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Characterization of *Pseudomonas aeruginosa* Chitinase, a Gradually Secreted Protein

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The gram-negative bacterium *Pseudomonas aeruginosa* secretes many proteins into its extracellular environment via the type I, II, and III secretion systems. In this study, a gene, *chiC*, coding for an extracellular chitinolytic enzyme, was identified. The *chiC* gene encodes a polypeptide of 483 amino acid residues, without a typical N-terminal signal sequence. Nevertheless, an N-terminal segment of 11 residues was found to be cleaved off in the secreted protein. The protein shows sequence similarity to the secreted chitinases ChiC of *Serratia marcescens*, ChiA of *Vibrio harveyi,* and ChiD of *Bacillus circulans* and consists of an activity domain and a chitin-binding domain, which are separated by a fibronectin type III domain. ChiC was able to bind and degrade colloidal chitin and was active on the artificial substrates carboxymethyl-chitin-Remazol Brilliant Violet and *p*-nitrophenyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose, but not on *p*-nitrophenyl-β-D-*N*-acetylglucosamine, indicating that it is an endochitinase. Expression of the *chiC* gene appears to be regulated by the quorum-sensing system of *P. aeruginosa*, since this gene was not expressed in a *lasIR vsmI* mutant. After overnight growth, the majority of the ChiC produced was found intracellularly, whereas only small amounts were detected in the culture medium. However, after several days, the cellular pool of ChiC was largely depleted, and the protein was found in the culture medium. This release could not be ascribed to cell lysis. Since ChiC did not appear to be secreted via any of the known secretion systems, a novel secretion pathway seems to be involved.

Chitin, a homopolymer of β-1,4-*N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant natural polymers. This polymer is present as a structural component in the exoskeletons of insects, in the shells of crustaceans, in the cell walls of many fungi and algae, and in nematodes. Recycling of chitin from disposed materials and dead organisms results mainly from the activity of chitinolytic microorganisms. Species of the genera *Serratia*, *Bacillus,* and *Vibrio* have been reported to secrete several chitinolytic enzymes and chitin-binding proteins, which are thought to degrade chitin synergistically, into the extracellular environment (2, 49, 51). The production of chitinases and chitin-binding proteins is often substrate regulated. Their synthesis is repressed when the bacteria are grown in rich medium and induced when the strains are grown in minimal medium supplemented with chitin (21, 49, 51).

Whereas many steps in the process from perception to catabolism of chitin by different bacteria have been elucidated (reviewed in reference 21), the transport of these metabolic proteins across the bacterial cell envelope has been studied in only a few cases. For example, the chitinase ChiA of *Vibrio cholerae* and the chitin-binding protein CbpD of *Pseudomonas aeruginosa* have been shown to be secreted into the extracellular medium via the type II secretion pathway (8, 11). Both ChiA and CbpD are synthesized with a typical N-terminal signal sequence, which is necessary for their transport across the cytoplasmic membrane via the Sec system (32, 36). Transport of these proteins across the outer membrane requires the type II secretion system. This secretion system, which is widely distributed among gram-negative bacteria, is composed of at least 12 proteins, encoded by *eps* genes in the case of *V. cholerae* and by *xcp* genes in the case of *P. aeruginosa* (31, 45). In *P. aeruginosa*, several other proteins, including elastase (LasB) and the staphylolytic protease LasA, are also secreted via the Xcp system (53).

In addition to the type II system, *P. aeruginosa* contains three other protein secretion systems. Alkaline protease is secreted via a type I secretion system, which directs the protein in one step across both membranes of the cell envelope (9). A type III secretion system is used for the transport of several proteins directly from the bacterial cytoplasm into eukaryotic target cells (54). Finally, an esterase was recently demonstrated to be secreted via an autotransporter system (52). Proteins secreted via such a system contain all the information required for their transport across the outer membrane in their primary structure, and they do not need any auxiliary proteins for this step in the secretion process.

In this study, we show that *P. aeruginosa* secretes, in addition to the chitin-binding protein, an endochitinase. This chitinase is not secreted via one of the previously identified secretion pathways and is therefore probably secreted via a novel pathway.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* strains were grown in Luria-Bertani broth (LB) (35) at 37°C unless stated otherwise, and *Pseudomonas putida* was grown in King's B medium (KB) (22) at 30°C. For plasmid maintenance or

TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	F⁻ Δ*(lacZYA-algF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA* ϕ80 *dlacZ*Δ*M15*	19
P. aeruginosa		
PAO25	PAO1 *leu arg*	18
F33	PAO1 *lasI lasR*	M. Holden
PAO1 exsA	PAO1 *exsA*Ω	12
PAN8	PAO1 *aprE lasB*::Km^r	4
PAN9	PAO1 *aprE lasB*::Km^r *xcpQ*::Gm^r	4
PAN10	PAO25 *lasB*::Km^r	4
PAN11	PAO25 *lasB*::Km^r *xcpR*	4
PAN20[b]	PAO25 *chiC*::Km^r	This study
PAN32[b]	F33 *vsmI*::Tn*501*Hg^r	This study
PDO110	PAO1 *vsmI*::Tn*501*Hg^r	M. Holden
P. putida		
WCS358	Wild type	16
Plasmids		
pUC18	Amp^r, ColE1, ϕ80d*lacZ, lac* promoter	30
pMMB67EH	Amp^r, RSF replicon (IncQ), *tac* promoter	13
pKNG101	Sm^r, *oriR6K mobRK2 sacBR*	20
pBSL99	Amp^r Km^r, kanamycin resistance cassette	1
pMP220	Tc^r IncP, promoterless *lacZ*	38
pRB1804	*lasB* in pUC18	3
pRK2013	Km^r Tra$^+$ Mob$^+$	10
pUWL13	*chiC* in pUC18	This study
pJA1	*chiC* in pMMB67EH	This study
pJA5	*chiC′-lacZ* transcriptional fusion in pMP220	This study
pPB107	*lasB′-lacZ* transcriptional fusion in pMP220	This study

[a] Abbreviations: Amp, ampicillin; Gm, gentamicin; Hg, mercury; Km, kanamycin; Sm, streptomycin; Tc, tetracycline.

[b] Constructed strains have been deposited in The Netherlands Culture Collection of Bacteria (www.cbs.knaw.nl/nccb) under collection numbers PC4317 (PAN20) and PC4318 (PAN32).

selection, the following antibiotics were used (concentration in micrograms per milliliter): for *E. coli*, ampicillin, 100, and gentamicin, 15; for *P. aeruginosa*, piperacillin, 75; kanamycin, 25; tetracycline, 40; and nalidixic acid, 25. To induce the expression of genes cloned behind the *lac* or *tac* promoter, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a concentration of 0.1 mM for *E. coli* and 0.5 mM for *P. aeruginosa*.

Plasmids and DNA manipulations. Plasmids used in this study are listed in Table 1. Chromosomal DNA, isolated from strain PAO25 as described (5), was used as the template in PCR. The putative chitinase gene, designated *chiC*, was amplified with PWO polymerase (Boehringer, Mannheim, Germany) and primers C1 (5′-G*GAATTC*CCCCGTGGTAGACGC-3′), which introduces an *Eco*RI site (italic), and C2 (5′-CCGAAAACGCATATGGATATG-3′). These primers hybridized approximately 120 bp upstream and 350 bp downstream of the open reading frame, respectively. The PCR product was digested with *Eco*RI and with *Hin*cII, for which a site was present 150 bp downstream of the stop codon, and the resulting fragment was cloned into the *Eco*RI and *Hin*cII sites of pUC18. The resulting plasmid was designated pUWL13.

To express the chitinase gene in *P. aeruginosa*, the *chiC* fragment was excised from pUWL13 with *Eco*RI and *Hin*dIII and ligated in *Eco*RI- and *Hin*dIII-digested pMMB67EH, resulting in plasmid pJA1. A chitinase-deficient mutant was created by cloning a kanamycin resistance cassette from pBSL99 into the unique *Xho*I site in the *chiC* gene on pUWL13. The *chiC*::Km allele was excised with *Eco*RI and *Hin*dIII, treated with the Klenow fragment of DNA polymerase, and cloned into the *Sma*I site of pKNG101. This plasmid was mobilized to PAO25 using pRK2013 as a helper plasmid (10), and double-crossover mutants were selected as described previously (11), yielding PAN20.

To construct a *chiC′-lacZ* transcriptional fusion, a 755-bp DNA fragment located directly upstream of the coding region of the *chiC* gene was amplified with PWO polymerase and primers C3 (5′-TGCGC*GAATTC*ACCCAACGCC-3′), which generates a site for *Eco*RI (italic), and C4 (5′-ATCCTGATCA*GGT ACC*GCTCCTC-3′), which generates a site for *Kpn*I (italic). This DNA fragment was cloned into the *Hin*cII site of pUC18, from which it was excised with *Eco*RI and *Kpn*I and ligated in the corresponding sites at the 5′ end of a promoterless *lacZ* gene carried by pMP220. The resulting plasmid was designated pJA5. Plasmid pPB107 carries a transcriptional *lasB′-lacZ* fusion and was constructed by the amplification of a 138-bp fragment upstream of the ATG start codon of *lasB* with PWO polymerase, plasmid pRB1804 as the template, primer LasB1 (5′-CTTGTTCAGTTCTCCTGG-3′), and the universal sequencing primer. This fragment was cloned into the *Hin*cII site of pUC18 and recloned as an *Eco*RI/*Kpn*I fragment into the corresponding sites of pMP220.

Cell fractionation and chitin binding. For fractionation experiments, cells from 3-ml overnight cultures were pelleted by centrifugation for 10 min at 8,000 × *g*. Cells remaining in the supernatant were removed by an additional centrifugation step for 3 min at 20,000 × *g*. Proteins in the cell-free supernatant were either precipitated with 5% (wt/vol) trichloroacetic acid or incubated with chitin as described below. The cell pellets were washed with 1 ml of 0.9% (wt/vol) NaCl, resuspended in 500 μl of sonication buffer (50 mM Tris-HCl [pH 7.4], 20 mM EDTA), and sonicated trice for 15 s to disrupt the cells. Soluble cellular proteins were obtained by removing unbroken cells and cell envelopes by centrifugation for 30 min at 20,000 × *g* at 4°C. Periplasmic proteins were obtained from cells of 3-ml overnight cultures. After washing, the cells were resuspended in 1 ml of 50 mM Tris-HCl (pH 7.4)–0.2 M $MgCl_2$, and spheroplasts and periplasmic fractions were obtained as described previously (28).

Chitin-binding proteins were obtained by incubating soluble proteins from the cells or the cell-free culture supernatant with 1.5 mg of colloidal chitin from crab shells (Sigma), which was prepared as described (34). After rotating the reaction tubes for 1 h at room temperature, the chitin with bound proteins was pelleted by centrifugation for 3 min at 20,000 × *g*, washed twice with 0.9% NaCl, and resuspended in 15 μl of 2× sample buffer (25). After boiling the suspension for 10 min, the released proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 11% (wt/vol) polyacrylamide. The amounts of cellular and secreted proteins were quantified by using Image Quant (Molecular Dynamics).

Enzyme activity assays. For β-galactosidase assays, cells were grown overnight in LB medium supplemented with the appropriate antibiotics. The cultures were diluted 50-fold in fresh medium, and, at various time intervals, the optical density at 600 nm (OD_{660}) and the β-galactosidase activity (35) were measured. Alternatively, the cells were incubated in 10-fold-diluted LB supplemented with the appropriate antibiotics and with 0.4% (wt/vol) colloidal chitin, GlcNAc (Sigma), or glycerol, and the β-galactosidase activities were measured after overnight growth.

To detect chitinase production, colonies were streaked on LB or KB plates containing 0.05% (wt/vol) colloidal chitin. The plates were incubated at 37°C, and at regular time intervals, the formation of halos around the colonies as a result of chitin degradation was evaluated. The activity and specificity of the chitinase were determined by subjecting cell-free culture supernatants to various substrates. In standard assays, 25-μl samples of culture supernatant were incubated at 37°C with 25 μl of 0.1 M sodium acetate buffer, pH 5.2, and 25 μl of the soluble chitin polymer carboxymethyl-chitin-Remazol Brilliant Violet (CM-chitin-RBV) (Blue Substrates, Göttingen, Germany). After terminating the reactions by adding 25 μl of 1 M HCl, the samples were incubated on ice for 10 min and centrifuged for 10 min at 20,000 × *g* at 4°C to precipitate nondegraded substrate. The supernatants were transferred to a 96-well microtiter plate to read the absorption at 550 nm. One unit of enzyme activity was defined as the amount of enzyme which increased the OD_{550} value by 0.001 unit per min per OD_{660} of the original culture under the specified conditions.

To determine the optimal pH for activity, the reactions were carried out at 37°C, and the sodium acetate buffer of pH 5.2 was replaced by 0.1 M of buffers ranging in pH from 3.2 to 8. Buffered sodium acetate was used for pH 3.2 to 6, and Tris-HCl was used for pH 7 and 8. To determine the temperature optimum, the reactions were carried out at pH 5.2 at temperatures ranging from 20 to 70°C.

Exochitinase and endochitinase activities were distinguished by measuring the release of *p*-nitrophenol from the substrates *p*-nitrophenyl-*N*-acetyl-β-D-glucosamine (pNP-GlcNAc) (British Drug Houses) and *p*-nitrophenyl-β-D-*N*,*N*′,*N*″-triacetylchitotriose [pNP-$(GlcNAc)_3$] (Sigma), respectively (46). Samples (32.5 μl) of cell-free culture supernatant were incubated with 5 μl of substrate (3 mg/ml dissolved in sterile demineralized water) and 37.5 μl of 0.1 M sodium acetate buffer (pH 5.2) at 50°C. After 2.5 h of incubation, the reactions were terminated by adding 50 μl of 0.4 M Na_2CO_3, and the absorbance was measured

at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that increased the OD_{405} value by 0.001 unit per min per OD_{660} of the original culture under the specified conditions.

The ability of the bacteria to inhibit the growth of *Rhizoctonia solani* Kühn (a gift of Y. Hadar, Hebrew University of Jerusalem) or *Fusarium oxysporum* Schlecht f. sp. *raphani* Kendrick & Snyder (26) was determined by inoculating KB agar plates containing 200 μM $FeCl_3$ with bacterial strains and, after 2 days, with the fungi, as described previously (11). Alternatively, 100- to 250-μl samples of bacterial culture supernatant were loaded in wells punched in the agar, and a plug of one of the fungi was immediately inoculated at the center of the plate. Inhibition of fungal growth around the bacteria or the wells was monitored for 1 week.

N-terminal amino acid sequencing. The chitin-bound proteins from cell extracts and from culture supernatant of strain PAO25 were separated on an 11% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The protein bands corresponding to the chitinase were excised and used to determine the N-terminal amino acid sequence with an automatic protein sequencer, model ABI 476A (Perkin-Elmer). The sequencing was performed at the Utrecht University Sequencing Centre.

Computer programs. Database searches were performed with the Blast 2.0 service from the National Center for Biotechnology Information (NCBI) World Wide Web server. DNA sequence analysis was performed with DNAsis V2.1 (Hitachi Software Engineering Co., Ltd.). Multiple amino acid sequence alignments were performed with ClustalW version 1.8 and optimized by hand.

Nucleotide sequence accession number. The nucleotide sequence data have been deposited in GenBank under accession no. AF279793.

RESULTS

Identification of a chitinase in *P. aeruginosa*. Previously, we reported that colonies of *P. aeruginosa* formed halos on colloidal chitin plates and that they were able to inhibit the growth of the fungi *F. oxysporum* and *R. solani* (11). Both properties could be attributed to the production of a chitinolytic enzyme. To identify possible chitinolytic enzymes of *P. aeruginosa*, several Blast searches against the *P. aeruginosa* genome bank were performed using the sequences of *S. marcescens* chitinases ChiA (AB015996), ChiB (AB015997), and ChiC (L41660) as probes. No significant homologies were found with ChiA and ChiB. However, an amino acid sequence deduced from an open reading frame (ORF) had significant homology to ChiC (77% identity) (Fig. 1). This ORF, tentatively designated *chiC*, putatively encodes a protein of 483 amino acid residues and is preceded by a possible Shine-Dalgarno sequence (data not shown).

Comparison of the amino acid sequence of the putative gene product with entries in the protein data banks revealed three other proteins with significant homology, ChiC of another *S. marcescens* strain (AB019238) (data not shown), ChiA of *Vibrio harveyi* (Fig. 1), and ChiD of *Bacillus circulans* (Fig. 1). These proteins consist of a catalytic domain and a chitin-binding domain separated by a fibronectin type III (Fn3)-like module, which has been suggested to maintain an optimal distance and orientation between the catalytic and binding domains (48). The positions of the catalytic domain and the chitin-binding domain of ChiD of *B. circulans* are reversed compared with those of the other chitinases. Regions with homology to these three domains were clearly identified in the putative chitinase of *P. aeruginosa* at residues 98 to 148 (catalytic domain), residues 338 to 430 (Fn3 module), and residues 435 to 482 (chitin-binding domain) (Fig. 1). The amino acid sequences of the catalytic and chitin-binding domains of the different chitinases are well conserved, whereas considerable variability, in length and sequence, is present in the Fn3 domains (Fig. 1).

The chitinases of *S. marcescens* and *B. circulans* have been detected in culture supernatants of these strains (15, 51). To investigate whether the *chiC* gene of *P. aeruginosa* is expressed and the protein is released into the medium, extracellular chitin-binding proteins of strain PAO25 were analyzed by SDS-PAGE. Two protein bands were detected on the gels, one corresponding to the previously identified 43-kDa chitin-binding protein CbpD (11) and a very faint band of 55 kDa (results not shown, but see Fig. 4). The N-terminal amino acid sequence of the 55-kDa protein was determined to be AREDAA. These residues are identical to residues 12 to 17 of the deduced amino acid sequence of the putative chitinase (Fig. 1). Since screening of the genomic bank revealed that this AREDAA sequence is not present in any other (putative) protein of *P. aeruginosa*, it confirms that the gene encoding the putative chitinase is expressed.

To study whether this 55-kDa protein is indeed a chitinolytic enzyme, the *chiC* gene was cloned behind the *lac* promoter, resulting in plasmid pUWL13, and expressed in *E. coli*. After a week, small halos were observed on colloidal chitin plates around colonies harboring pUWL13, but not around colonies carrying the empty vector (data not shown). This result indicates that the ChiC protein has chitinolytic activity.

To investigate the presence of ChiC-mediated chitinolytic activity in the culture supernatant of *P. aeruginosa*, the *chiC* mutant PAN20 was constructed. The 55-kDa protein was not detected among the chitin-binding proteins in the culture supernatant of this mutant (data not shown). Furthermore, with CM-chitin-RBV as the substrate, hardly any chitinolytic activity was detected in the culture medium of PAN20 (Table 2). In contrast, such activity was detected in the culture medium of strain PAN11 (*lasB xcpR*), which is deficient in the production of elastase and in the secretion of type II exoproteins (Table 2), and which had an activity comparable to that of PAO25 (results not shown). Moreover, the activity in the culture medium of PAN11 was increased when the strain harbored plasmid pJA1 carrying the *chiC* gene (Table 2). Together, these results demonstrate that the *chiC* gene is expressed in *P. aeruginosa* and that it encodes an active chitinase.

Enzymatic characterization of ChiC. To determine the optimal pH and temperature for the activity of the chitinase, we used cell-free culture supernatant of strain PAO25 overproducing the chitinase from plasmid pJA1. With CM-chitin-RBV as the substrate, the chitinase activity appeared to be optimal between pH 4.5 and 5.0 and at 50°C. To determine whether ChiC is an exo- or an endochitinase, the activity of culture supernatants was tested on the appropriate substrates. For these experiments, the culture supernatant of the elastase-deficient strain PAN11 was used to prevent extracellular degradation of ChiC. The culture supernatant of PAN11 harboring pJA1 exhibited significant activity when pNP-$(GlcNAc)_3$ was used as the substrate (Table 2). In contrast, when pNP-GlcNAc was used as a substrate for exochitinase activity, hardly any activity was detected in the culture supernatants (Table 2). These data indicate that ChiC has endochitinase activity.

Endochitinase activity has been suggested to correlate with antifungal activity. However, PAO25 and PAN20 appeared to inhibit the growth of *F. oxysporum* and *R. solani* in vitro to a similar extent, probably as a result of antibiotic production. In

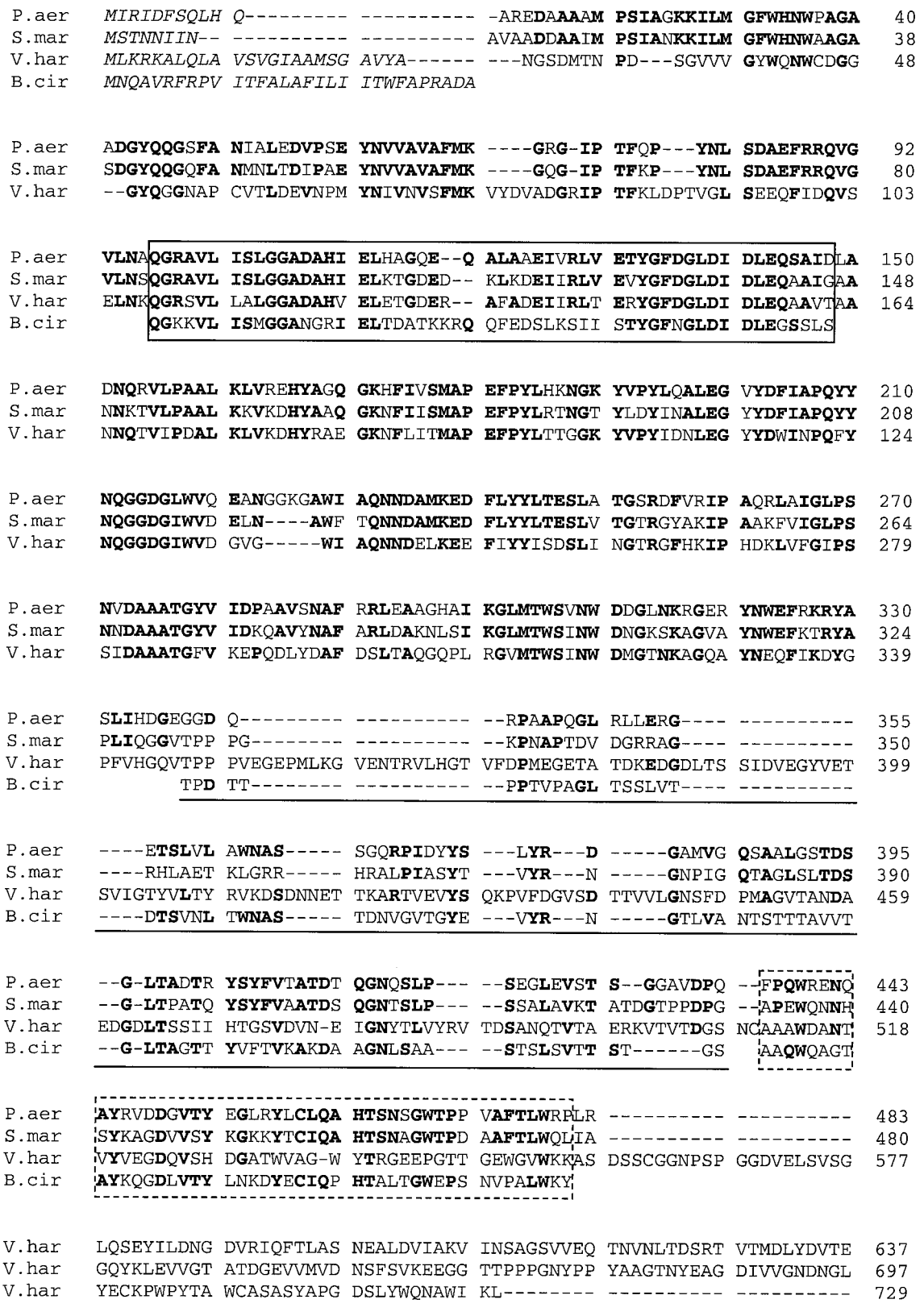


FIG. 1. Alignment of the amino acid sequence of ChiC of *P. aeruginosa* (P.aer) with ChiC of *S. marcescens* (S.mar) (accession. no. L41660), ChiA of *V. harveyi* (V.har) (U81496), and the relevant domains of ChiD of *B. circulans* (B.cir) (D10594). Amino acids identical to *P. aeruginosa* ChiC are indicated in bold. Cleaved N-terminal residues are indicated in italics. Boxed residues indicate the catalytic domain (solid) and the chitin-binding domain (dashed). The Fn3 modules are underlined. The catalytic, Fn3, and chitin-binding domains of ChiD comprise residues 269 to 322, 87 to 181, and 31 to 76, respectively.

TABLE 2. Chitinase activity in cell-free culture supernatants of *P. aeruginosa* strains on various substrates after growth for 24 h in LB medium

Strain	Genotype	Activity (U)[a]		
		CM-chitin-RBV	pNP-$(GlcNAc)_3$	pNP-GlcNAc
PAN11	*xcpR lasB*	29	ND	ND
PAN11(pJA1)	*xcpR lasB*	420	10.3 ± 2.1	0.6 ± 0.2
PAN20	*chiC*	1	1.2 ± 0.3	0.8 ± 0.2

[a] When standard deviations are given, the results are from three independent measurements. Otherwise, the results are representative of two independent experiments. ND, not determined.

contrast, concentrated culture supernatants of either bacterial strain, after dialysis to remove antibiotics and siderophores, did not inhibit the growth of *F. oxysporum* and *R. solani* at all (data not shown). These data suggest that the chitinase of *P. aeruginosa* has no antifungal activity under the conditions tested. However, the possibility that the enzyme lost activity during the assay, which lasts for several days, cannot be excluded.

Expression of *chiC*. In *S. marcescens*, expression of chitinases is induced when this strain is grown in yeast extract-supplemented minimal medium containing colloidal chitin, whereas the expression is repressed in the presence of GlcNAc (29, 49). Therefore, we investigated the promoter activity of *chiC* using the reporter construct pJA5, which carries *lacZ* under the control of the *chiC* promoter. After overnight growth of strain PAO25 carrying pJA5 in LB medium, high β-galactosidase activity comparable to that of strain PAO25 carrying pPB107 (*lasB′-lacZ*) was detected (Table 3). However, supplemention of 10-fold-diluted LB medium with either colloidal chitin, GlcNAc, or glycerol did not greatly affect β-galactosidase activity (Table 3). Thus, the *chiC* promoter activity was neither induced nor repressed when colloidal chitin or GlcNAc was present as a carbon source.

In *P. aeruginosa,* quorum sensing regulates the secretion of exoproteins, such as elastase (LasB). Once the concentration of autoinducers has reached a certain threshold level, generally in the late logarithmic phase, the expression of genes encoding exoproteins and secretion systems is induced (44). To investigate whether the expression of *chiC* is also controlled by quorum sensing, we determined the β-galactosidase activity in cultures of PAO25 harboring pJA5 during growth in LB medium. The β-galactosidase activity increased about 10-fold when the cultures entered the late logarithmic phase, similar to what was observed for a culture of PAO25 carrying pPB107 (*lasB′-lacZ*) (Fig. 2). These results indicate that quorum sensing regulates the expression of *chiC*. This notion was underscored by the observation that expression of *chiC′-lacZ* was strongly reduced in PAN32 (*lasIR vsmI*), a mutant which does not produce homoserine lactone autoinducers (Table 3). The expression levels of *chiC* and *lasB* were twofold higher in the late logarithmic phase (Fig. 2) compared to stationary-phase cultures (Table 3), suggesting that the expression of both genes decreases after prolonged incubation. However, we did not study this further.

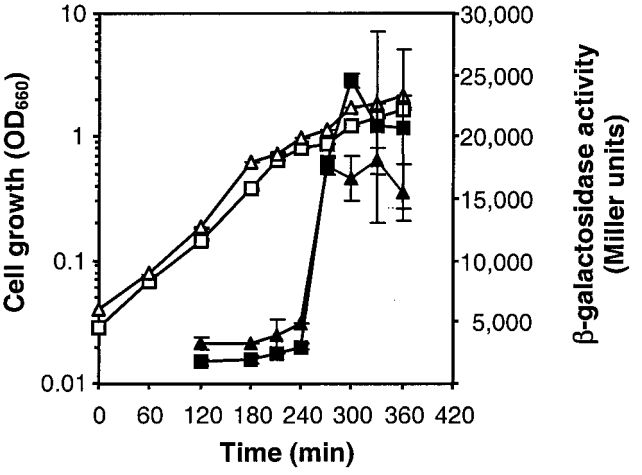


FIG. 2. Growth curves (open symbols) and β-galactosidase activities (solid symbols) of *P. aeruginosa* strain PAO25 harboring pJA5, which carries a *chiC′-lacZ* fusion (triangles), or pPB107, which carries a *lasB′-lacZ* fusion (squares). At 0 h, the cultures were diluted 1:50 in fresh LB medium. At various time points, cell growth and β-galactosidase activity were measured.

Localization of ChiC. Based on the β-galactosidase activities measured with the promoter-reporter fusions, the promoter activity of *chiC* appeared comparable to that of *lasB*. However, the amount of protein produced from *chiC* detected in the culture medium after overnight growth was much lower than that of elastase and of CbpD, suggesting either that the protein is unstable or that very little is secreted and it accumulates intracellularly. To distinguish between these possibilities, the localization of ChiC was studied in cultures of strains PAN8 (*aprE lasB*) and PAN10 (*lasB*). These strains, which are deficient in the production of the extracellular protease elastase and, in the case of PAN8, of alkaline protease as well, were chosen to diminish the chance of extracellular degradation of ChiC.

TABLE 3. β-Galactosidase activity in *P. aeruginosa* PAO25 cells expressing the *chiC′-lacZ* or *lasB′-lacZ* fusion

Plasmid	Fusion	β-Galactosidase activity (Miller units)[a]					
		PAO25 (wild-type)					PAN32 (*lasIR vsmI*), LB
		LB	1/10 LB	1/10 LB + glycerol	1/10 LB + GlcNAc	1/10 LB + chitin	
pMP220		203 ± 28	225	63	58	44	169 ± 72
pJA5	*chiC′-lacZ*	7,758 ± 960	1,590	1,259	1,119	1,044	152 ± 42
pPB107	*lasB′-lacZ*	12,383 ± 2,570	ND	ND	ND	ND	104 ± 51

[a] When standard deviations are given, the results are from at least four independent measurements. Otherwise, the results of a representative experiment are shown. ND, not determined. Cells of PAO25 and PAN32 were grown overnight in LB or in 10-fold-diluted LB (1/10 LB) supplemented with a carbon source.

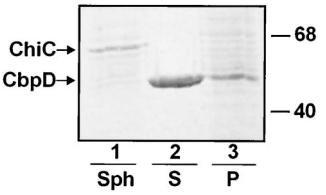
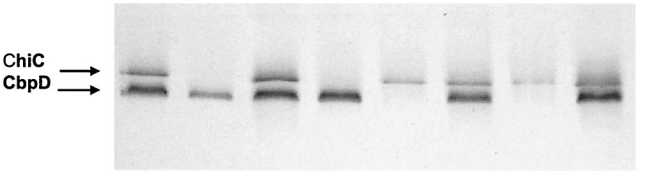


FIG. 3. Localization of ChiC in an overnight culture of *P. aeruginosa* strain PAN8 (*aprE lasB*). The cells were separated from the culture supernatant (S) and fractionated further into spheroplasts (Sph) and periplasmic proteins (P). The proteins that bound to chitin were analyzed on Coomassie blue-stained polyacrylamide gels. For each fraction, equivalents of 3 ml of culture were analyzed. The positions of the chitin-binding protein CbpD and chitinase ChiC are indicated at the left. The positions of molecular mass protein markers (in kilodaltons) are shown at the right.



	C	S	C	S	C	S	C	S
	day 1		day 2		day 3		day 4	
% ChiC out	2		5		45		69	
% CbpD out	31		54		94		96	
viable cells ($*10^9$/ml)	1.7		2.3		3.2		2.8	

FIG. 4. Coomassie blue-stained polyacrylamide gel containing chitin-bound proteins from cells (C) and culture supernatant (S) of *P. aeruginosa* strain PAN8 (*lasB aprE*). The strains were grown for 4 days in LB at 30°C, and every 24 h samples were taken. For each fraction, equivalents of 2 ml of culture were loaded on the gels. The positions of ChiC and CbpD are indicated at the left. The amounts of ChiC and CbpD were quantified, and the amount of secreted protein is shown as a percentage of the total amount of the proteins present in the C and S fractions at each time point. The total number of viable cells is expressed in 10^9 CFU.

Chitin-binding proteins were isolated from various cell fractions and from the culture supernatants. The amounts and the localization of ChiC were similar for both strains, and only the results for PAN8 are shown. After overnight growth, hardly any ChiC was detected in the culture supernatant (Fig. 3, lane 2), but a considerable amount was present as a soluble protein in the spheroplast fraction (Fig. 3, lane 1). These results suggest that ChiC is a cytoplasmic protein. In contrast, most of the CbpD was present in the culture supernatant, with the remainder being in the periplasmic fraction (Fig. 3, lane 3), as expected for a protein secreted via the type II pathway. Because the intracellularly detected ChiC bound chitin, this protein seems to fold properly within the cytoplasm. From these experiments, it is not clear whether the presence of small amounts of extracellullar chitinase results from active secretion or from cell lysis.

Secretion of ChiC. The results presented in the previous paragraph suggested that ChiC is a cytoplasmic rather than a secreted protein. However, it was reported that the chitinolytic activity in the culture medium of *S. marcescens* increases over a growth period of several days (49). Therefore, we investigated the possibility that the secretion of ChiC occurs only after a longer growth period. To limit the amount of cell lysis, this experiment was performed at 30°C.

Indeed, the amount of ChiC in the culture medium of *P. aeruginosa* strain PAN8 (*aprE lasB*) increased substantially over a growth period of 4 days, whereas the amount of intracellular ChiC decreased steadily (Fig. 4). Usually, 60 to 90% of the total pool of ChiC was present in the culture supernatant after 4 days. Apparently, ChiC first accumulates in the cytoplasm and is then slowly but efficiently secreted into the medium. In contrast, half of the amount of the type II exoprotein CbpD was already secreted after overnight growth, and after 3 days, secretion was essentially complete (Fig. 4). This shows that CbpD is secreted efficiently after initial accumulation in the periplasm.

Cell growth was analyzed by counting the number of CFU (Fig. 4). This parameter increased during the first 3 days of growth and decreased slightly on day 4. In addition, Coomassie brilliant blue-stained gels, on which cells and culture supernatants were analyzed, revealed that 80 to 90% of the total amount of cellular proteins was still found in the cell pellet after 4 days of growth (data not shown). These data indicate the absence of severe cell lysis during the 4-day growth period. As an additional control for cell leakage, we studied the localization of the specific cytoplasmic marker β-galactosidase, which was placed under the control of the *chiC* promoter in chitinase-expressing *Pseudomonas* cells. After 4 days of growth at 30°C, on average only 11% of the total β-galactosidase activity could be detected in the supernatant. Therefore, it can be concluded that ChiC is actively secreted rather than being released into the culture medium as a result of cell lysis.

Extracellular ChiC does not contain the N-terminal 11 amino acids (see above). To analyze whether this protein is processed only upon secretion, the N-terminal amino acid sequence of isolated cytoplasmic chitinase was determined. This sequence, MIRIDF, showed that intracellular ChiC is not processed at the N terminus. Therefore, the full-length form is a soluble, cytoplasmic protein, which is able to bind chitin. It should be mentioned that the putative N-terminal leader fragment does not resemble a classical signal peptide.

Since strain PAN8 carries an *aprE* mutation, the type I secretion system required for the secretion of alkaline protease cannot be involved in the secretion of ChiC. To investigate whether the chitinase is secreted via one of the other known secretion pathways, the intra- and extracellular protein profiles of various mutant derivatives of *P. aeruginosa* were analyzed over 4 days of growth. Strain PAN9 (*lasB aprE xcpQ*), which is defective in the type II secretion system because of an *xcpQ* mutation, and PAN8 produced and secreted similar amounts of ChiC (data not shown). Therefore, ChiC is not secreted via the type II secretion system either. Similarly, a type III secretion mutant of PAO1, strain PAO1*exsA*, secreted normal amounts of ChiC into the culture medium (data not shown), indicating that the type III secretion system is also not involved.

Since the known secretion systems did not appear to be involved in the secretion of ChiC, we investigated the possibility that chitinase itself contains all the functions required for its secretion within its primary structure. In that case, one would expect that the protein would also be secreted when expressed

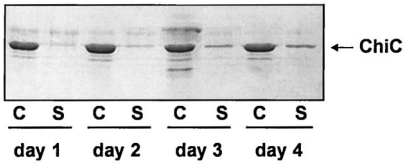


FIG. 5. Coomassie blue-stained polyacrylamide gel containing chitin-bound proteins from cells (C) and culture supernatant (S) equivalent to 3 ml of culture of *P. putida* WCS358(pJA1). The strain was grown for 4 days in LB medium at 30°C, and each day samples were taken. The position of ChiC is indicated at the right.

in a heterologous host. To test this possibility, plasmid pJA1, encoding *chiC*, was introduced into *Pseudomonas putida* WCS358 and into *E. coli* DH5α. In these organisms, ChiC was produced efficiently. However, only minor amounts of ChiC were detected in the culture medium of *P. putida* (Fig. 5) or *E. coli* (data not shown), even after 3 or 4 days of growth. The small amount of extracellular chitinase may be due to cell lysis. These results indicate that ChiC is not actively secreted when expressed in *P. putida* WCS358 or *E. coli* and that secretion of ChiC by *P. aeruginosa* requires a specific, hitherto unidentified secretion system.

DISCUSSION

In this study, we identified a chitinase (ChiC) in *P. aeruginosa* with homology to the chitinases ChiC of *S. marcescens*, ChiA of *V. harveyi,* and ChiD of *B. circulans*. ChiC of *P. aeruginosa* is most homologous to ChiC of *S. marcescens*, which has been identified in two *S. marcescens* strains (15, 42). ChiC of *P. aeruginosa* also resembles ChiC of *S. marcescens* in molecular weight, substrate specificity, and temperature and pH optima. However, because the *chiC* gene has a high G+C content (67%) and the codon usage is characteristic of *P. aeruginosa*, the gene was probably not recently acquired by horizontal gene transfer. The G+C content of ChiC of *S. marcescens* is much lower (50% G+C) than that of the *P. aeruginosa* ChiC and therefore does not support a recent reciprocal gene transfer either.

In *S. marcescens*, part of the extracellular ChiC is processed, since both the full-length and a C-terminally truncated protein lacking the Fn3 and chitin-binding domains were detected in the culture medium (15, 42). In *P. aeruginosa*, such processing was not observed. However, as was also reported for ChiC of *S. marcescens* (15, 42), N-terminal processing resulting in cleavage of the first 11 residues of ChiC was found to occur. Importantly, the N-terminally cleaved fragments of the two chitinases lack the typical features of a signal sequence (47), indicating that these chitinases are not secreted via a Sec-dependent pathway. In contrast, ChiA of *V. harveyi* and ChiD of *B. circulans* each contain a typical signal sequence (43, 50) (see also Fig. 1). In gram-positive bacteria, such as *B. circulans*, the Sec system is sufficient for the transport of exoproteins into the extracellular medium, since they only need to cross the cytoplasmic membrane. In gram-negative bacteria, an additional transport system, most likely a type II secretion system in the case of the chitinase of *V. harveyi*, is required for translocation of proteins across the outer membrane.

Analysis of the secretion of ChiC in mutants of *P. aeruginosa* excluded the involvement of the Apr type I system, the *xcp*-encoded type II system, and the type III system in the secretion process. This raises the question of how the protein is secreted. Of course, additional conventional secretion machineries could be encoded by the pseudomonad genome. Genome analysis revealed genes for neither an additional type III secretion system nor a type IV system (examples of which are involved in pertussis toxin secretion and DNA transfer) in the *P. aeruginosa* genome sequence (39).

In addition to the *xcp* genes, the *P. aeruginosa* genome does contain another set of genes with homology to type II secretion genes. However, since ChiC lacks a typical N-terminal signal sequence, it is unlikely to be secreted via this putative second type II secretion system. Besides the type I secretion system involved in the secretion of alkaline protease, the genome appears to encode four additional (putative) type I systems (39), which do not have to be involved in protein secretion. One of these type I systems is involved in the secretion of a heme-binding protein (27), whereas no proteins have been found so far to be secreted via one of the other systems.

We did not test the secretion of ChiC in mutants deficient in these other type I secretion systems. Therefore, it remains a possibility that ChiC is secreted via one of these systems. However, a number of considerations do not support this idea. First of all, most genes encoding type I exoproteins are clustered with their cognate secretion genes. This is not the case for *chiC*, as deduced from the genome sequence (http://pseudomonas.bit.uq.edu.au). Moreover, type I exoproteins usually contain a secretion motif within their C-terminal end, which consists of a negatively charged residue followed by several hydrophobic residues (17). This motif is present at the extreme C-terminal end of proteases, lipases, and the heme-binding protein and has been reported to be essential for the secretion of proteases (17). ChiC does not contain this C-terminal amino acid motif and does not have any obvious sequence homology to type I secreted proteins, either in the last 50 residues, where the secretion signal is supposedly located, or in the rest of the protein (data not shown). Moreover, cytoplasmic ChiC is able to bind chitin, which suggests that the protein is folded in the cytoplasm. Since the outer membrane component of type I secretion systems is a trimeric complex with a channel that is not large enough to allow the passage of folded proteins (24), type I exoproteins are believed to be translocated in an unfolded conformation. Therefore, cytoplasmic folding argues against secretion via a type I secretion pathway. However, the possibility that folding of the cytoplasmic chitinase occurred during the isolation procedure cannot be totally excluded. On the other hand, it is highly unlikely that an unfolded protein is stable for the long period of time required for ChiC secretion. Finally, extracellular ChiC is present in a processed form, missing the N-terminal 11 amino acids. This region could function as a specific leader peptide, directing the protein to the secretion machinery. Subsequently, this peptide will be removed during transport into the medium. Alternatively, this domain could be removed in the medium by extracellular proteases, although this N-terminal processing also takes place in strain PAN8. This strain is deleted for the genes encoding the two most prominent extracellular proteases, elastase and alkaline protease.

Secretion of ChiC via an autotransporter system (23) can

also be excluded, since ChiC possesses neither a classical N-terminal signal sequence nor a putative β-barrel structure at the C terminus with the characteristic signature of outer membrane proteins (40). Besides, ChiC was not secreted when expressed in *E. coli* or *P. putida*, as would be expected if all the secretion functions required are contained within the protein itself. Altogether, these results and considerations indicate that ChiC is probably secreted via a novel secretion system.

Since ChiC of *S. marcescens* is very homologous to ChiC of *P. aeruginosa* and also lacks a classical signal sequence, *S. marcescens* may contain a similar secretion system. Remarkably, it has been reported that this *S. marcescens* chitinase could be isolated from the culture medium when the gene was expressed in *E. coli* (15). However, the amount of protein secreted was not quantified and could have resulted from cell lysis. In our experiments, some release of ChiC was also evident from the degradation of colloidal chitin around colonies of *E. coli* expressing *chiC*. Moreover, Suzuki et al. (42) expressed *chiC* from another *S. marcescens* strain in *E. coli* and observed that this ChiC also accumulated intracellularly.

S. marcescens produces at least three chitinases (ChiA, -B, and -C) and a chitin-binding protein (CBP21) (41), which together have been proposed to degrade chitin synergistically. Although the synthesis of these proteins seems to be regulated similarly (41), they are probably secreted via different secretion pathways. Only ChiA and CBP21 possess a typical signal sequence and are likely to be secreted via a type II system. As described, ChiC has an atypical leader peptide, whereas ChiB is processed at the C terminus (14). The secretion mechanisms of these proteins remain to be elucidated. *P. aeruginosa* produces, in addition to the chitinase ChiC, the chitin-binding protein CbpD, which is secreted via the type II system (11). CBP21 of *S. marcescens* has no chitinolytic activity, and its role in chitin degradation is not clear. Similarly, CbpD has no chitinolytic activity, and it did not seem to support the activity of ChiC on the substrates colloidal chitin and CM-chitin-RBV (11).

In *P. aeruginosa*, the regulation of the expression of exoproteins that use different secretion pathways, such as elastase and alkaline protease, is controlled by the same quorum-sensing system (44). Although expression of *chiC* is also dependent on this quorum-sensing system, ChiC is secreted very slowly compared to elastase and CbpD. Why are the secretion rates of these proteins different and how are they regulated? Possibly a signal (intra- or extracellular) is required for secretion of exoproteins, and this signal may be different for ChiC and the type II exoproteins. The difference in secretion profiles suggests that ChiC is needed in another growth phase than CbpD and elastase, at least in vitro.

To verify this, the biological role of ChiC needs to be clarified. Probably, this role of ChiC in *P. aeruginosa* is different from that of ChiC in *S. marcescens*. *S. marcescens* secretes several chitinases and a chitin-binding protein into the extracellular medium, which enables this bacterium to degrade colloidal chitin very efficiently and to utilize chitin as the sole carbon source (49, 51). However, *P. aeruginosa* is not able to utilize chitin as the sole carbon source (unpublished observation), probably because it lacks a transport system for the uptake of the products released from chitin by ChiC. This enzyme cannot release GlcNAc efficiently from chitin, as shown with the artificial substrates (Table 2). On the contrary, *P. aeruginosa* has a transporter for monomeric GlcNAc residues, but not for chitobiose [$(GlcNAc)_2$] (39). This is in agreement with the observation that *P. aeruginosa* can grow on minimal medium with GlcNAc but not with chitobiose as the sole carbon source (unpublished results). Furthermore, enzymes that can hydrolyze chitobiose into GlcNAc seem to be lacking in *P. aeruginosa*, a conclusion which is based on the absence of annotation and on Blast searches with the chitobiases of *E. coli* (accession no. P17411) and *S. marcescens* (L43594) as probes. This suggests that ChiC (and CbpD) is not involved in hydrolysis of colloidal chitin for its utilization as a carbon source.

Interestingly, ChiC was also produced by clinical isolates of *P. aeruginosa* (unpublished observation), which also produced CbpD (11), suggesting that these proteins might play a role in pathogenicity. Human macrophages have also been reported to secrete a chitinolytic enzyme and a chitin-binding protein as well (33). The chitin-binding protein has also been found in patients with recurrent breast cancer and was suggested to play an important role in tumor invasion. The physiological function of these human proteins is unknown, but they were proposed to have a role in morphogenetic events by rearrangement of the extracellular matrix (33). Involvement of chitinases in developmental processes has also been documented in plants (reviewed in reference 7) and zebrafish (37). Interestingly, water-soluble chitin, which can be hydrolyzed by chitinases, has been reported to accelerate wound healing (6). Since *P. aeruginosa* is known to infect (burn) wounds, ChiC (and also CbpD and elastase) might have a function in retarding wound healing, thus enabling the bacteria to establish infection. To gain evidence for this idea, the role of these proteins in humans needs further clarification.

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