Applied and Environmental Microbiology, Aug. 1997, p. 2989–2996 Vol. 63, No. 8
0099-2240/97/$04.00+0
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Use of a Promoterless *lacZ* Gene Insertion To Investigate Chitinase Gene Expression in the Marine Bacterium *Pseudoalteromonas* sp. Strain S9

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Received 17 December 1996/Accepted 20 May 1997

Sequence data for genes encoding 16S rRNA indicated that the marine strain previously named *Pseudomonas* sp. strain S9 would be better identified as a *Pseudoalteromonas* sp. By use of transposon mutagenesis, a chitinase-negative mutant of S9 with a *lacZ* reporter gene insertion was isolated. Part of the interrupted gene was cloned and sequenced. The deduced amino acid sequence had homology to sequences of bacterial chitinases. Expression of the chitinase gene promoter was quantified by measuring the *lacZ* reporter gene product, β-galactosidase. β-Galactosidase production was induced 10-fold by *N*-acetylglucosamine and 3-fold by chitin in minimal medium. Repression of β-galactosidase synthesis was observed in rich medium either with or without chitin but was not observed in minimal medium containing glucose. The chitinase gene promoter was induced by starvation and higher-than-ambient levels of carbon dioxide but not by cadmium ion, heat or cold shock, or UV exposure.

The marine environment is variable in nutrient composition but is generally nutrient depleted in that most marine waters contain relatively little phosphate and are limited in carbon and nitrogen (35, 44). In such an environment, chitin, mainly from zooplankton, provides a carbon and nitrogen source for marine organisms (14). The marine environment would be completely depleted of carbon and nitrogen in a relatively short time if the insoluble form of chitin were not returned to the ecosystem in biologically useful forms. Conversion of the highly complexed chitin into a utilizable form is a recycling process which provides carbon, nitrogen, and energy in the environment (58). Marine chitinolytic bacteria are responsible for biological recycling of chitin and therefore play a major role in the ecology of marine environments.

The marine bacterium strain S9 was first characterized by Humphrey et al. (17) as a gram-negative, heterotrophic, rod-shaped, polarly flagellated, nonpathogenic bacterium which secretes several extracellular enzymes including chitinase to the external medium (2). Physiological studies of exoprotease activity during starvation as well as the starvation survival response of S9 have been investigated (1, 55, 56). More recently, genes specifically induced by carbon dioxide and not by other environmental signals known to stimulate regulators of bacterial gene expression have been characterized for strain S9 (46). Strain S9 was initially identified as a marine *Pseudomonas* strain from its biochemical data (17). Since studies on the phylogenetic relationships of different marine bacterial strains have shown that 16S rRNA sequences are a useful tool with which to identify phenotypically closely related bacteria (21), the sequence of the genes encoding 16S rRNA (16S rDNA) of S9 was determined in this study. The phylogenetic relationship of strain S9 was found to be closest to the genus *Pseudoalteromonas.*

Chitinase-encoding genes in nonmarine bacteria such as *Serratia marcescens* (10) and *Bacillus circulans* (52) and in marine strains such as *Alteromonas* sp. strain O7 (48), *Vibrio harveyi* (45), and *Vibrio vulnificus* (54) have been cloned and sequenced. However, the expression of such chitinase genes in response to environmental conditions has been little studied. In the present study, an S9 chitinase-negative mutant containing a promoterless *lacZ* reporter gene was isolated by insertion of the transposon mini-Tn*10:lac:kan* (2) and was used to investigate expression of a chitinase gene promoter in response to various environmental conditions. DNA sequence analysis of part of the transposon-interrupted gene from the chitinase-negative mutant revealed similarity with other bacterial chitinase genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. S9 is a gram-negative marine bacterium producing intensely orange-colored colonies and was originally described as a *Pseudomonas* strain (17). S91 is a spontaneous Sm^r mutant of S9 (2) and was used to generate S91CX, a transposon-interrupted chitinase-negative LacZ$^+$ mutant. *Escherichia coli* SM10λpir (15), carrying plasmid pLBT::mini-Tn*10:lac:kan* (2), was used in transposon mutagenesis. *E. coli* DH5α (Gibco-BRL) was used as a host of recombinant plasmids. Plasmid pUC119 (50) was used as a cloning vector. Plasmid pCX1 contained the pUC119 vector with a 5.2-kb insert of the transposon-interrupted chitinase gene from S91CX.

Strains S91 and S91CX were grown in tryptone soya broth (TSB; Oxoid) supplemented with 20 g of NaCl liter^{-1}, 1 mM $MgCl_2$, and 300 μM $CaCl_2$ or in marine minimal medium (MMM) (34) supplemented with 20 mM glutamic acid (MMMglt) at 30°C. *E. coli* was grown at 37°C on Luria-Bertani broth (LB) medium (26). Agar plates contained 15 g of Bitek agar (Difco) liter^{-1}. Where appropriate, antibiotics (Sigma) were used at the following concentrations: for S91, streptomycin at 200 μg ml^{-1} and kanamycin at 600 μg ml^{-1}; for *E. coli*, ampicillin at 50 μg ml^{-1} and kanamycin at 50 μg ml^{-1}. Colloidal chitin was prepared from practical-grade chitin (Sigma) as described by Shihamara and Takiguchi (43).

Sequencing of S9 16S rRNA. 16S rRNA sequence determination was carried out by direct sequencing of a PCR product of the 16S rDNA gene amplified from a boiled cell lysate of S9. Universal primers 10-30F and 1492-1510R (23) were used to amplify the 16S rRNA-encoding gene. PCR conditions followed a standard protocol (18) and consisted of initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, and annealing at 50°C for 30 s and extension at 72°C for 2 min, followed by a further 10-min extension at 72°C. Prior to direct sequencing, the PCR product was purified with a Prep-A-Gene kit (Bio-Rad). Manual DNA sequencing (42) of the PCR product was performed with primers complementary to eubacterial conserved 16S rRNA

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sequences (23, 53) and a modified T7 polymerase protocol (8). A Sequenase (U.S. Biochemicals, Cleveland, Ohio) T7 DNA polymerase sequencing kit employing [^{35}S]dATP was used as described in the manufacturer's instructions. Both DNA strands were sequenced in duplicate.

Sequence comparison and phylogenetic analysis. Comparison with other 16S rRNA sequences was performed with the FastA program (36) with GenBank and EMBL databases. Phylogenetic analysis of the S9 16S rRNA sequence with the *Pseudoalteromonas-Alteromonas* group was carried out by the distant matrix method (19) followed by neighbor-joining analysis (41) to create an evolutionary dendrogram for illustration of genetic relatedness. The reliability of each tree node was confirmed by bootstrapping of 100 trees, and the consensus tree was constructed by use of the SEQBOOT and CONSENSE program (9). These phylogeny programs are part of the Phylip package provided through the Australian National Genomic Information Service (Sydney, Australia).

Transposon mutagenesis. Transposon mutagenesis of strain S91 was carried out as described previously (2). Transconjugants were screened for a chitinase-negative phenotype by patching on MMMglt supplemented with 0.1% yeast extract and 0.1% colloidal chitin. Plates were incubated at 30°C for at least 15 days; colonies which produced no clearing zone at this time were designated chitinase negative. A clearing zone in the chitin agar around the wild-type colony was detected after overnight incubation. Chitinase-negative mutants were grown on chitin-MMMglt plates with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg ml^{-1}) to identify LacZ$^+$ mutants as blue colonies. A chitinase-negative LacZ$^+$ mutant, S91CX, was isolated and used for chitinase gene expression studies. Isolation of S91 and S91CX genomic DNA, labelling of the probe, and Southern hybridization were carried out by standard methods (42) to check for single insertion of the transposon.

Chitinase and chitobiase activity test. Chitinase activity was tested against fluorescent chitin oligomer analogs, 4-methylumbelliferyl-*N*,*N*′-diacetylchitobiose [4-MU-(GlcNAc)$_2$; exochitinase] and 4-methylumbelliferyl-*N*,*N*′,*N*″-triacetylchitotriose [4-MU-(GlcNAc)$_3$; endochitinase] (37). The chitobiase activity test was carried out with a fluorogenic substrate, 4-methylumbelliferyl-β-D-*N*-acetylglucosamine (4-MU-GlcNAc) (32).

Protein electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% gels by the procedure of Laemmli (22). A set of low-range molecular size markers (Bio-Rad; 14 to 97 kDa) was used. After electrophoresis was completed, renaturation of the enzymes (47) and the detection of chitinase activity were carried out. Briefly, this involved incubation for 2 h at 4°C with shaking in 1% (vol/vol) Triton X-100 and then incubation at 30°C for 15 min with 250 μM 4-MU-(GlcNAc)$_3$ overlaid on top. Fluorescent bands in gels were visualized under UV light and photographed. Total protein in culture supernatants was concentrated by ultrafiltration (Centripep-3 concentrator; Amicon). Chitinase proteins from culture supernatants were purified by the chitinase affinity method as described by Roberts and Cabib (39).

Determination of bacterial attachment. Cultures of S91 and S91CX were grown in MMMglt to an absorbance at 610 nm (A_{610}) of 0.1 (approximately 2 × 10^8 to 3 × 10^8 cells ml^{-1}). To measure bacterial attachment, approximately equal areas (about 4.84 cm^2) of sterile glass coverslip and pieces of sterile squid pen were added separately to S91 and S91CX cultures. Squid pen, 40% chitin and 60% (wt/wt) protein (14), was collected from a fish market in Sydney. The cultures were incubated for 60 min at 30°C without shaking. The number of attached bacterial cells was determined as the total CFU by vortexing the glass coverslip and squid pen in MMM (1 ml) and plating appropriate serial dilutions of cell suspensions on TSB plates. Each treatment was done in duplicate. After the squid pen and coverslip were vortexed, both surfaces were observed under a microscope to check for cells remaining on the surfaces.

Cloning of the transposon-interrupted, chitinase-negative gene from S91CX. DNA cloning procedures were carried out by use of standard protocols (42). Isolated genomic DNA of S91CX, digested with restriction enzymes *Eco*RI and *Sal*I, which do not cut within the kanamycin resistance gene carried on the transposon (2), was ligated to pUC119 also digested with *Eco*RI and *Sal*I. *Eco*RI cuts within the *lacZ* gene in the transposon. The ligation mixture was transformed into competent *E. coli* DH5α cells. Transformants were plated on LB-ampicillin-kanamycin plates. The DNA sequence adjacent to the insertion point of the transposon in plasmid pCX1 was sequenced by designing a primer (5′-CACTGATGAATGTTCCGTTGC-3′) reading outward at the end of the Km^r gene. Approximately 740 bp of the transposon-interrupted gene was then sequenced downstream of the Km^r gene by directed sequencing with progressive oligonucleotide primers (42). The complementary strand was similarly sequenced beginning with the reverse primer (5′-TGTTTGTGGGAAGTTTGAA-3′). Both DNA strands were sequenced with an automated fluorescence sequencer (Applied Biosystems model 373) at a DNA sequencing facility at Westmead Hospital, Sydney, Australia. Comparison of amino acid sequences with protein sequences available in databases was performed by use of programs in the Genetic Computer Group software package (University of Wisconsin). The computation was performed through a computer link to the Australian National Genomic Information Service with the BLAST program (3).

Chitinase gene expression studies. Expression of the interrupted chitinase gene promoter was quantitated by measuring levels of β-galactosidase specific activity (26) in S91CX. An overnight culture was diluted 1:100 in the following media: MMMglt, MMMglt with 0.1% chitin, MMMglt with 0.1% *N*-acetylglucosamine (Sigma), TSB with 0.1% chitin, and MMM with 0.1% chitin and glucose (20 mM) as a carbon source. The cultures were grown overnight at 30°C with shaking. β-Galactosidase activity was assayed by the same procedure as that described in detail by Stretton et al. (46).

To test the effects of various environmental conditions, S91CX was grown in MMMglt at 30°C until the culture reached an A_{610} of 0.2. The culture was divided into aliquots of equal volume and subjected to the following conditions. Carbon starvation was carried out by pelleting and resuspending cells in MMM without an added carbon source (34). UV treatment was performed by exposing the shaking culture to UV light at a distance of 15 cm (Oliphant Ultraviolet Product; 253 nm) (46). The effect of a heavy metal, cadmium ion ($3CdSO_4 \cdot 8H_2O$), was studied at different concentrations (10, 50, and 100 μg ml^{-1}). The effect of temperature was investigated by exposing cultures to 4 and 37°C with shaking.

To study the response of the chitinase gene promoter to CO_2, S91CX was grown on MMMglt plates containing X-Gal under normal atmospheric conditions. Following 16 h of incubation, the plates were (i) placed in a 10% CO_2–20% O_2 chamber (GasPak; catalog no. 70308; BBL), (ii) wrapped with Nescofilm, and (iii) wrapped with Nescofilm after the addition of a vial of KOH (1 ml, 20%) to act as a -CO_2 sink (46). CO_2 was removed from liquid cultures by placing a glass tube containing KOH (5 ml, 20%) inside flasks made airtight with a rubber stopper. A cotton-topped flask was used as a control for normal atmospheric conditions (46).

Tests for statistical significance. The statistical significance of the differences between the means of numbers of attached cells to surfaces, as well as β-galactosidase activities of the mutant subjected to varying conditions, was determined with a two-sample two-tailed *t* test.

Nucleotide sequence accession number. The 16S rDNA sequence of S9 has been deposited in the GenBank nucleotide sequence data library under the accession number U80834.

RESULTS

16S rDNA sequence of S9. The alignment results of S9 16S rRNA sequences obtained from both GenBank and EMBL databases confirmed the highest similarity to the *Pseudoaltero-monas-Alteromonas* group (11) (Fig. 1). The similarities ranged from 92.8 to 97.2% for *Pseudoalteromonas* sp. but only 88.8% for *Alteromonas* sp. The most similar organism in this group was *Pseudoalteromonas luteoviolacea*, with 97.2% DNA sequence homology to strain S9.

Generation of chitinase-negative mutants. By use of transposon mutagenesis, four chitinase-negative mutants were isolated from approximately 3,500 transconjugants screened on chitin overlay agar plates. The chitinase-negative mutants showed no clearing zone around colonies on chitin-MMMglt plates in contrast to S91, which produced a clearing zone after overnight incubation. All four mutants, however, were shown to have exo- and endochitinase activity when 4-MU-(GlcNAc)$_2$ and 4-MU-(GlcNAc)$_3$, respectively, were used. In addition, each mutant was shown to have chitobiase activity when 4-MU-GlcNAc was used. The four mutants were patched onto chitin-MMMglt plates containing X-Gal, and one mutant developed blue colonies. This chitinase-negative, LacZ$^+$ mutant, S91CX, was used further to study regulation of chitinase gene expression in this organism. In contrast to S91, S91CX was unable to grow in MMM with chitin as the sole carbon source. Southern hybridization of S91CX genomic DNA showed that a single insertion of the transposon had occurred (data not shown).

SDS-PAGE analysis showed that two protein bands were missing from the culture supernatants of S91CX. These bands corresponded to 76- and 64-kDa proteins present in S91 culture supernatants and purified chitinase from S91 supernatants (Fig. 2A). In addition, similar-sized proteins on chitinase activity gels were also missing in the supernatant of S91CX that were present in supernatants of S91 and purified chitinase (Fig. 2B).

Attachment of the S91 wild type and the S91CX chitinase-negative mutant to surfaces. Since the 76- and 64-kDa chitinase proteins were missing from the chitinase-negative mutant and these two proteins were shown to bind specifically to chitin particles, an adhesion test was carried out to determine whether interruption of the chitinase gene affected initial at-

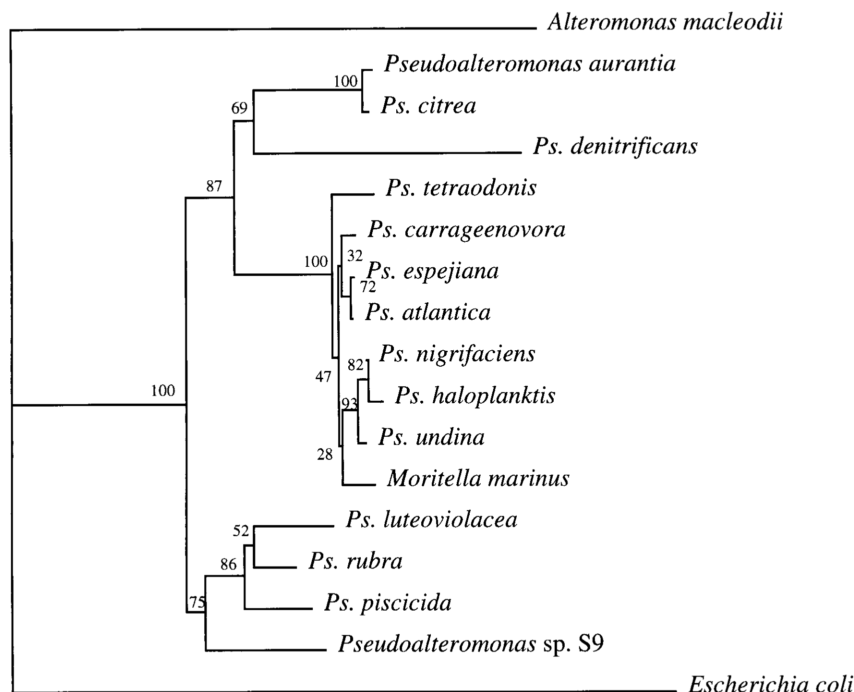

FIG. 1. Phylogenetic relationship of the 16S rDNA sequence of strain S9 to that of other gram-negative marine bacteria. The phylogenetic tree was based on the alignment of complete 16S rRNA sequences. The 16S rRNA sequence of *E. coli* was chosen as an outgroup. The distant matrix method (19) was used to infer phylogeny, and the neighbor-joining method (41) was used to construct the tree. Bootstrap values obtained from 100 resamplings are given in percentages and indicated at the corresponding nodes. The distance between two species was calculated by combining the length of the connecting horizontal branches by use of the scale shown. Ps., *Pseudomonas.*

tachment of S91CX, compared to that of S91, to chitin. A glass slide was included to determine also if there was a difference in cell attachment between a biodegradable substrate (squid pen) and an inert one (glass). As shown in Fig. 3, the numbers of attached cells of S91CX to squid pen and the glass surface, incubated without agitation for 1 h, were the same as those of S91. Attachment of S91 and S91CX cells to squid pen was about 10-fold higher than that to the glass surface ($P < 0.05$) (Fig. 3). After being vortexed, both surfaces were examined microscopically; attached cells (8.4×10^2 cells/cm^2) remained on squid pen, while glass slides were clear of cells.

Cloning of the interrupted, chitinase-negative gene from S91CX. *Eco*RI-and-*Sal*I-digested pUC119 and S91CX genomic DNA were ligated. After transformation of the ligation mixture into competent DH5α cells, plasmid DNA of several Ap^r and Km^r transformants was analyzed on an agarose gel. One plasmid with an insert of 5.2 kb was named pCX1 and investigated further. Approximately 1.5 kb was from part of the transposon including the complete kanamycin gene, and 3.7 kb was S91CX genomic DNA. Approximately 740 bp of the interrupted DNA downstream of the Km^r gene from plasmid pCX1 was determined. The nucleotide sequences and the deduced amino acids of the interrupted gene are shown in Fig. 4A. Comparison of the deduced amino acid sequence of a portion of the interrupted gene with protein sequences available in databases showed homology to bacterial chitinases belonging to family 18 of glycosyl hydrolases (33). Alignment of the partial sequence of deduced amino acids of the interrupted gene revealed a region of similarity to chitinases in gram-positive bacteria as shown for the three best matches in Fig. 4B, i.e., the chitinase A of *Clostridium thermocellum* (58% similarity, 37% identity), the chitinase A precursor of *B. circu-*

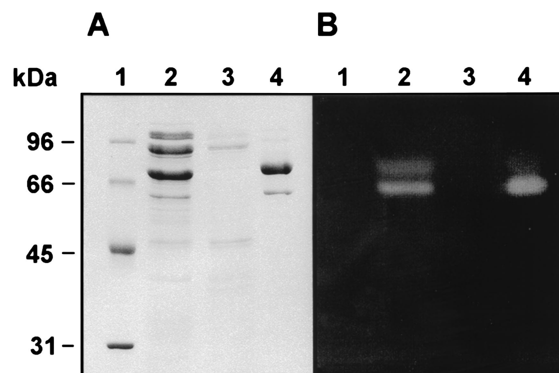

FIG. 2. SDS-PAGE analysis of culture supernatants from the wild type, S91, and S91CX. (A) Coomassie brilliant blue-stained SDS-polyacrylamide gel of culture supernatants; (B) chitinase activity detected on the polyacrylamide gel with 4-MU-(GlcNAc)$_3$. Samples were supernatants from S91 (lane 2, 2.5 μg of total protein), S91CX (lane 3, 2.5 of μg total protein), and purified chitinase protein (lane 4). Molecular size standards (lane 1) consisted of rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), and bovine carbonic anhydrase (31.0 kDa).

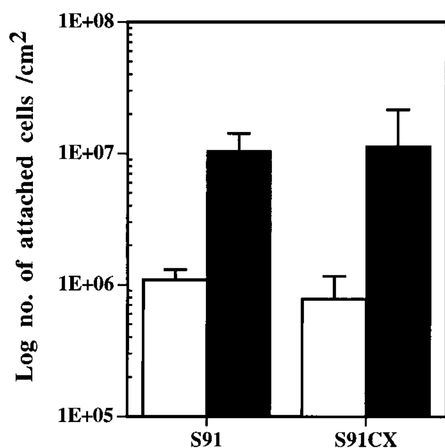

FIG. 3. Attachment of S91 and S91CX to squid pen (■) and a glass surface (□). Data represent the means of two independent experiments measured in duplicate. Error bars represent standard deviations of means.

lans (54% similarity, 34% identity) (52), and a chitinase of *Kurthia zopfii* (53% similarity, 34% identity).

Investigation of chitinase gene expression. Investigation of the effect of external environmental stimuli on expression of the chitinase gene promoter was carried out. β-Galactosidase activity was induced 3-fold by chitin and 10-fold by *N*-acetylglucosamine in stationary-phase cells (16 h) compared to the low basal level of β-galactosidase produced when cells were grown in MMMglt without chitin ($P < 0.05$) (Fig. 5A). Induction of β-galactosidase began rapidly after S91CX cells grown in MMMglt were exposed to *N*-acetylglucosamine, whereas induction by chitin was slower (data not shown). Catabolite repression was not observed when glucose was added as a carbon source in minimal medium (Fig. 5B). However, β-galactosidase production was reduced when S91CX was grown in TSB either with or without chitin compared to when it was grown in MMMglt without chitin (Fig. 5B). SDS-PAGE analysis of protein profiles of the concentrated culture supernatants from S91 showed increased production of chitinase enzymes in minimal medium containing either glutamine or glucose plus either chitin or *N*-acetylglucosamine, compared to low levels from cells grown in MMMglt without chitin or grown in TSB with or without chitin (Fig. 6). Exposure of S91CX cultures to 4°C, 37°C, UV light, or cadmium ion had no effect on β-galactosidase activity (data not shown). During the first 3 h of carbon starvation, S91CX cells showed about a three- to fourfold increase of β-galactosidase synthesis compared to that of cells growing in MMM with glutamate as a carbon source ($P < 0.05$) (Fig. 7).

When S91CX was grown on plates under normal atmospheric conditions, colonies were pale blue. Colonies of S91CX turned dark blue on plates incubated in a 10% CO_2 chamber or wrapped with Nescofilm. Plates wrapped with Nescofilm containing a KOH/CO_2 sink also produced pale blue colonies. Levels of β-galactosidase production were measured during growth in liquid cultures. In the presence of a KOH/CO_2 sink, levels of β-galactosidase in stationary-phase (16 h) cultures were decreased about two- to threefold compared to that of stationary-phase cells grown under normal atmospheric conditions ($P < 0.05$) (Fig. 8). Cell densities at 16 h were equivalent for all cultures.

DISCUSSION

Identification of marine bacteria based on biochemical tests alone has not been able to distinguish between similar strains of marine *Pseudomonas* and *Alteromonas* species (11). Recent studies have shown 16S rRNA sequence determination to be more useful than G+C content assays (11). In this study, determination and alignment of the 16S rDNA sequence of a marine strain, S9, previously identified as *Pseudomonas* sp. strain S9, revealed a high similarity of S9 to *Pseudoalteromonas* spp., a new genus proposed by Gauthier et al. (11). This genus contains 12 species, including those previously identified as *Alteromonas* spp. and others previously assigned as marine *Pseudomonas* strains. Only one species, *Alteromonas macleodii*, is retained in the genus *Alteromonas*, for which it also serves as the type species. S9 branched deeply within the same cluster of three other pigmented *Pseudoalteromonas* strains, *Pseudoalteromonas piscicida* (yellow-orange), *Pseudoalteromonas luteoviolacea* (violet), and *Pseudoalteromonas rubra* (red) (7, 12, 13). S9 produces a distinct orange pigment. It is interesting that pigmented strains of *Pseudoalteromonas* have common characteristics in their own group and may have evolved from the same or very closely related ancestors. Since phylogenetic analysis of the 16S rDNA sequence of S9 showed high relatedness to the *Pseudoalteromonas* genus, S9 would be better designated as *Pseudoalteromonas* sp. strain S9. This nomenclature is used in the current study.

The presence of multiple chitinase genes in bacteria is common, as found in *S. marcescens* (27) *V. harveyi* (45), and *B. circulans* (51). This could be the case for S9 since the transposon-interrupted chitinase-negative mutants produced no clearing zone when grown on minimal medium plates containing chitin, but when the more sensitive fluorescent substrate assay (16) was used, chitinase activity was shown to be present. It is possible that cell-associated chitinase could catalyze hydrolysis of the fluorescent chitin oligomer analogs, giving fluorescence in colonies as reported previously for *S.marcescens* BJL200 (6). In *S. marcescens* BJL200, chitinase is only exported to the periplasm, not secreted to the medium (6). It is also possible that, in S91, another chitinase was secreted but at levels too low to be detected in culture supernatants. Two chitinase proteins (76 and 64 kDa) were found in the culture supernatant of S91 on chitinase activity gels. Since both proteins were absent from the culture supernatant of S91CX, it is possible that the 64-kDa chitinase protein was derived from the 76-kDa chitinase protein by proteolytic cleavage after secretion, as has been shown previously for the chitinase system of *B. circulans* (51). The deduced amino acid sequence analysis of part of the transposon-interrupted gene revealed a similarity to other chitinase genes. This region corresponded to the catalytic domain of chitinase A1 in *B. circulans* (52). Cloning and sequencing of the wild-type gene is under way to obtain more information about regulation of chitinase genes in *Pseudoalteromonas* sp. strain S9.

Previous studies by Montgomery and Kirchman (28, 29) have demonstrated that attachment of *V. harveyi* to chitin involves specific chitin-binding proteins. They found that attachment of a chitinase-overproducing mutant to chitin was about twice as much as that of the wild type (29). In the present study, no difference in initial attachment behavior was found between S91 and S91CX on glass and squid pen surfaces. However, the number of bacterial cells attached to the natural biodegradable substrate, squid pen, after 1 h was significantly greater than the number attached to the inert substrate, a glass slide, for both S91 and S91CX. This suggests that *Pseudoalteromonas* sp. strain S9 attachment to chitin may also involve specific adhe-

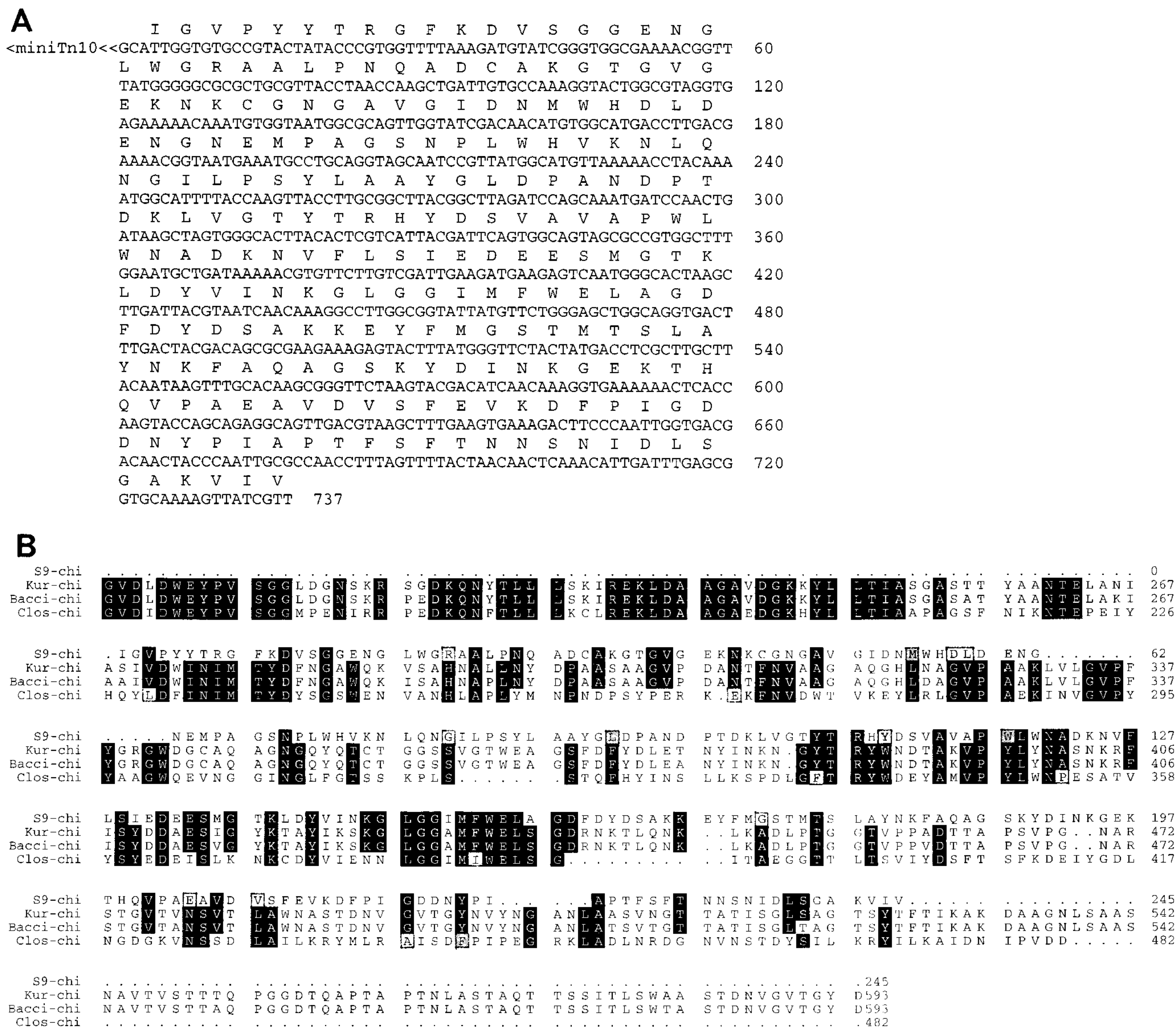

FIG. 4. (A) Nucleotide sequence and deduced amino acid sequence of 740 bp of the interrupted gene downstream from mini-Tn*10:lac:kan*. (B) Multiple amino acid sequence alignments of the deduced amino acid sequence of the interrupted gene of S91CX (S9-chi) with the chitinase of *Kurthia zopfii* (Kur-chi), the chitinase A precursor of *B. circulans* (Bacci-Chi), and the chitinase A of *Clostridium thermocellum* (Clos-chi). ■, identical amino acids; □, semiconserved or conserved amino acids.

sion mechanisms and that the 76- and 64-kDa chitinase proteins missing in S91CX were not solely responsible for initial cell attachment to squid pen. The 76- and 64-kDa chitinase proteins were able to bind specifically to chitin as they were able to be isolated by the chitinase affinity purification method (39). Nonspecific attachment of these cells to surfaces may also occur, as in the case of glass.

Previous work on regulation of chitinase enzymes in different organisms has shown that chitinase enzymes can be induced by chitin and can be either induced or repressed by chitosan, chitobiose, *N*-acetylglucosamine, or glucosamine (5, 27, 38). *N*-Acetylglucosamine acts as an inducer of chitinase in several bacteria such as *Alteromonas haloplanktis* (24), *Vibrio furnissi* (5), and *V. harveyi* (29). In *V. furnissi*, *N*-acetylglucosamine has been shown to cause specific adhesion of cells to chitin and to act as a chemoattractant (4, 57). Montgomery and Kirchman (29) have demonstrated that production of chitin-binding proteins, chitinase activity, and attachment to chitin are inducible in *V. harveyi* by the addition of chitin and *N*-acetylglucosamine oligomers. It has been shown that *N*-acetylglucosamine represses chitinase production in *S. marcescens* (27), *Streptomyces lividans* (30), and in the fungus *Trichoderma harzianum* (49). These studies have been based mostly on total chitinase enzymatic activities which may have included more than one enzyme, with the level of induction or repression of individual enzymes not known. In this study, by use of the S91CX chitinase-negative mutant with a *lacZ* reporter gene, it was possible to investigate the regulation of a single chitinase-encoding gene promoter. This promoter was induced by chitin and *N*-acetylglucosamine and was not subject to catabolite repression by glucose, although it was repressed by rich medium, which suggested that catabolite repression may have been caused by nutrient components of the medium. Chitin must be hydrolyzed to smaller molecules before transport

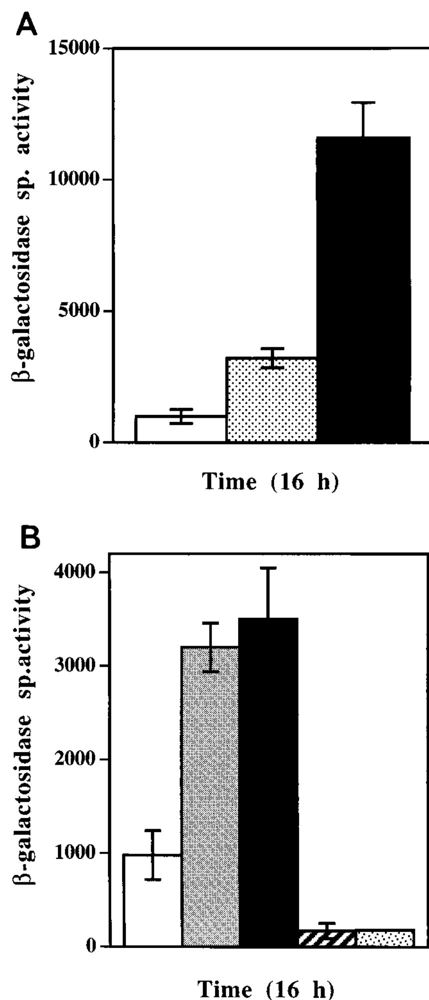

FIG. 5. Induction of β-galactosidase synthesis in S91CX at 16 h postinoculation. (A) Symbols: □, MMMglt; ▨, MMMglt plus 0.1% chitin; ■, MMMglt plus 0.1% *N*-acetylglucosamine. (B) Symbols: □, MMMglt; ▨, MMMglt plus 0.1% chitin; ■, MMMglucose plus 0.1% chitin; ▨, TSB; ▨, TSB plus 0.1% chitin. β-Galactosidase specific activity was measured per milligram of protein. Data represent the means of two independent experiments measured in duplicate. Error bars represent standard deviations of means.

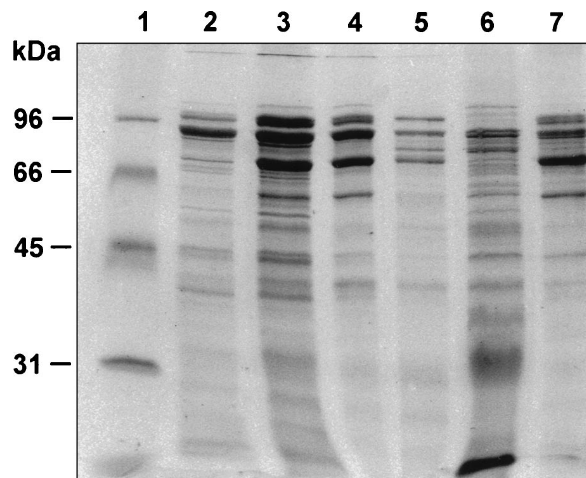

FIG. 6. SDS-PAGE analysis of culture supernatants from the wild type, S91. A Coomassie brilliant blue-stained SDS-polyacrylamide gel of culture supernatants is shown. Samples were supernatants from S91 grown in MMMglt (lane 2; 2.5 μg of total protein), MMMglt with 0.1% chitin (lane 3; 2.5 μg of total protein), MMMglt with 0.1% *N*-acetylglucosamine (lane 4; 2.5 μg of total protein), TSB (lane 5; 2.5 μg of total protein), TSB with 0.1% chitin (lane 6; 2.5 μg of total protein), and MMMglucose with 0.1% chitin (lane 7; 2.5 μg of total protein). Molecular size standards (lane 1) consisted of rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin 45.0 kDa), and bovine carbonic anhydrase (31.0 kDa).

across the bacterial cytoplasmic membrane can occur. It appeared that the gene encoding chitinase was expressed at a low basal level in S91. The basal level of chitinase activity may have been sufficient to initiate chitin degradation and to release soluble oligomers, including the end product of chitin biodegradation, *N*-acetylglucosamine, which then acted as the inducer(s) of expression of the chitinase-encoding gene and thus led to increased chitinase production.

Increasing evidence has shown that bacterial gene expression is often complex and can be regulated by multiple environmental signals, global regulators, and gene-specific elements (25, 40). There is little information on the specific responses of chitinase-encoding genes to physiochemical conditions. In this study, we have shown that levels of CO_2 in stationary-phase (16 h) culture can induce the *lacZ*-interrupted chitinase gene promoter in S9. Accumulation of CO_2 presumably occurred in the liquid culture as a result of increased cell density. β-Galactosidase activity was decreased in the stationary phase by removing CO_2 from the culture with a KOH/CO_2 sink. S9 is known to have other CO_2-inducible genes which are not induced by other environmental conditions, including starvation, as reported previously (46). It is interesting that this chitinase gene promoter was also induced during the first 3 h of carbon starvation. Increased production of exoproteases dur-

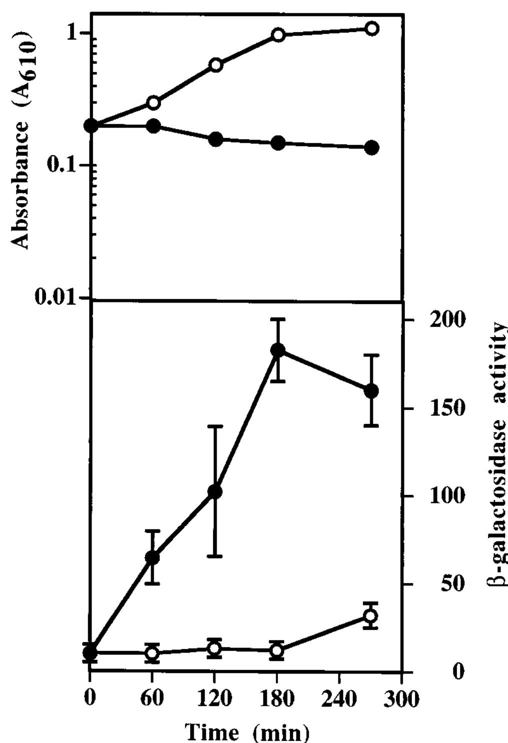

FIG. 7. Induction of β-galactosidase in S91CX by carbon starvation. Aliquots of S91CX grown to an A_{610} of 0.2 were pelleted and resuspended in MMMglt (○) and in MMM (●). β-Galactosidase activity was measured per A_{610} of the culture. Data represent the means of three independent experiments, each assayed in duplicate. Error bars represent standard deviations of means.

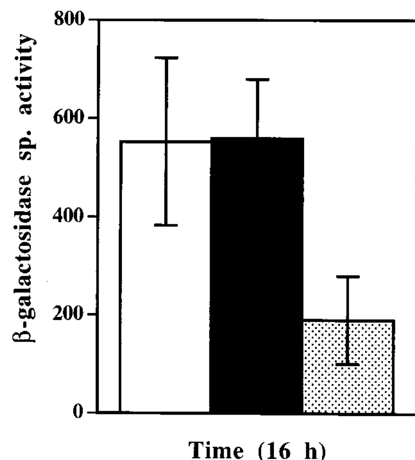

FIG. 8. Effect of CO_2 on β-galactosidase synthesis in S91CX. Cultures grown in MMMglt to an A_{610} of 0.2 were grown further with the following treatments: (i) with a cotton stopper (□), (ii) with a rubber stopper (■), and (iii) with a KOH-CO_2 sink and rubber stopper (▨). Samples were taken at the stationary phase (16 h) for the β-galactosidase assay. Data represent the means of three independent experiments, each assayed in duplicate. Error bars represent standard deviations of means.

ing the early stage of starvation has been demonstrated in S9 and *Vibrio* sp. strain S14 (1, 31). Increased production of exoproteases and chitinases at the onset of starvation conditions would provide a tactic for improving nutrient scavenging over a larger area, particularly in low-nutrient environments. This response would provide cells within a population potential opportunities to escape the starvation process (31). The chitinase gene promoter was not induced by other environmental stresses such as heat or cold shock or exposure to UV or cadmium ion, all which are known to induce genes encoding stress and starvation-inducible proteins (20, 31).

ACKNOWLEDGMENTS

This work was supported in part by The Flinders University of South Australia and the Australian Research Council. S. Pongpattanakitshote and S. Techkarnjanaruk are supported by Royal Thai Government Scholarships.

Thermal cyclers used for PCR were supplied by Corbett Research Australia.

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