Chitinolytic Activity in *Chromobacterium violaceum*: Substrate Analysis and Regulation by Quorum Sensing

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Quorum sensing control mediated by N-acyl homoserine lactone (AHL) signaling molecules has been established as a key feature of the regulation of exoenzyme production in many gram-negative bacteria. In *Chromobacterium violaceum* ATCC 31532 a number of phenotypic characteristics, including production of the purple pigment violacein, hydrogen cyanide, antibiotics, and exoproteases are known to be regulated by the endogenous AHL *N*-hexanoyl-1-homoserine lactone (HHL). In this study we show that *C. violaceum* produces a set of chitinolytic enzymes whose production is regulated by HHL. The chitinolytic activity was induced in strains grown in the presence of chitin as the sole carbon source and quantitated in the secreted proteins by using p-nitrophenol analogs of disaccharide, trisaccharide, and tetrasaccharide oligomers of *N*-acytetylglucosamine. By using 4-methylumbelliferyl analogs of the same oligomers of *N*-acytetylglucosamine as substrates for proteins separated and renatured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, at least six enzymes were detected: a chitobiase with high specificity to a dimeric substrate of 87 kDa, two *N*-acytetylglucosaminidases with apparent molecular masses of 162 and 133 kDa, two endochitinases of 108 and 67 kDa, and a chitobiosidase of 56 kDa. In addition, two unidentified bands of >205 kDa were found where a tetrameric chitin derivative was used as a substrate. A pleiotropic mini-Tn*5* mutant of *C. violaceum* (CV026) that is defective in HHL production and other quorum-sensing-regulated factors was also found to be completely deficient in chitinolytic activity. Growth of this mutant on minimal medium with chitin supplemented with culture supernatant from the *C. violaceum* wild-type strain or 10 μM synthetic HHL restored chitinase production to the level shown by the parental strain. These results constitute the most complete evidence so far for regulation of chitinolytic activity by AHL signaling in a gram-negative bacterium.

Many species of bacteria are known to synthesize enzymes capable of degrading chitin, an insoluble linear polymer consisting of β-1,4-linked N-acetylglucosamine (GlcNAc) units that forms the main structural component of cell walls of most fungi and arthropods (33, 43). Among gram-negative bacteria, chitinolytic activity has been described for strains from the genera *Aeromonas* (3, 16), *Alteromonas* (53), *Enterobacter* (5), *Pseudomonas* (11, 54, 57), *Serratia* (20, 29, 39), *Ewingella* (17), and *Vibrio* (1, 18, 60). Although studies on chitinolytic activity in *Vibrio* (1, 21, 46) and *Pseudoalteromonas* (49) spp., have shown that soluble oligosaccharides liberated by the action of extracellular chitinase on chitin elicit the induction of expression of a number of proteins, little is known about the genetic regulation of chitinolytic enzyme expression in gram-negative bacteria.

In strain *Pseudomonas fluorescens* BL915, studied by Gaffeney et al. (11), expression of uncharacterized chitinolytic activity is regulated by a two-component system consisting of a transmembrane environmental sensor protein (LemA) and a cytoplasmic response regulator protein (GacA) (7, 25, 38). Cloning of the gacA regulatory region from strain BL915 in certain heterologous soil isolates of *P. fluorescens* was found to stimulate expression of otherwise latent chitinase genes (11), indicating that global regulation by two-component regulators may be a common feature of the regulation of chitinase expression.

It has emerged over the last few years that expression of many phenotypic characteristics in late-growth-phase bacterial cultures, including cell differentiation and the production of secondary metabolites and exoenzymes, is a cell density-dependent phenomenon mediated by intercellular communication in a process known as quorum sensing (10). In gram-negative bacteria, quorum sensing control is typically regulated by N-acyl homoserine lactone molecules (AHLs). These signal molecules have been reported to control a multitude of characteristics (reviewed in references 9 and 48), including extracellular enzyme production in *Pseudomonas aeruginosa* (19, 24, 32, 57), *Erwinia carotovora* (19, 35), and *Chromobacterium violaceum* (50, 51). At the core of the majority of AHL-based quorum sensing systems so far described are genes encoding protein products with homology to LuxI and LuxR from *Vibrio fischeri* (8, 10). Proteins with homology to LuxI are responsible for AHL biosynthesis, whereas LuxR homologs act as transcriptional activators, interacting with the accumulated AHL small molecule signals to stimulate gene expression (for reviews see references 9, 28, and 48). There is good evidence to suggest that in *Pseudomonas* AHL-mediated regulation may in turn be controlled by a global GacA-LemA regulation system (37). Thus far, more than 16 genera of gram-negative bacteria have been reported to utilize AHL regulation in the control of a variety of metabolic traits (9, 48). However, despite the widespread...
nature of this means of communication in gram-negative bacteria, the involvement of AHL signaling in the regulation of chitinase(s) has only been demonstrated in Pseudomonas aeruginosa PA01, where butanoyl-L-homoserine lactone synthesized by the VsmI (RhlII) protein was shown to restore uncharacterized extracellular chitinolytic activity in the P. aeruginosa pleiotropic mutant PAN067 (57).

A strain of C. violaceum, ATCC 12472, selected from a variety of chitin-utilizing bacterial species as the most active in chitin degradation, has previously been shown to grow on crystalline or colloidal chitin as its sole carbon and nitrogen source (45). More recently, in C. violaceum ATCC 31532 (a strain originally isolated as a monocambact antibiotic producer [55]), the production of a variety of factors, including violacein pigment, antibiotic hydrogen cyanide, and proteases, has been shown to be controlled by the endogenous AHL inducer molecule N-hexanoyl-L-homoserine lactone (HHL) (26, 50, 56). We describe here the existence of a number of chitinolytic enzymes in C. violaceum ATCC 31532 and demonstrate that chitinolytic activity is controlled by quorum sensing regulation mediated by the endogenous AHL HHL.

MATERIALS AND METHODS

Cultures and growth media. Three related strains of C. violaceum were used in this work: the wild-type C. violaceum ATCC 31532 (CVWT) (HHL producer) and two mutants affected in quorum sensing regulation obtained after mini-Tn5 mutagenesis of a spontaneous streptomycin-resistant mutant of CVWT, CV017 (Smr mini-Tn5 Hgr) produces HHL and carries a genetically uncharacterized mutation causing derepression of the HHL-inducible violacein pigment production at 30°C; CV026 (Smr mini-Tn5 Hgr cvi::Tn5xylE) is a non-HHL producer derived from CV017 as a result of mini-Tn5 insertion in the cvi gene encoding the LexA homolog, CviI. This mutant is unpigmented unless provided with exogenous AHL and thus acts as a biosensor (26, 56). The Enterobacter agglomerans strains IC1270 and 40b were described previously (4, 47), were used as controls. For bacterial growth, liquid or solid (1.5% [wt/vol] agar) LB were used. To induce chitinolytic activity, bacteria were grown in SM with 0.2% (wt/vol) colloidal chitin and 10% (vol/vol) LB. The chitinolytic enzymes appeared as fluorescent bands under UV light because of enzymatic hydrolysis of fluorescent 4-methylumbelliferyl-β-D-glucosaminide (4-MU-GlcNAc) or 4-methylumbelliferyl-β-D-nitrophenyl-GlcNAc (4-MU-[GlcNAc]0). The standard reaction mixture contained ca. 10 mg of the proteins tested and bovine serum albumin as a standard.

Detection of chitinolytic enzymes after SDS-PAGE. Proteins concentrated in the Micro-ProDiCon system were used in sample buffer (22) without 2-mercaptoethanol (except when specifically indicated) and incubated for 10 min at room temperature prior to loading. Where sample buffer containing 2-mercaptoethanol was used, the samples were boiled for 4 min prior to loading. The proteins were separated by sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gel electrophoresis (PAGE). Enzymes were reactivated in the gels by removing SDS by the casein-EDTA procedure (27) as modified by Haran et al. (13). Enzyme activity was detected on gels by using fluorescent substrates as described by Troumenn and Hamran (52). The molecular weights of the chitinolytic enzymes were estimated by using range prestained standards (Bio-Rad Laboratories). Proteins separated by SDS-PAGE were stained with Coomassie brilliant blue G-250 prepared as described by Neuhoff et al. (30). Each lane contained about 30 µg of protein.

RESULTS

Chitinolytic activity. C. violaceum CVWT and CV017 hydrolysed colloidal chitin after 72 to 96 h of growth on minimal agar (SM plus LB at a 1:10 ratio) supplemented with colloidal chitin as the sole carbon source. Large zones of clearing around the growing bacteria were observed (Fig. 1). Two additional strains were tested on the same plate as the controls. Enterobacter agglomerans IC1270 was previously shown to produce chitinolytic activity containing β-1,4-linkage to the GlcNAc monosaccharide (in the case of 4-MU-GlcNAc) or oligosaccharides [in the case of 4-MU-(GlcNAc)2 and 4-MU-(GlcNAc)3] (Sigma). These compounds served as analogs of disaccharide, trisaccharide, and tetrasaccharide chitin derivatives, respectively, with the 4-methylumbelliferyl group linked by β-1,4 linkage to the GlcNAc monosaccharide (in the case of 4-MU-GlcNAc) or oligosaccharides [in the case of 4-MU-(GlcNAc)2 and 4-MU-(GlcNAc)3] (Sigma). These compounds served as analogs of disaccharide, trisaccharide, and tetrasaccharide chitin derivatives, respectively, with the 4-methylumbelliferyl group linked by β-1,4 linkage to the GlcNAc monosaccharide (in the case of 4-MU-GlcNAc) or oligosaccharides [in the case of 4-MU-(GlcNAc)2 and 4-MU-(GlcNAc)3] (Sigma).

Chitinase activity was detected on gels by using fluorescent substrates as described by Troumenn and Hamran (52). The molecular weights of the chitinolytic enzymes were estimated by using range prestained standards (Bio-Rad Laboratories). Proteins separated by SDS-PAGE were stained with Coomassie brilliant blue G-250 prepared as described by Neuhoff et al. (30). Each lane contained about 30 µg of protein.

These results suggested that the chitinolytic activity secreted into the culture medium by C. violaceum is subject to quorum sensing regulation stimulated by the HHL signal molecule. To investigate this chitinolytic activity further with a quantitative assay, supernatants from 72-h cultures of the C. violaceum wild-type, CV017, and CV026 strains grown in liquid SM with chitin and of the CV026 strain grown in the same medium supplemented with HHL (10 µM) were assayed. The chitinolytic activity of extracellular proteins was examined by assessing the release of the chromophore pNP in reaction mixed with chromogenic analogs of di- and trioligomers of GlcNAc.
The activity was observed with both of these substrates (Fig. 3), but the level was found to be strain specific; in the extracellular proteins of strain CVWT it was higher in the reaction with the pNP monosaccharide derivative (pNP-GlcNAc) and lower with pNP-(GlcNAc)₂ (Fig. 3, columns 1 and 2), while proteins secreted by strain CV017 exhibited almost the same level of activity with both substrates (Fig. 3, columns 3 and 4). Almost no activity was found in secreted proteins of the mutant CV026 (Fig. 3, columns 5 and 6) grown on SM with chitin. However, when CV026 was grown on SM with chitin supplemented with HHL, the chitinolytic activity was restored to a level comparable to that of strain CV017 (Fig. 3, columns 7 and 8). The same restoration of chitinolytic activity was observed in a culture of CV026 when a filtrate of conditioned culture medium of strain CVWT was added to SM plus chitin medium (data not shown). These results indicate that when grown in chitin-containing medium, *C. violaceum* CVWT and CV017 produce chitinolytic enzyme(s) in their culture supernatants which exhibit activity against di- and trioligomers of GlcNAc. Only very weak chitinolytic activity was detected in fractions containing intracellular proteins. The chitinolytic activity was restored in culture supernatants of the HHL-deficient and chitinase-deficient mutant CV026 by growth in the presence of either synthetic HHL or culture supernatant from CVWT. None of these strains showed constitutive chitinolytic activity when the chitin in the medium was replaced with glucose or sucrose, independent of HHL supplementation (data not shown).

**Identification of chitinolytic enzymes.** Preliminary experiments with CM-chitin-RBV to detect endochitinase activity directly in *C. violaceum* wild-type and mutant culture supernatants and after SDS-PAGE separation of extracellular proteins showed endochitinase activity in CV017 (data not shown). To determine the specific chitinolytic activities of extracellular proteins which had been renatured following their separation by SDS-PAGE, a set of three fluorescent chitin derivatives were used. Chitinolytic enzymes appear as fluorescent bands under UV light as a result of hydrolysis of the fluorescent substrate 4-methylumbellifereone from the GlcNAc mono- and oligosaccharides. Although no fluorescent bands were seen with extracellular proteins of mutant CV026 growing on medium containing chitin (Fig. 4, lanes 1), supplementation of the same culture medium with 10 μM HHL resulted in a band pattern similar to that obtained for CV017 (Fig. 4, lanes 2 and 3). Extracellular proteins exhibiting chitinolytic activity were designated according to their apparent molecular masses. Bands of
Chit133 and Chit162 were detected with all three substrates used; bands of Chit67 and Chit108 were detected with analogs of trimeric \([4\text{-MU-(GlcNAc)}_2]\) and tetrameric \([4\text{-MU-(GlcNAc)}_3]\) chitooligosaccharides, and a band of Chit87 appeared only with the dimeric analog \(4\text{-MU-(GlcNAc)}\). Chit56 activity was revealed only with the trimeric analog, although the activity was lower than for Chit67.

Most of these bands of chitinolytic activity were found to be similarly nonrenatured by casein-EDTA after being boiled in sample buffer with 2-mercaptoethanol (Fig. 4, lanes 4). However, bands of Chit56 and Chit67 were just visible with 4-MU-(GlcNAc)_3 after this treatment (Fig. 4C, lane 4), and only the band of 67 kDa could be observed with the 4-MU-(GlcNAc)_2 after extended (up to 1 h instead of a few minutes) incubation of the gel with the substrate. Two additional bands of chitinolytic activity, each with a molecular mass higher than 200 kDa, were observed in secreted proteins when the tetrameric, but not the dimeric or trimeric, chitin derivatives were used as substrates. However, these bands were absent from preparations boiled in the presence of 2-mercaptoethanol (Fig. 4C, lane 4) or heated at 55°C for 3 min (data not shown).

Bands corresponding to the chitinolytic enzymes described above could be observed by SDS-PAGE after Coomassie blue staining of extracellular proteins produced by strains CV017 and CVWT grown in the presence of colloidal chitin or mutant CV026 grown on SM plus chitin supplemented with HHL. These proteins were not visible or were very weakly visible when strains CV017 and CVWT were grown on glucose (or sucrose) instead of chitin or when the mutant CV026 was grown on SM plus chitin without the addition of HHL (data not shown).

**DISCUSSION**

A number of phenotypic traits of *C. violaceum* are known to be controlled by quorum sensing regulation mediated by an AHL molecule. The results presented here extend this plethora of factors to include the most complete example of quorum-sensing-regulated control of chitinolytic activity enabling a gram-negative bacterium to utilize chitin as a growth source.

In this system, quorum sensing regulation is mediated by HHL synthesized by *C. violaceum*. Two of the test strains (CVWT and its derivative CV017) produce HHL molecules and release them into the culture medium, while CV026, a derivative of CV017 with a mini-Tn\(_5\) insertion in the autoinducer synthase gene, is deficient in HHL production. In the presence of HHL, either produced by the CVWT or CV017 strain or supplied exogenously for CV026, all of the strains exhibited strong chitinolytic activity, as determined by the formation of clearing zones on chitin agar, by degradation of colloidal chitin fine particles in liquid medium, and by the release of pNP from the chromogenic chitooligosaccharide analogs. No activity or only very weak activity was found for intracellular protein of the strains, suggesting that the chitinolytic enzymes are almost completely extracellular.

We used a set of fluorescent 4-MU glucosides of GlcNAc mono- and oligosaccharides as substrates to identify the chitinolytic activity of extracellular proteins renatured after their separation by SDS-PAGE. The same pattern of bands showing chitinolytic activity of extracellular proteins was observed when the activity in the latter was restored by induction with HHL in the growth medium. Chitinolytic activity was detected only when the bacteria were grown in an inducing medium containing colloidal chitin. The enzymes detected differed in their substrate specificities. Those designated according to their apparent molecular masses as Chit162 and Chit133 released fluorescent 4-MU from all three substrates. Fluorescent bands corresponding to Chit108 and Chit87 appeared as a result of the release of 4-MU from trimeric \([4\text{-MU-(GlcNAc)}_2]\) and tetrameric \([4\text{-MU-(GlcNAc)}_3]\) chitin analogs, but not from the dimeric analog 4-MU-GlcNAc. The band of Chit56 was detected only when 4-MU-(GlcNAc)_2 was used, and its activity with this substrate was apparently weaker than that of Chit67. Finally, the band of Chit87 was detected only with the dimeric...
The results for growth of the wild-type and mutant strains of C. violaceum, T. harzianum, and A. caviae are released. In previous tetrameric [4-MU-(GlcNAc)₃], and higher analogs of chitin, including chitotriose and biose and higher analogs of chitin, including chitotriose and chitobiose, are released. This type of activity, specifically hydrolyzing the dimeric chitin analog, has been observed previously in some acaropathogenic fungi (4).

The enzymes of 108 and 67 kDa can be classified as chitinases with an endo-mode of chitin splitting. They produced a fluorescent product from 4-MU-GlcNAc, but not from trimeric [4-MU-(GlcNAc)₃] or tetrameric [4-MU-(GlcNAc)₄], analogs of chitin, can be regarded as a chitobiose. This type of activity, specifically hydrolyzing the dimeric chitin analog, has been observed previously in some acaropathogenic fungi (4).

The enzymes of 108 and 67 kDa can be classified as chitinases with an endo-mode of chitin splitting. They produced a fluorescent product from 4-MU-(GlcNAc)₂ and 4-MU-(GlcNAc)₃, indicating random cleavage at internal sites along the entire length of the chitin analog, but they did not hydrolyze 4-MU from the dimeric analog. Chit56 produced 4-MU only from the trisaccharide analog 4-MU-(GlcNAc)₂, and consequently we defined this enzyme to be a chitobiosidase. Chitobiosidase activity has been reported for several bacteria, including E. agglomerans (5), Serratia marcescens (2), and Bacillus cereus (36). Unlike the other chitinases produced by the C. violaceum strains studied, the 56- and 67-kDa proteins retained chitinolytic activity after renaturation irrespective of prior treatment with 2-mercaptoethanol. Similar characteristics have been reported for E. agglomerans enzymes of 50 and 58 kDa (5, 6). However, at present it is unclear why Chit56 renatured after treatment with 2-mercaptoethanol should be active against the tetrameric substrate when it exhibits only weak activity for a trimeric substrate known to be specific for chitobiosidase.

Experiments with C. violaceum grown in SM medium containing glucose, sucrose, or 10% LB instead of colloidal chitin as the carbon source indicated that the chitinolytic activity was not induced under these growth conditions either in the presence or absence of HHL. These results support the assumption that colloidal chitin, or more specifically low-molecular-weight breakdown products of chitin, are required for the induction of chitinolytic enzyme production in C. violaceum. The colloidal chitin used in these experiments was prepared by partial hydrolysis with 10 N HCl and sterilized by autoclaving. Under these conditions α-glucosamine and GlcNAc, known inducers for the synthesis of chitinase (44), are released. In previous studies colloidal chitin prepared by this technique has been shown to induce a wide spectrum of chitinolytic enzymes, including N-acetylglucosaminidase, endochitinase, and chitobiosidase in the bacterial species Aeromonas caviae (16), E. agglomerans (5, 6), and Bacillus cereus (36) and in the fungal species Trichoderma harzianum (13) and Hirsutella sp. (4). Moreover, the results for growth of the wild-type and mutant strains of C. violaceum on colloidal chitin clearly indicate that induction with an AHL (HHL) is likewise an absolute requirement for the expression of chitinolytic activity, even in the presence of the substrate-level inducers. It is significant that induction with HHL stimulates the coordinate expression of a combination of chitinolytic enzymes with different specificities for polymeric chitin and degradation products, as this enables C. violaceum to use the resultant GlcNAc subunits as a sole source of carbon and nitrogen for growth. A similar situation has previously been observed in a mutant of E. agglomerans, in which a single Tn5 insertion was found to downregulate the coordinate expression of several chitinases (5).

The experimental conditions used here, with colloidal chitin as the sole carbon source and in the presence of chitin breakdown products and synthetic or endogenous HHL, were designed to be optimal for expression of AHL-regulated chitinolytic activity. However, although we used up-to-date and sensitive methods for the determination of chitinolytic enzymes (2, 52), the possibility remains that some chitinase activity may go undetected because of denaturation by treatment with SDS. In this analysis we have therefore restricted our interpretation to the enzymes which could be observed through their functional activity against the particular substrates used.

There is a wealth of data supporting the important role of chitinolytic enzymes in microbial antagonism, including antagonism between different bacteria and between bacteria and fungi in the rhizosphere (for reviews see references 12 and 23). Although C. violaceum usually constitutes only a minor component of the total microflora found in soil and water, some strains isolated from rhizosphere soil of maize and used for the inoculation of maize seeds were found to significantly increase plant yield (15). This could be the result of antagonism towards other soilborne bacterial and fungal plant pathogens. It has been suggested that quorum sensing signaling by AHLs may contribute to the success of a bacterium in competition with other rhizosphere inhabitants (34, 59). As C. violaceum also produces antibiotics and hydrogen cyanide under AHL-mediated control (56), a combination of these factors may be important. Results from preliminary in vitro experiments show that C. violaceum is able to suppress the growth of the fungal phytopathogens Pythium aphidiamondatum and Rhizoctonia solani and that in the HHL-deficient mutant this correlates with supplementation of HHL to the growth medium (58). Future studies will focus on how other bacterial regulatory systems interact with quorum sensing signaling to modulate the expression of a number of factors, including chitinolytic enzyme production, which may contribute to successful biocontrol of plant pathogens.

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