ORIGINAL ARTICLE

Nutrient regime regulates complex transcriptional start site usage within a *Pseudoalteromonas* chitinase gene cluster

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The chitinase gene cluster of the marine bacterium *Pseudoalteromonas* sp. S91, *chiABC*, which produces the major chitinases of this sp., was transcribed as an operon and from each individual gene. *chiA*, *chiB* and *chiC* were found to possess multiple transcriptional start points (TSPs), the use of which was determined by the nutrient regime used for S91 growth. In minimal medium containing glutamate, *chiA*, *chiB* and *chiC* each used 3, 1 and 1 TSP, respectively. Upon the addition of the chitin monomer *N*-acetylglucosamine, the number of *chiA* TSPs was unaffected. However, *chiB* used an additional 4 TSPs, and *chiC* used four new TSPs excluding the TSP used in glutamate only. In addition, the cluster was transcribed as an operon from TSP A1 of *chiA*. All TSPs were potentially associated with either a σ^{70} - or σ^{54} -dependent promoter. Under the growth conditions used, no TSPs were detected for *chiB* or *chiC* in S91CX, a *chiA* transposon mutant. The transcription of the S91 *chiABC* gene cluster produced at least four polycistronic mRNAs. In addition, the occurrence of operon transcription of *chiABC*, and identification of an additional 12 putative TSPs within the gene cluster, gave an indication that each gene appeared to be transcribed from more than one promoter region upstream of each in-frame translation start codon. Questions arose regarding the reason for this complexity of transcription within the gene cluster, leading to a re-evaluation of the Chi protein domains. By bioinformatic review, ChiA, ChiB and ChiC were found to potentially possess additional putative domains.

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Introduction

Chitin is an insoluble polysaccharide of *N*-acetylglucosamine (GlcNAc) monomers. After cellulose, chitin is the most abundant biopolymer in the biosphere, with over 10^{11} tonnes produced annually in the marine environment from animals such as plankton and the exoskeletons of crustaceans and copepods (Keyhani and Roseman, 1999). The process of chitin assimilation is essential in understanding oceanic carbon, nitrogen and energy recycling (Azam, 1998) through to comprehending the spread and persistence of pathogens such as *Vibrio cholerae*, which endures in the environment on chitinous exoskeletons and particles (Nalin *et al.*, 1979) where its natural competence is induced (Meibom *et al.*, 2005). Chitinase secreting marine

bacteria play an essential role in chitin recycling (Gooday, 1990); extracellular chitinases secreted from cells degrade chitinous particulate organic matter into small oligosaccharides and GlcNAc subunits (Wetzel, 1991).

Many bacterial chitinase genes have been identified and sequenced, however, little is known about their transcriptional organization or regulation. Chitinase gene expression is regulated by growth in different nutrient regimes. Catabolite repression can be caused by rich medium (Techkarnjanaruk *et al.*, 1997) and carbon sources such as glucose (Morimoto *et al.*, 2007; Leisner *et al.*, 2008). GlcNAc, the monomer of chitin, can induce (Techkarnjanaruk *et al.*, 1997; Meibom *et al.*, 2004) or repress (Monreal and Reese, 1969; Robbins *et al.*, 1992; Miyashita *et al.*, 2000) chitinase production. The observation of gene transcriptional patterns in the presence or absence of GlcNAc, however, has not been investigated.

Pseudoalteromonas S91 is a non-pathogenic Gram-negative marine bacterium in which three adjacent genes, *chiABC*, in the same transcriptional direction on the chromosome, encode two chitinases with catalytic activity (ChiA and ChiC) and a

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chitin-binding protein (ChiB) (Techkarnjanaruk *et al.*, 1997; Techkarnjanaruk and Goodman, 1999). The transcriptional organization of this cluster is not known. Strain S91CX, a *chiA* interrupted transposon mutant, has no ChiA or ChiC chitinolytic activity, indicating that the gene cluster may be an operon, with transcription initiating at *chiA* (Techkarnjanaruk *et al.*, 1997). Sequencing of *chiABC*, however, revealed a potential promoter region for each gene, and when cloned into *Escherichia coli*, *chiC* was expressed from its own promoter (Techkarnjanaruk, 1998; Techkarnjanaruk and Goodman, 1999). Techkarnjanaruk *et al.* (1997) found that the *chiA* promoter was active at a basal level when S91CX was grown in marine minimal medium plus glutamate (MMM glt) and was induced three-fold and ten-fold on the addition chitin and GlcNAc, respectively. Techkarnjanaruk *et al.* (1997) proposed that the significantly large induction by GlcNAc may be indicative of S91's scavenging lifestyle.

The aim of this study was to determine the transcriptional organization of S91 *chiABC* and determine if the addition of GlcNAc, an inducer of *chiA* and nutrient source found in nature, would alter the cluster's transcriptional patterns. Dependent on the nutrient regime, *chiABC* appeared to be transcribed as an operon as well as from individual promoters within the *chiABC* sequence. Each *chi* gene possessed both potential σ^{70} - and σ^{54} -dependent promoters, the use of which depended on the nutrient regime, indicative of the type of regulation controlling expression. Bioinformatic review of the ChiA, B and C protein sequences revealed additional putative domains, suggesting that the production of multiple mRNAs enabled the independent synthesis of these domains.

Materials and methods

Strains, growth conditions and RNA extraction

Pseudoalteromonas sp. S91 wt and S91CX (Techkarnjanaruk *et al.*, 1997) were grown in 10 ml of marine minimal medium (MMM) (Östling *et al.*, 1991) supplemented with 40 mM glutamate (glt) or 40 mM glt + 5 mM (0.1% w/v) GlcNAc with shaking at 30 °C. Cultures of optical density (595 nm) 0.7–0.8 were treated with RNA protect (Qiagen, Venlo, Netherlands) and RNA was isolated using an RNeasy mini kit (Qiagen). An additional DNaseI digestion (RNase free DNaseI) of the isolated RNA was performed (appropriate amounts of DNaseI and 5 × DNaseI buffer were added to the RNA which was incubated at 37 °C for 60 min and then inactivated by heating at 75 °C for 5 min in the presence of 2 mM EDTA). The RNA sample was purified by application to a fresh RNeasy mini column and eluted two times, first with 50 µl nuclease-free H₂O and second with the 50 µl eluate, to concentrate the sample. The isolated RNA was quantified spectrophotometrically, and stored at –80 °C.

RT-PCR

Reverse transcription (RT)-PCR of RNA was performed using Thermoscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). A 20 µl reaction contained 2 pmol gene-specific primer (GSP)-either *chiA*-R895 (*chiA*), *chiB*-R4055 (*chiB*) or *chiC*-R5712 (*chiC*) (Table 1), in addition to 1 µg RNA, 1 mM of each deoxynucleotide triphosphate and nuclease-free H₂O to a final volume of 12 µl. The mixture was incubated at 65 °C for 5 min then chilled on ice for 2 min. cDNA synthesis buffer, 5 mM DTT, 2 µl nuclease-free H₂O and 15 U (1 µl) Thermoscript were then added. Tube contents were pipette mixed, briefly microfuged and incubated at 60 °C for 60 min. RT-PCR negative controls were always included and contained 1 µl nuclease-free H₂O in place of Thermoscript. Heating at 85 °C for 5 min terminated cDNA synthesis. Untranscribed RNA was removed from cDNA by alkaline hydrolysis, as per Tillett *et al.* (2000). Following glycogen precipitation, cDNA was resuspended in 20 µl TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0).

PCR was performed on recovered cDNA to confirm that the RT-PCR was successful, and on the RT-PCR negative controls to check that no genomic DNA was present in the original RNA sample. S91 genomic DNA was included as a positive control. Primer pairs used were *chiA*-F776 plus *chiA*-R895 (*chiA*), T4G6 plus *chiB*-R4014 (*chiB*) and *chiC*-F5513 plus *chiC*-R3 (*chiC*) (Table 1). The forward primer was located downstream of each *chi* translation start codon. The reverse primer was located upstream of the RT-PCR GSP used to synthesize the cDNA.

PCR across chi transcriptional junctions

chiB cDNA (generated using GSP *chiB*-R4055) was used as the template for amplification across *chiAB*, using primers CH8T4G1 and T8G10. *chiC* cDNA (generated using GSP *chiC*-R5712) was the template used across *chiBC* with primers T4G6r1 and *chiC*-R4. The forward primer in each case was located upstream of the *chiA* (for *chiAB*) or *chiB* (for *chiBC*) stop codon. The reverse primer was located downstream of the adjacent *chi* gene's translation start codon (*chiB* for *chiAB* and *chiC* for *chiBC*).

Ligation-anchored PCR (LA-PCR)

Ligation-anchored PCR, also known as rapid amplification of 5' cDNA ends (5' RACE), as described by Tillett *et al.* (2000), was used to map potential transcriptional start points (TSPs). The 5'-phosphorylated, 3'-end cordycepin-blocked (3' phosphate) anchor oligonucleotide DT88 (Tillett *et al.*, 2000; Table 1) does not have any sequence homology with the S91 *chiABC* sequence. Five picomoles of DT88 were ligated to 5 µl purified cDNA in a reaction mix also containing 20 U T4 RNA ligase (Promega, Madison, WI, USA), 1 × T4 RNA ligase buffer and nuclease-free H₂O to 10 µl.

Table 1 Primers, and their sequences (5'–3'), used in this study

Specificity	Primer	Sequence (5'–3')	Genbank position number
	DT88	*GAAGAGAAGGTGGAAATGGCGTTTTGG ^b	
	DT89	CGCCATTTCCACCTTCTCTTC	
<i>chiA</i>	chiA-R895	TGCAATCGCCACACCATCAACCAGTA	495
	chiA-R1	ACTACTTCAGCCACAGTCACACTATT	629
	chiA-R2	GTAAAAGAACTACCGCTCATAGGTGATA	401
	chiA-R3	TGATTGCTTACTTGACCATCACTAC	357
	chiA-R1519	AATATCATCTACTAGGTAAGACGGTTGG	1119
	chiA-F472	TCCATTAGGGGGAAGTATGAGTAACA	72
	chiA-F776	TCACCTATGAGCGGTAGTTCTTT	376
	T8G17Eco-F	CTCGAATTCATCTTGTTTTTATTTTCTCG	–25
	CH8-T4G1-F	CGTCGTGTCATTGGTTATT	1063
	T4G1-F	GGTGAATTAGCTGGTAAACA	1657
	T4G2-F	AGCTTGGTTAGGTAACGCA	2052
	T4G3-F	GCTGTCGTAGTCAAAGTCA	2454
	T8G10-R	ACCAATGTTGATGCGACC	1968
	T8G9-R	GTGCCCATGACTCTTCAT	2375
	T8G7-R	TGTTTGTGGGAAGTTTGA	3111
<i>chiB</i>	chiB-R4055	TGCCAATGCGCTGACGGTAAATTCATAC	456
	chiB-R4014	ACTACCCGCAGCGCACAAAGTA	415
	chiB-R3	GCAAACATCATGCTCTTGAATAAACT	336
	T4G6-F	TGGATAGCCCTAAAGCAC	189
	T4G6r1-F	TTGACTGGTTCCCGCTCG	801
<i>chiC</i>	chiC-R5712	ACTGATGACGGCCGATTGCGTGATG	613
	chiC-R3	GTACCTTCATTTACTACTACACCGTCAA	548
	chiC-R4	TTTACTTTGTAGCTATCGCCACCTT	518
	chiC-F5513	CGGTCAAGGTTCTGTATAAGTCA	414

The position number of the first base of each primer on the S91 *chiA* (Genbank Accession number AF007894), *chiB* (AF007895) and *chiC* (AF007896) sequences are detailed. DT88 is *5' phosphate, and ^b3' cordycepin blocked, at the positions marked. Primers containing a capital R are in the reverse direction.

Reactions were performed at 16 °C overnight and heat inactivated at 65 °C for 15 min.

Nested touchdown LA-PCR was performed on DT88 ligated cDNA. For LA-PCR1 a *chi* GSP (*chiA* with chiA-R2; *chiB* with chiB-R4014; and *chiC* with chiC-R3) was used as the reverse primer with DT89 (DT88-specific primer) (Table 1). For LA-PCR2, a GSP located within the LA-PCR1 product (*chiA*, chiA-R3; *chiB*, chiB-R3; *chiC*, chiC-R4) was used as the reverse primer with DT89 (Table 1). LA-PCR1 contained 1 µl DT88 ligated cDNA, 10 pmol LA-PCR1 GSP, 0.2 mM deoxynucleotide triphosphates, 1 × reaction buffer, 2 mM MgCl₂, 0.625 U HotStarTaq (Qiagen) and nuclease-free H₂O to 25 µl. PCR cycling was performed at: 95 °C 15 min; (94 °C 30 s, 64 °C 60 s (then –1 °C every second cycle), 72 °C 90 s) × 20; (94 °C 30 s, 54 °C 60 s, 72 °C 90 s) × 10; 72 °C 10 min, 4 °C ∞. LA-PCR2 contained 0.5 µl LA-PCR1 product, 10 pmol LA-PCR2 GSP, and the same reaction mix as LA-PCR1. Cycling conditions were as described for LA-PCR1. All experiments were carried out at least twice.

LA-PCR2 products were visualized by electrophoresis on 3% (w/v) agarose gels in Tris acetate EDTA buffer. Fragments, each corresponding to a separate LA-PCR2 product, potentially a TSP for the corresponding gene, were excised from 2% (w/v) low melting point agarose (for fragments <1500 bp; Progen, Darra, Qld, Australia) in Tris acetate EDTA

buffer. Excised fragments were Wizard Cleaned (Promega), cloned into a pGEM-T vector (Promega) and sequenced by the ABI BigDye Terminator method at the Australian Genome Research Facility (University of Queensland, Brisbane, Australia).

Sequences were analyzed using BioManager by ANGIS (<http://www.angis.org.au>). Primers were designed using Primer3 (Rozen and Skaletsky, 1998) within BioManager. All nucleic acid sequence alignments were performed using Local Pair Alignment (Huang and Miller, 1991) within BioManager. BL2seq (Tatusova and Madden, 1999) and BLASTP (Altschul *et al.*, 1997) (<http://www.ncbi.nlm.nih.gov/BLAST/>) were used for amino acid sequence alignments and analyses, respectively. Multiple amino acid sequence alignments were performed using ClustalW2 (Chenna *et al.*, 2003). The Conserved Domain Database (Marchler-Bauer *et al.*, 2003; <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) was used for the investigation, identification and nomenclature of chitinase protein domain architecture.

Results

chiABC is an operon

PCR products spanning the *chiAB* and *chiBC* junctions were obtained from cDNA of S91 grown

in MMM glt with or without added GlcNAc (data not shown). As this result indicated that *chiABC* was being transcribed as a single mRNA from the *chiA* promoter, determination of the operon TSP was attempted using LA-PCR. The expected size of the operon transcript was about 5 kb. Attempts to isolate large (>1 kb) LA-PCR2 fragments with reverse primers internal to *chiC* (or to *chiB*) were unsuccessful. PCR walking in a 5' direction using cDNA reverse transcribed from *chiC* with the *chiC*-R5712 primer (Table 1) was therefore carried out to determine if one *chiABC* transcript was produced. PCRs were performed using *chiA* gene-specific primers on *chiC* cDNA to locate the TSP from which transcription of *chiABC* was occurring (Table 2, PCRs 3–8). A PCR product was generated each time using primer pair numbers 3–7 (Table 2). However, no PCR product was generated from primer pair number 8 (T8G17Eco-F and *chiA*-R1) indicating that the TSP was located downstream of the forward primer-binding site. The TSP was in the vicinity of TSP A1 (Tables 3 and 4). The same result was obtained for S91 grown in MMM glt with or without added GlcNAc. PCR testing of reverse transcribed cDNA negative control (RNA only) confirmed that no DNA was present in the RNA samples.

Each *chi* gene has individual transcriptional start points in S91

LA-PCRs were performed separately in the vicinity of the translational start points of *chiA*, *chiB* and *chiC* (Techkarnjanaruk, 1998) to determine if transcription from each gene's putative promoter region occurred. LA-PCRs were progressively performed from the 3' to the 5' end of the *chiABC* operon and LA-PCR2 products were sequenced to determine the base pair number for each potential TSP.

chiA

Three potential TSPs were identified for *chiA* when S91 was grown in glt with or without GlcNAc

Table 2 Reactions performed to PCR walk in a 5' direction (from *chiC* to *chiA*)

Primer pair number	<i>chi</i> region	Forward primer	Reverse primer	Product length (bp)
1	<i>chiB</i> – <i>chiC</i> transcriptional junction	T4G6r1	<i>chiC</i> -R4	1217
2	<i>chiA</i> – <i>chiB</i> transcriptional junction	T4G3	<i>chiB</i> -R3	1099
3	<i>chiA</i>	T4G2	T8G7	1077
4	<i>chiA</i>	T4G1	T8G9	718
5	<i>chiA</i>	CH8-T4G1	T8G10	923
6	<i>chiA</i>	<i>chiA</i> -F774	<i>chiA</i> -R1519	743
7	<i>chiA</i>	<i>chiA</i> -F472	<i>chiA</i> -R1	557
8	<i>chiA</i>	T8G17Eco-F	<i>chiA</i> -R1	no product

(Tables 3 and 4). A LA-PCR2 fragment of 310 bp corresponded to TSP A1 at bp 54 of *chiA* (Table 4). The other PCR generated fragments were approximately 200 bp and 70 bp in length corresponding to 184 bp (A2) and 299 bp (A3), respectively, of the S91 *chiA* sequence (AF007894; Tables 3 and 4). TSP A1 was in the vicinity of the putative TSP used for *chiABC* operon transcription.

chiB

When grown in the presence of the chitinase gene inducer GlcNAc, three potential TSPs, B1–B3, were identified for S91 *chiB* (Table 3). The three LA-PCR2 fragments identified were approximately 300, 250 and 100 bp, respectively (data not shown). The TSPs were located at 54 bp (B1), 115 bp (B2) and 259 bp (B3) (GenBank accession no. AF007895; Table 4). In addition, two other *chiB* LA-PCR2 fragments of approximately 1000 and 500 bp were present following S91 growth in MMM glt GlcNAc (data not shown). The TSPs were located at 2419 bp (B_{A1}) and 3052 bp (B_{A2}), within the ORF of *chiA* (GenBank accession no. AF007894; Tables 3 and 4). mRNAs from these putative TSPs were therefore at least bicistronic. TSPs were named to indicate which cDNA was used for their identification, *chiB* or *chiC*. The subscript letter indicates the *chi* gene in which the TSP was located.

Following the growth of S91 in glt alone, one *chiB* TSP was detected (Table 3). Sequencing of the 500 bp LA-PCR2 fragment (data not shown) showed this TSP was located at 3052 bp, and was the same as TSP B_{A2} (Table 4).

chiC

Three LA-PCR2 fragments were isolated from S91 *chiC* cDNA during growth in the presence of GlcNAc, approximately 400 (TSP C1), 280 (TSP C3) and 120 (TSP C4) bp in length (Tables 3 and 4).

Table 3 Putative TSPs used by S91 for the transcription of *chiABC* following growth in MMMglt with or without GlcNAc (✓ indicates transcription, – indicates no transcription)

TSP used	Growth in MMM+	
	Glt	<i>glt</i> +GlcNAc
A1	✓	✓
A2	✓	✓
A3	✓	✓
B _{A1}	–	✓
B _{A2}	✓	✓
B1	–	✓
B2	–	✓
B3	–	✓
C _{B1}	–	✓
C1	–	✓
C2	✓	–
C3	–	✓
C4	–	✓

Table 4 Sections of the S91 *chiABC* sequence

TSP	5' → 3' promoter sequence
(a)	
A1	15 ATTTCACTA ATTTGTTAATCATGTGTT AGTT AATAAATAGTCG54N(28)GGAAGTATG 90
B _{A1}	2388 CGTAATCA ACAAAGGCCTTGGCGGTAT TTAT GT2419N(44)AAGAGTACTTTATG 2475
B1	20 CAT TCCGCCG ATTGGGATAGTGTGTG TACTTT AATAA54N(35)GGAGAAGATTATG 101
B2	85 TG TTTTGG AGAAGATTATGAATACCA AAATTT AA115N(64)ACACGGTTATATG 191
C _{B1}	752 C TTTTATA ACTGTAGCGATATTAT TATTGAG CGCGA787N(359)GCAGATCTGGTAATG 1160
C2	175 TTAAATTT AATCGGCTAAAGCGAGCC ATAAGTT 204N(65)AGAGAAACATTATG 292
C4	387 AA AAATAT TCATTGTGTGATGTAAAT TATTGAC G416N(335)GATCCATTACCAATG 715
<i>E. coli</i> σ ⁷⁰	TTGACA TATAAT
consensus	-35 -10
(b)	
A2	152 GGGTGGGCAGCCCATGCAGCAGTGGACTGTACAA184N(182)AGAGGTTTTATCACCTATG 384
A3	262 AAGT GCTGG ACACAAAGCCGATCCAGTAACCCAT TCAGG 299N(67)AGAGGTTTTATCACCTATG 384
B _{A2}	3019 GGTGGCAATGATGGCGGCGGTAA ACCG 3052N(94)GTGACTTGATG 3156
B3	230 AGCGCGTATTGCTGGCCAGAAAGACGGCAG259N(164)AAGCGCGGTATG 434
C1	121 GCTGGTGGACCAAAGCGGATGAGCCAGGTA150N(129)AGAGAAACATTATG 292
C3	238 CGCGCGCTTGCTTGGCGACGTTGCGGGTT TTTAA 271N(10)AGAGAAACATTATG 292
<i>E. coli</i> σ ⁵⁴	TGGCACGNNNNNTTGC
consensus	-24 -12

Sections of the S91 *chiABC* sequence highlighting the potential promoter region upstream of each putative TSP with homology to: (a) the -10 and -35 sites of the *E. coli* σ⁷⁰-dependent promoter consensus sequence (bold and underlined; Hawley and McClure 1983); (b) the -12 and -24 sites of the *E. coli* σ⁵⁴-dependent promoter consensus sequence (underlined; Barrios *et al.*, 1999) (the most conserved residues in the -12 and -24 positions are bold and underlined). Numbers, before and after each sequence, indicate the base number in each GenBank sequence (*chiA*, AF007894; *chiB*, AF007895; *chiC*, AF007896; Techkarnjanaruk and Goodman, 1999). Each base in bold and double underlined is that at which transcription initiates and the subscript numeral beside it indicates its base number, as found in GenBank. N with bracketed numeral indicates the number of bases to the putative ribosome binding site (italics) located upstream of a putative in-frame translation initiation codon (ATG; underlined).

Potential TSPs C1, C3, C4 were located at bp 150, 271 and 416, respectively, on the *chiC* sequence (GenBank accession no. AF007896; Table 4). A further large PCR fragment (>1 kb) was observed in agarose gels at a low concentration (data not shown) and could not be isolated from the higher concentration smaller fragments. GSP *chiC*-R5220, which bound further upstream of *chiC* and thereby excluded TSPs C1–C4, was used in place of GSP *chiC*-R4 in LA-PCR2. This resulted in a shorter LA-PCR2 product being amplified, and subsequently the isolation and identification of TSP C_{B1}, located half way through the *chiB* ORF at 787 bp (GenBank accession no. AF007895; Tables 3 and 4).

When S91 was grown in glt alone, a LA-PCR2 fragment of approximately 350 bp was produced from *chiC* cDNA. This TSP (C2; Table 4) was located at 204 bp (GenBank accession no. AF007896; Table 4) and was not used for the transcription of *chiC* when S91 was grown in the presence of GlcNAc.

S91CX

S91 *chiABC* was transcribed as an operon as well as from individual TSPs within each gene. The location of TSPs in S91CX *chiABC* was therefore investigated following growth of S91CX in glt with or without GlcNAc. As found for S91, the same three *chiA* LA-PCR2 fragments were produced by S91CX during growth in either medium. DNA sequencing showed that these *chiA* TSPs were indeed the same as those found for S91; A1, A2 and A3 (Table 4). No

LA-PCR2 fragments were detected for S91CX *chiB* or *chiC* in either medium despite repeated testing.

TSP potential promoters

The DNA sequence just upstream of each putative TSP was assessed for the presence of an appropriately positioned promoter. Both σ⁷⁰- (Table 4a) and σ⁵⁴- (Table 4b) dependent promoter sequences were identified, each with homology to their respective consensus sequences (Hawley and McClure, 1983; Barrios *et al.*, 1999) and those reported earlier in *E. coli* (Dombroski *et al.*, 1996), *Myxococcus xanthus* (Keseler and Kaiser, 1995) and *Pseudomonas aeruginosa* (Medina *et al.*, 2003). In addition, a putative in-frame translation initiation codon and corresponding potential ribosome-binding site were identified for each transcript arising from a potential TSP (Table 4).

Re-evaluation of the domains within *ChiA*, *ChiB* and *ChiC*; a bioinformatics approach

The positions of potential translation start codons, downstream of each TSP, were mapped onto a diagram of the *chiABC* sequence showing the various domains within each protein sequence (Figure 1). The sequence was also analyzed to see if domains in addition to those reported previously (Techkarnjanaruk and Goodman, 1999) could be identified (Table 5 and Figure 1). The amino acid positions correspond to those recorded

Figure 1 The relative position of each putative translation start codon of the S91 *chiABC* sequence. Boxes represent labeled domains: ChtBD3, chitin-binding domain type 3 (smart00495); glyco-18, glyco-18 catalytic domain (smart00636); ChiC refers to a type of domain commonly found adjacent to Glyco-18 (Pfam06483) and involved in chitin-binding; CBD, chitin-binding domain Pfam03067. A black inverted triangle shows the point of insertion of miniTn10:*lac:kan* in S91CX. Translation start (ATG) and stop codons are indicated by arrows and asterisks, respectively. Each ATG is in frame with the protein open reading frame in which it is located. Hatched boxes show domains identified by Techkarnjanaruk and Goodman (1999); white boxes show domains identified in this study; the box located immediately downstream of B_{A2} represents the ChtBD3-like region, identified in this study, containing the highly conserved ChtBD3 stWWst motif (AKWWTS) (see text). Diagram is not to scale.

for each protein in the Entrez Protein Database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein>) (ChiA, AAC79665; ChiB, AAC79666; ChiC, AAC79667).

After the Glyco-18 catalytic domain (smart 00636) of ChiA (Techkarnjanaruk and Goodman, 1999), there was a chitinase C (ChiC) domain, involved in chitin-binding (Pfam 06483; Watanabe *et al.*, 1994), of 128 residues, Glu⁷⁹³–Gly⁹⁸⁰ (Table 5 and Figure 1). Also, ChiA was found to have a chitin-binding type 3 domain (ChtBD3; smart 00495) at its N-terminal end of 52 residues, Cys³¹–Cys⁸² (Table 5 and Figure 1). At the C-terminal end of ChiA, an additional putative chitin-binding domain was identified of 62 residues, Cys⁹⁹¹–Cys¹⁰⁵² (Figures 1 and 2a). It possessed the highly conserved stWWst (AKWWTS; Ala¹⁰³⁴–Ser¹⁰³⁹) region and was flanked by two cysteines (Cys⁹⁹¹ and Cys¹⁰⁵²) which possibly formed a disulfide bridge (Brun *et al.*, 1997) (Figure 2a).

At the N-terminal end of ChiB there was a chitin-binding domain (Chitin_bind_3; Pfam 03067; Table 5 and Figure 1) of 215 residues, Ser¹³ to Ile²²⁷, similar to that in the *Serratia marcescens* chitin-binding protein CBP21 (Suzuki *et al.*, 1998); CBP21 is reported to have strong binding activity towards squid pen (β-chitin), the backbone of squid (Suzuki *et al.*, 1998). At the C-terminal end of ChiB from amino acids Ala⁴⁸⁵ to Leu⁵²⁵, there was a second ChtBD3 domain of 41 residues (Table 5 and Figure 1), only eight amino acids downstream of the one reported earlier (41 residues, Ala⁴³⁸–Leu⁴⁷⁸; Table 5 and Figure 1) (Techkarnjanaruk and Goodman, 1999).

S91 ChiC possesses a glyco-18 catalytic domain (Val¹⁵⁸–Asp⁵⁷²) and a chtBD3 of 38 residues, Ala⁸³⁰ to Trp⁸⁶⁷ (Techkarnjanaruk, 1998; Techkarnjanaruk and Goodman 1999). In addition, another ChtBD3 domain of 41 residues, Thr⁷⁸¹ to Val⁸²¹, just 10 amino acids upstream of the earlier reported ChtBD3 was identified. Figure 2(a) shows an alignment of all

Table 5 The amino acids and their positions, with respect to each S91 Chi protein sequence in the Entrez protein database, in forming domains within ChiA, ChiB and ChiC

Protein	Accession number	Domain	Amino acids
ChiA	AAC79665	ChtBD3	Cys ³¹ –Cys ⁸²
		Glyco-18	Arg ³²⁰ –Asp ⁷⁸⁴
		ChiC	Glu ⁷⁹³ –Gly ⁹⁸⁰
		ChtBD3-like	Cys ⁹⁹¹ –Cys ¹⁰⁵²
ChiB	AAC79666	CBD	Ser ¹³ –Ile ²²⁷
		ChtBD3	Ala ⁴³⁸ –Leu ⁴⁷⁸
		ChtBD3	Ala ⁴⁸⁵ –Leu ⁵²⁵
ChiC	AAC79667	Glyco-18	Val ¹⁵⁸ –Asp ⁵⁷²
		ChtBD3	Thr ⁷⁸¹ –Val ⁸²¹
		ChtBD3	Ala ⁸³⁰ –Trp ⁸⁶⁷

ChtBD3, chitin-binding domain type 3; ChiC, chitin-binding domain commonly found adjacent to Glyco-18; ChtBD3-like, similar to ChtBD3; Glyco-18, Glyco-18 catalytic domain.

conserved domain database identified ChtBD3 domains in S91 ChiABC with the similar domains in ChiD, Cbp1 and ChiA of *Alteromonas* sp. O-7 (Tsujiro *et al.*, 2002). The S91 C-terminal end ChiA ChtBD3-like domain was included in Figure 2(a) along with similar domains identified in other bacterial chitinase genes, including ChiD of *Alteromonas* sp. O-7, to demonstrate the homology of these unrecognized potential chitin-binding domains to that of ChtBD3. Figure 2(b) shows the homology between the N-terminal ends of these ChtBD3-like domains. Figure 2(c) shows the high homology between the ChtBD3 domains at the N-terminal ends of the ChiA and ChiD proteins of S91 and *Alteromonas* sp. O-7, respectively.

Discussion

The investigation of the transcriptional patterns and organization of bacterial chitinase genes under

Figure 2 Amino acid sequence alignments of: (a) the ChtBD3 type 3 domains from *Alteromonas* sp. O-7 Cbp1 (O_7_Cbp1, Ala⁴³⁵–Ile⁴⁷⁵), O-7 ChiA (O_7_ChiA, Thr⁷⁷⁸–Val⁸¹⁶), *Pseudoalteromonas* sp. S91 ChiC (ChiC_2, Ala⁸³⁰–Trp⁸⁶⁷), S91 ChiB (ChiB_2, Ala⁴⁸⁵–Leu⁵²⁵, ChiB_1, Ala⁴³⁸–Leu⁴⁷⁸), S91 ChiA (ChiA_1, Cys³¹–Cys⁸²), O-7 ChiD (O_7_ChiD, Cys³⁰–Cys⁸¹) and S91 ChiC (Chi_1, Thr⁷⁸¹–Val⁸²¹). In addition, the S91 C-terminal end ChiA ChtBD3-like domain (Pse_S91, Cys⁹⁹¹–Cys¹⁰⁵²) amino acid sequence is shown aligned with sequences from *M. degradans* CdxA (Mic_2_40, Cys¹⁰⁸⁸–Cys¹¹⁴⁴), *Alteromonas* sp. O-7 ChiD (Alt_O_7, Cys⁹⁸²–Cys¹⁰³⁵), *V. parahaemolyticus* chitodextrinase (Vib_par, Cys⁹⁹⁶–Cys¹⁰⁵²) and *V. harveyi* ChiB (Vib_har, Cys⁹⁹⁵–Cys¹⁰⁵¹). (b) The N-terminal ends of ChtBD3-like domains of *V. harveyi* ChiB (Vib_har), *V. parahaemolyticus* chitodextrinase (Vib_par), *M. degradans* CdxA (Mic_2_40), S91 C-terminal ChiA (Pse_S91) and *Alteromonas* sp. O-7 ChiD (Alt_O_7). (c) The ChtBD3 domains of S91 ChiA (ChiA_1) and *Alteromonas* sp. O-7 ChiD (O_7_ChiD) at the N-terminal ends of the proteins. An asterisk represents identical residues; a colon represents conserved residues; a period represents semi-conserved residues.

different nutrient regimes has not been widely reported. Such an approach aids in understanding their expression in nature. Transcriptional analysis of S91 showed that the *chiABC* cluster was transcribed as an operon from at least one TSP, A1, in MMM glt in the presence and absence of the *chiA* inducer GlcNAc. Dependent on the nutrient regime in which S91 was grown, a further 12 potential TSPs were identified within the gene cluster, indicating that each gene could have been transcribed from more than one potential promoter region. Each TSP identified possessed a promoter of high homology to either a σ^{70} - or σ^{54} -dependent promoter (Hawley and McClure, 1983; Barrios *et al.*, 1999). Such an arrangement has not previously been reported for any bacterial chitinase system, however, individual genes within functional operons can be transcribed separately from their own promoters as well as being transcribed as part of the operon mRNA. The use of internal promoters enables bacteria to control protein production from an operon (Condon, 2003). Using LA-PCR, Kaebernick *et al.* (2002) observed that the microcystin synthase, *mcy*, gene cluster of *Microcystis aeruginosa* is transcribed as two polycistronic operons from a central bi-directional promoter; the leader gene of each operon possesses two TSPs, the use of which depends on light intensity, and within operon *mcyDEFGHIJ*, each gene possesses a functional TSP. In *E. coli*, Plumbridge (1996) showed that in addition to

being transcribed as part of the GlcNAc catabolic operon *nagBACD*, *nagC* expression is directed from two promoters located within the upstream *nagA* gene.

It is possible that LA-PCR2 fragments may have been products of mRNA processing rather than individual transcripts, as acknowledged by Kaebernick *et al.* (2002) who used LA-PCR for transcriptional analysis of the *mcy* gene cluster. Post-transcriptional processing of a primary transcript is possible, resulting in the production of mRNAs with differing stability and varying translational efficiency (Meinken *et al.*, 2003). However, if this were the case, mRNA processing of S91 *chiABC* would have been regulated under the different nutrient regimes tested, producing processed transcripts that were required in response to specific environmental conditions; this is a phenomenon that does not appear to have ever been reported in the literature. It must be noted that none of the references cited herein that report the analysis of TSPs has eliminated the possibility of mRNA processing. It is likely that each *chiABC* TSP is indeed a site of transcription initiation, as a putative promoter with homology to *E. coli* consensus sequences (Hawley and McClure, 1983; Barrios *et al.*, 1999) was identified for each TSP (von Hippel *et al.*, 1984).

Nutrient regime determined which potential TSPs were used for the transcription of S91 *chiB* and *chiC*. The presence of GlcNAc appeared to induce the

expression of *chiB* and *chiC*, because five potential TSPs were used for the transcription of each gene. In the absence of GlcNAc, each gene used only one potential TSP. In addition, GlcNAc may have had a repressive effect on transcription from *chiC* TSP C2; this TSP was not detected. GlcNAc is known both to repress chitinase gene expression in *Serratia marcescens* (Monreal and Reese, 1969), *Streptomyces plicatus* (Robbins *et al.*, 1992) and *St. lividans* (Miyashita *et al.*, 2000) and induce it in *Vibrio cholerae* (Meibom *et al.*, 2004) and S91 (Techkarnjanaruk *et al.*, 1997). This pattern of transcription is indicative of multiple regulatory systems controlling chitinase gene expression in S91 (McGowan *et al.*, 2003). The use of one nutrient regime alone would not have revealed this transcriptional complexity.

chiABC operon transcription was observed from a TSP in the vicinity of TSP A1. In S91CX, however, no TSPs were detected for *chiB* or *chiC*. This is consistent with the loss of S91CX ChiC activity reported earlier by Techkarnjanaruk and Goodman (1999). Although *chiB* and *chiC* appeared to have individual promoters from which transcription may have initiated in S91, it appears that transcription was not possible once *chiA* was transposon interrupted. A DNA conformational arrangement may have been required for transcription from potential σ^{54} -dependent promoters of *chiB* and *chiC*. The S91CX *chiABC* gene cluster conformation would have changed upon insertion of the 5 kb mini-Tn10:*lac:kan* into *chiA*, preventing transcriptional activator(s) to interact appropriately with their target, the enhancer-binding site of σ^{54} -dependent promoters. Activators must bind to the correct position to activate transcription otherwise repression may result (Collado-Vides *et al.*, 1991). Similarly, it is possible that the σ^{70} -dependent promoters of *chiB* and *chiC* may have also required activators with correct conformational placement, as reported for the arabinose operon in *E. coli* (Plumbridge and Pellegrini, 2004).

chiABC each appear to possess both σ^{70} - and σ^{54} -dependent promoters. Regulation of gene expression by different regulatory mechanisms has been previously reported; the *P. aeruginosa* quorum-sensing transcriptional regulatory gene, *rhlR*, possesses both σ^{70} - and σ^{54} -dependent promoters. *rhlR* possesses 4 TSPs from which transcription occurs following growth in a rich medium (Medina *et al.*, 2003); three TSPs possess a σ^{70} -dependent promoter and the other, a σ^{54} -dependent promoter. In minimal medium, however, only one of the four TSPs is used (possessing a σ^{70} -dependent promoter) (Medina *et al.*, 2003). The presence of putative σ^{70} - and σ^{54} -dependent promoters for each S91 *chi* gene suggests that there is complex and tight regulation of transcription and additionally, multiple pathways or levels of control are involved in the regulation of S91 chitinase gene expression (McGowan *et al.*, 2003).

To date there has only been one report of a potential chitinase gene possessing a conserved σ^{54} -dependent promoter; whole genome macroarray analysis of a *Listeria monocytogenes* *rpoN* mutant revealed that gene expression of *lmo0105*, a gene sequence similar to *L. monocytogenes* chitinase B, was different to that of the wild type (Arous *et al.*, 2004). Transcription from σ^{54} -dependent promoters is tightly regulated by environmental conditions, which can be sensed through regulatory domains in the enhancer-binding protein or through interaction with regulatory proteins (Studholme and Dixon, 2003). The former can include two-component system response regulator receiver domains such as that possessed by NtrC, the nitrogen assimilation regulatory protein (Studholme and Dixon, 2003). Regulation of bacterial chitinases by nitrogen has not been previously reported. NtrC is a possible candidate as the activator for the σ^{54} -dependent promoters, as S91 *chiA* promoter activity appears to be regulated by nitrogen (Delpin and Goodman, 2009). Growth in varying nitrogen concentration and source regulated S91 *chiA* transcriptional patterns and the collective *chiA* promoter activity of S91CX (Delpin and Goodman, 2009).

From the transcriptional complexity observed, questions arose regarding why such complex patterns of *chiABC* transcription would exist whereby half, or less, of a gene is transcribed. Re-evaluation of S91 ChiA, ChiB and ChiC protein sequences by bioinformatic analysis identified previously unreported potential protein domains (Figure 1). Upon examination of the potential transcripts produced, in-frame translated products, ceasing at the translational stop codon of that gene, could be produced giving rise to functional domains (Figure 1). BLASTP searches found that each S91 ChiA, ChiB and ChiC protein had highest amino acid identity to its equivalent gene in *Alteromonas* sp. O-7; S91 ChiA, ChiB and ChiC had 66%, 53% and 66% identity with O-7 ChiD, Cbp1 and ChiA, respectively. The *Alteromonas* sp. O-7 *chiD*, *cbp1* and *chiA* (Genbank accession no. AB063629; Tsujibo *et al.*, 2002) genes are also arranged in this order in a cluster, and the domains identified within each gene are similarly located in S91 *chiABC* (Figure 1 and Table 5; Techkarnjanaruk and Goodman, 1999). The GC content of each gene cluster is similar (46% for S91 and 45% for O-7). Although highly homologous, the two gene clusters are not identical. S91 ChiB and ChiC each possess an additional chitin-binding domain type 3, ChtBD3, at the C-terminal end (Figure 2a). The end C-terminal ChtBD3 domains of ChiB and ChiC were not direct repeats of the adjacent ChtBD3 domains, previously identified (Techkarnjanaruk, 1998; Techkarnjanaruk and Goodman, 1999).

S91 ChiA possessed two regions with homology to the highly conserved stWWst motif of the chitin-binding domain ChtBD (Brun *et al.*, 1997); at the N-terminal (Cys³¹–Cys⁸²) and C-terminal (Cys⁹⁹¹–

Cys¹⁰⁵²) end of ChiA (Figure 2a). Multiple sequence alignment has shown that this consensus sequence is shared by 12 chitinases (Brun *et al.*, 1997). The C-terminal ChtBD domain, however, is distinctive, differing to the ChtBD domain at the N-terminal of ChiA and to the ChtBD domains of S91 ChiB and ChiC (Figure 2a).

The S91 ChiA C-terminal end ChtBD3-like domain (amino acid sequence Cys⁹⁹¹–Cys¹⁰⁵²) had 66% identity to amino acids Cys¹⁰⁸⁸–Cys¹¹⁴⁴ of *Microbulbifer degradans* chitodextrinase, CdxA (Entrez protein database accession number AAO47607), 54% identity to Cys⁹⁹⁵–Cys¹⁰⁵¹ of *Vibrio harveyi* ChiB (AAM94336), 53% identity to Cys⁹⁹⁶–Cys¹⁰⁵² of *Vibrio parahaemolyticus* chitodextrinase (NP_800342) and 45% identity to Cys⁹⁸²–Cys¹⁰³⁵ of *Alteromonas* sp. O-7 ChiD (BAB79618) (Figure 2b). It is interesting to note that there was high homology in the N-terminal region of the ChtBD3 domain of these five marine bacterial proteins (Figure 2b). In addition, each peptide sequence possesses the highly conserved stWWst amino acid sequence (Figure 2a) characteristic of ChtBD3 domains (Brun *et al.*, 1997). Interestingly, in all five organisms this domain is located two amino acids from the translation stop codon of the *chi* gene in which it is located and has a cysteine at the beginning and the end, as originally described by Brun *et al.* (1997). The ChtBD3s of the S91 ChiA, Cys³¹–Cys⁸² and the *Alteromonas* sp. O-7 ChiD, Cys³⁰–Cys⁸¹ (to which S91 ChiA is highly homologous, Figure 2c) also possess these boundary cysteines, whereas none of the other recognized ChtBD3 domains in S91 ChiB or ChiC was bounded by cysteines (Table 5 and Figure 2a). Owing to these differences, the S91 ChiA ChtBD3 domains could potentially have different chitin-binding properties, as Ikegami *et al.* (2000) found for the *Bacillus circulans* ChiA1 chitin-binding domain. The product of a transcript generated at TSP B_{A2} would solely possess this domain (Figure 1).

Putative TSPs B_{A1}, B_{A2} and C_{B1} were located within the coding region of the upstream *chiA* and *chiB* genes, respectively. Potentially, these multiple TSPs within a single gene may give rise to smaller functional proteins consisting of fewer domains than the whole-gene encoded protein (Figure 1). Interestingly, TSPs B_{A1} and B_{A2} have different promoters, σ^{70} - and σ^{54} -dependent, respectively, indicating that their expression is differentially regulated. It is possible that translation could initiate at each in-frame translation start codon for these transcripts, and the others which initiate in the coding regions of *chiABC* (indicated in Figure 1 and Table 4), and terminate at the first translational stop codon on the mRNA; *chiABC* mRNA contains translational stop codons at the end of each gene (Figure 1; Techkarnjanaruk, 1998). Dependent on the nutrient regime ($\pm$ GlcNAc) several different ChiA, ChiB and ChiC 'subproteins' would thereby be produced and possess one or multiple domains.

The presence or absence of a chitin-binding domain affects chitinolytic activity (Svitil and Kirchman, 1998). Different chitin-binding domains enable attachment to various types of chitin (Svitil and Kirchman, 1998). Svitil and Kirchman (1998) found that endochitinase activity from the *V. harveyi* *chiA* gene increased upon removal of the chitin-binding domain, as this changed the enzyme's accessibility to glycosidic bonds within a chitin strand. In *B. circulans*, when the *chiA1* chitin-binding domain was cloned separately from the gene in which it is located and expressed, the ChiA1 domain protein maintained its structure and function (Ikegami *et al.*, 2000). It is interesting to hypothesize therefore that putative S91 subproteins, possessing chitin-binding domains, may be functional and potentially assist in the rapid dissemination of chitin particles that is observed in the marine environment (Gooday, 1990).

The N-terminal end of family 18 chitinases characteristically contain the catalytic domain (Merzendorfer and Zimoch, 2003). The location of chitin-binding domains at the C-terminal end of many family 18 chitinases suggests that the transcriptional patterns observed for S91 *chiABC*—which could potentially result in the production of small subproteins possessing chitin-binding domains—could be widespread in many chitinase genes. It is possible that such subproteins are produced under different environmental conditions, for example in the presence of different types of chitin or in cells hydrolyzing chitin in a biofilm, and will be investigated further.

In conclusion, this study has reported that S91 *chiABC* appears to be transcribed as an operon as well as from individual promoters within the gene cluster. Bioinformatic analysis of the potential products of these transcripts revealed that they possessed putative domains. Ideally, future work could determine if such translated transcripts are functional. The presence of potential σ^{54} -dependent promoters for the expression of *chiA*, *chiB* and *chiC*, indicate a possibility that expression of these chitinase genes could be regulated by nitrogen, as many σ^{54} -dependent promoters are part of the nitrogen assimilation regulatory system (Zimmer *et al.*, 2000). Delpin and Goodman (2009) describe the work investigating this hypothesis for the regulation of the S91 *chiA* gene.

Abbreviations

CBD, chitin-binding domain; ChtBD, chitin-binding domain; ChtBD3, chitin-binding domain type 3; GlcNAc, N-acetylglucosamine; glt, glutamate; GSP, gene-specific primer; LA-PCR, ligation-anchored PCR; LA-PCR1, ligation-anchored PCR number 1; LA-PCR2, ligation-anchored PCR number 2; MMM, marine minimal medium; RBS, ribosome-binding

site; RT-PCR, reverse transcription; TSP, transcriptional start point; TSPs, transcriptional start points.

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