction of chitobiase, *chiR* and *chbC* mutants were grown in medium containing GlcNAc or (GlcNAc)₂. As shown in Fig. 3, the *chiR* mutant (N1) produced chitobiase normally, indicating that chitobiase is not regulated by ChiR. Since the *chbC* mutant grew very slowly on (GlcNAc)₂ (Fig. 2), chitobiase production was compared in medium containing both 0.1 % glycerol and 0.1 % (GlcNAc)₂, in which the wild-type strain and the *chbC* mutant grew equally (Uchiyama *et al.*, 2003). Glycerol does not repress chitobiase production of the wild-type strain, as shown in Fig. 3. On the other hand, chitobiase activity of the *chbC* mutant was very low, indicating that uptake of (GlcNAc)₂ is important for the production of chitobiase. Both mutants produced chitobiase on GlcNAc at the same level as the wild-type strain.

DISCUSSION

Many chitinolytic bacteria have β -N-acetylglucosaminidases, which hydrolyse (GlcNAc)₂ and chito-oligosaccharides to GlcNAc (Bassler *et al.*, 1991; Tsujibo *et al.*, 2000;

Fig. 2. Growth of *S. marcescens* 2170 strains on YEM medium containing (a) 0.1 % (GlcNAc)₂ or (b) 0.1 % GlcNAc. ○, Wild-type; △, *ctb* mutant; □, *nagE* mutant; ◇, *chbC* mutant; ● no carbon source control. Each point represents the mean ± SD of two to four independent experiments.

Fig. 3. Total chitobiase activity. Wild-type and mutant strains were grown to mid-exponential phase in YEM medium containing 0.1 % glucose, 0.1 % glycerol, 0.1 % GlcNAc, 0.1 % (GlcNAc)₂ or 0.1 % glycerol + 0.1 % (GlcNAc)₂. Total chitobiase activities were measured using 4-MU-GlcNAc. Data are means $\pm$ SD of two or three independent experiments.

Matsuo *et al.*, 1999; Yang *et al.*, 2006). This study showed that chitobiase is the main β -N-acetylglucosaminidase for chitin utilization in *S. marcescens* 2170, because activity against 4-MU-GlcNAc was hardly detectable in the *ctb* mutant, and (GlcNAc)₂ is the main product of *S. marcescens* chitinases. When *S. marcescens* 2170 was cultivated on chitin, chitobiase activity was detected both inside and outside the cells. *S. marcescens* chitobiase possesses a signal sequence in its precursor form and is secreted in the periplasm when cloned in *E. coli* (Tews *et al.*, 1996). Therefore chitobiase inside the cells of *S. marcescens* 2170 is considered to be localized in the periplasm.

Uchiyama *et al.* (2003) showed that (GlcNAc)₂ molecules are taken up by a (GlcNAc)₂-specific permease into the cytoplasm. In addition to uptake as (GlcNAc)₂, a certain portion of (GlcNAc)₂ must be converted to GlcNAc by chitobiase, mainly in the periplasm, and incorporated into the cytoplasm by the GlcNAc-specific enzyme II permease, because *ctb* and *nagE* are both required for normal growth on (GlcNAc)₂. Therefore, uptake of both (GlcNAc)₂ and GlcNAc is important for the utilization of (GlcNAc)₂ in *S. marcescens*.

Both *ctb* and *nagE* mutants are assumed to essentially take up only (GlcNAc)₂ molecules when they are grown on (GlcNAc)₂. However, the two mutants grew differently on (GlcNAc)₂, as shown in Fig. 2(a). The *nagE* mutant initially grew slowly at a similar rate to the *ctb* mutant on (GlcNAc)₂, and then the growth rate was greatly reduced. This observation suggests that most of the (GlcNAc)₂ in the periplasm was degraded to GlcNAc by chitobiase when the growth rate was reduced. In the case of *V. furnissii*,

mutation of *NagE*, the sole permease of GlcNAc, resulted in an increase in the generation time from 1.4 to 2.5 h on (GlcNAc)₂ (Bassler *et al.*, 1991). The effect of *nagE* mutation appeared to be smaller than with *S. marcescens*. The difference in the effect of the two mutations may be explained by the difference in (GlcNAc)₂ permeases (ABC transporter in *V. furnissii*; PTS permease in *S. marcescens* 2170). The affinity for (GlcNAc)₂ of the ABC transporter is much higher than that of the PTS permease (Keyhani *et al.*, 2000). The high-affinity transporter may enable more effective transport under the conditions in which (GlcNAc)₂ concentration is reduced by the action of β -N-acetylglucosaminidases.

The *ctb* mutant, which takes up only (GlcNAc)₂ molecules on (GlcNAc)₂, grew more slowly on (GlcNAc)₂ than on GlcNAc. This means that (GlcNAc)₂ is less efficiently fermented by *S. marcescens* 2170 in the absence of chitobiase. Slower fermentation of (GlcNAc)₂ than GlcNAc was also demonstrated with *E. coli* (Keyhani & Roseman, 1997), which belongs to the *Enterobacteriaceae*, as does *S. marcescens*. *E. coli* takes up GlcNAc and (GlcNAc)₂ by respective PTS permeases (Keyhani & Roseman, 1997), just like *S. marcescens*, but lacks chitobiase (Yang *et al.*, 2006). Therefore, it is plausible to conclude that *S. marcescens* possesses chitobiase for efficient utilization of (GlcNAc)₂ by converting it to the more readily fermentable sugar GlcNAc.

Chitobiase was induced by GlcNAc and (GlcNAc)₂, and (GlcNAc)₂ is a more effective inducer. (GlcNAc)₂ uptake and ChiR are essential for the induction of chitinases and CBP21 (Uchiyama *et al.*, 2003; Suzuki *et al.*, 2001). The *chiR* mutant N1 produced chitobiase normally on (GlcNAc)₂. In contrast, the *chbC* mutant, defective in the (GlcNAc)₂-specific enzyme IIC component of the PTS, did not show any chitobiase activity in a medium containing (GlcNAc)₂ and glycerol. These results indicate that uptake of (GlcNAc)₂ is important in the induction of chitobiase as well as chitinases and CBP21. The requirement of (GlcNAc)₂ uptake for chitobiase production suggests that a certain substrate generated during or after the uptake, such as (GlcNAc)₂-6P, is involved in regulation of the *ctb* gene. The importance of (GlcNAc)₂ uptake for chitobiase production also means that the *chbC* mutant essentially cannot utilize either GlcNAc or (GlcNAc)₂ when supplied with (GlcNAc)₂ as carbon source, due to the absence of chitobiase. This would explain the very slow growth of the *chbC* mutant on (GlcNAc)₂, as shown in Fig. 2. Unlike chitinases and CBP21, chitobiase is not regulated by ChiR. In *S. marcescens*, the *ctb* gene is clustered with the *ybfM* and *ybfN* genes located upstream of *ctb*. Yang *et al.* (2006) predicted that *ybfM* is the regulon of NagC, which is a regulator of the *nag* operon (Vogler & Lengeler, 1989), because of the presence of a possible NagC-binding site upstream from the *ybfM* gene in *S. marcescens*. It has been shown that NagC regulates not only the *nag* operon, but also the *chb* operon, responsible for utilization of (GlcNAc)₂, and the *glmUS* genes, involved in the GlcNAc

Fig. 4. A model of the catabolic pathway of degradation products from chitin in *S. marcescens*. Solid arrows indicate the pathways supported by experimental data. Dashed arrows denote the hypothetical pathways with no experimental data. OM, outer membrane; IM, inner membrane.

biosynthetic pathway, in *E. coli* (Plumbridge & Pellegrini, 2004; Plumbridge, 1995). The involvement of NagC in chitinase induction is suggested by the fact that chitinase is induced by GlcNAc. In addition to NagC, other regulators must be involved in chitinase induction because chitinase was more strongly induced by (GlcNAc)₂ than by GlcNAc.

Fig. 4 illustrates a model of the catabolic pathway of degradation products from chitin in *S. marcescens* deduced from previously obtained results and the present study. (GlcNAc)₂ is the main product of the action of *S. marcescens* chitinases on chitin. A proportion of (GlcNAc)₂ molecules enter the periplasm and are taken up into the cytoplasm by the (GlcNAc)₂-specific PTS permease. The remainder of the (GlcNAc)₂ is converted to GlcNAc either outside the cells or in the periplasm by chitinase, which is induced by (GlcNAc)₂ uptake. GlcNAc molecules are taken up via the GlcNAc-specific PTS permease. Both (GlcNAc)₂ and GlcNAc are phosphorylated during translocation across the inner membrane. (GlcNAc)₂-6P in the cytoplasm is presumably hydrolysed by phosphochitinase to GlcNAc-6P and GlcNAc, and further catabolized. This model should be confirmed in the near future by uptake studies using radioactively labelled substrates.

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