JOURNAL OF BACTERIOLOGY, Feb. 2001, p. 1369–1375 Vol. 183, No. 4
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.4.1369–1375.2001

Regulation of Metalloprotease Gene Expression in *Vibrio vulnificus* by a *Vibrio harveyi* LuxR Homologue

CHUNG-PING SHAO AND LIEN-I HOR*

Department of Microbiology and Immunology, College of Medicine, National Cheng-Kung University, Tainan 701, Taiwan, Republic of China

Received 11 September 2000/Accepted 29 November 2000

Expression of the *Vibrio vulnificus* metalloprotease gene, *vvp*, was turned up rapidly when bacterial growth reached the late log phase. A similar pattern of expression has been found in the metalloprotease gene of *Vibrio cholerae*, and this has been shown to be regulated by a *Vibrio harveyi* LuxR-like transcriptional activator. To find out whether a LuxR homologue exists in *V. vulnificus*, a gene library of this organism was screened by colony hybridization using a probe derived from a sequence that is conserved in various *luxR*-like genes of vibrios. A gene containing a 618-bp open reading frame was identified and found to be identical to the *smcR* gene of *V. vulnificus* reported previously. An isogenic SmcR-deficient (RD) mutant was further constructed by an in vivo allelic exchange technique. This mutant exhibited an extremely low level of *vvp* transcription compared with that of the parent strain. On the other hand, the cytolysin gene, *vvhA*, was expressed at a higher level in the RD mutant than in the parent strain during the log phase of growth. These data suggested that SmcR might not only be a positive regulator of the protease gene but might also be involved in negative regulation of the cytolysin gene. Virulence of the RD mutant in either normal or iron-overloaded mice challenged by intraperitoneal injection was comparable to that of the parent strain, indicating that SmcR is not required for *V. vulnificus* virulence in mice.

Vibrio vulnificus, an opportunistic human pathogen, causes severe wound infection and primary septicemia (36, 38). This organism produces a few extracellular products implicated in bacterial virulence and pathogenesis, including cytolysin (12, 39), metalloprotease (11, 19), phospholipase (37), and siderophores (31). Only a single extracellular metalloprotease (designated Vvp) has been identified (11, 19). This protease has been shown to increase vascular permeability and edema through activating the Hageman factor-plasma kallikrein-kinin cascade (20, 21, 22) and to cause hypodermic hemorrhage in guinea pigs (23). It can also facilitate iron acquisition by the organism by digesting heme proteins, transferrin, and lactoferrin (24, 25). Based on these observations, Vvp was thought to be important in bacterial growth and disease development. Nevertheless, a Vvp-deficient mutant has been shown to be even more virulent than the wild-type strain, probably because of overexpression of the cytolysin in the absence of Vvp (30).

Vibrio cholerae hemagglutinin/protease (HA protease) (8) and *Vibrio anguillarum* EmpA (17), like Vvp of *V. vulnificus*, are members of the metalloprotease family in vibrios. The regulators of the genes of these two proteases have been identified: HapR for HA protease (10) and VanS for EmpA (D. Milton, U. Hope, M. Camara, and P. Williams, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. H-193, p. 366, 1999). Both HapR and VanS are members of the *Vibrio harveyi* LuxR family, with sequences and function similar to those of LuxR (10). The LuxR protein is a transcriptional activator which controls expression of the *lux* operon of *V. harveyi* at high bacterial cell density (34). Gene regulation by LuxR is a part of the quorum-sensing (cell density-dependent) regulatory system.

In this study, we investigated the transcription of *vvp* under various culture conditions, including different temperatures, osmotic pressures, iron levels, and oxygen levels. The results indicated that *vvp* was transcribed in a growth phase-dependent manner similar to that of the *Pseudomonas aeruginosa* elastase gene (*lasB*) (26) or the *V. cholerae* HA protease gene (*hap*) (10). Expression of LasB has been demonstrated to be regulated by a quorum-sensing regulatory system (26). A *luxR* homologue was identified in *V. vulnificus* by PCR with a pair of degenerate primers derived from sequences conserved in the genes of members of the *V. harveyi* LuxR family. The deduced amino acid sequence of this gene was identical to that of the *smcR* gene of *V. vulnificus* reported previously (15). We further demonstrated that SmcR is involved in the regulation of transcription of both *vvp* and the cytolysin gene by isolating and characterizing a mutant that was disrupted in *smcR*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *V. vulnificus* and *Escherichia coli* strains and the plasmids used in this study are listed in Table 1.

Cultivation and storage of bacteria. All strains were grown at 37°C with vigorous aeration in Luria broth (LB), in which ampicillin (100 μg/ml), polymyxin B (50 U/ml), or tetracycline (15 μg/ml) was added as appropriate. They were maintained at −70°C in LB medium containing 17% glycerol.

DNA preparation and manipulation. Plasmid DNA was extracted from the bacterial cells by the alkaline lysis method of Birnboim and Doly (3). Standard techniques were used to construct recombinant plasmids (28). DNA restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. DNA fragments were purified from the agarose gels by using the GeneClean Glassmilk kit (Bio 101, Inc.).

PCR was performed with a thermocycler (GeneAmp PCR system 9600; Perkin-Elmer Cetus) as described previously (30). The degenerate primers used in PCR for amplifying the *luxR* homologue from the chromosome of *V. vulnificus*

* Corresponding author. Mailing address: Department of Microbiology and Immunology, College of Medicine, National Cheng-Kung University, Tainan 701, Taiwan, Republic of China. Phone: 886-6-2353535 ext. 5635. Fax: 886-6-2082705. E-mail: h061453@mail.ncku.edu.tw.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
V. vulnificus		
YJ016	Clinical isolate	Laboratory collection
YJ024	Translucent mutant of YJ016	9
CP080	YJ016 Δ*vvp*	30
CP154	YJ016 *smcR*::pVR19	This study
CP156	YJ016 Δ*smcR*	This study
CP194	CP156/pVR21	This study
E. coli		
DH5α	*supE44 lacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	7
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir* Km^r Nal^r	16
LLM1956	DH5α/pRS205	14
Plasmids		
pRS205	pUC19 with the *lux* operon of *V. harveyi*	14
pJRD215	Shuttle vector; Km^r	5
pUC19	Cloning vector; Ap^r	Laboratory collection
pCVD442	Suicide vector; Ap^r	6
pVR11	pBR322 inserted with a 3.3-kb fragment containing the intact *smcR* gene	This study
pVR18	pVR11 with a 445-bp deletion in *smcR* (Δ*smcR*)	This study
pVR19	pCVD442 with the *Nhe*I-*Nhe*I fragment that contains Δ*smcR* cloned from pVR18	This study
pVR21	pJRD215 with the *Nhe*I-*Nhe*I fragment that contains the intact *smcR* cloned from pVR11	This study
pVR22	pJRD215 with the *Nhe*I-*Nhe*I fragment that contains Δ*smcR* cloned from pVR18	This study

[a] Abbreviations: Ap^r, Km^r, and Nal^r are resistance to ampicillin, kanamycin, and nalidixic acid, respectively.

were 5′-GGTGGTCACGCG(A)GATATTG and 5′-CCGTGGAAT(C)AGG (A)T(C)TT(G/C)GCC. Nucleotide sequence was determined by an autosequencer (ABI Prism 377 DNA Sequencer; Applied Biosystems).

RNA slot, colony, and Southern hybridization. Total RNA was prepared with an RNA isolation kit (RNeasy MiniKit; Qiagen GmbH, Hilden, Germany). Whole-cell DNA was prepared as described previously (30). Probes used in colony, Southern, and RNA slot hybridization were labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech Asia Pacific Ltd., Hong Kong, People's Republic of China) by random priming with a kit (Megaprime DNA labeling system; Amersham Pharmacia Biotech) using either the PCR products or fragments excised from the recombinant plasmids as the templates. The nylon membranes with DNA or RNA were prehybridized with the hybridization buffer (ExpressHyb Hybridization solution; Clontech Laboratories, Inc.) for 30 min at 68°C, hybridized for 1.5 h at the same temperature, washed, and visualized by autoradiography.

Primer extension. Fifty micrograms of total RNA was used as the template in the primer extension reaction. A primer (5′-GCCACGACGAGCAAACACTT CCAG-3′) complementary to the coding strand 98 bp downstream of the start codon of *smcR* was end labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech). Primer extension was performed by the method of Wu et al. (40) with modifications. Superscript II RNase H^- reverse transcriptase (GIBCO BRL Life Technologies, Inc.), instead of avian myeloblastosis virus (AMV) reverse transcriptase, was used and the reaction mixture was incubated at 50°C for 1.5 h. Plasmid pVR11 was primed with the same primer for a sequencing reaction by the dideoxy chain termination procedure of Sanger and Coulson (29) with the Sequenase 2.0 kit (United States Biochemicals) and [α-^{35}S]dATP (Amersham Pharmacia Biotech). The primer extension product was then subjected to electrophoresis on a 7 M urea–6% polyacrylamide gel in parallel with the DNA sequencing products.

Bioluminescence assay. The level of bioluminescence in a bacterial overnight culture was determined with a luminometer (Minilumate LB9506; Laboratorium Prof. Dr. Berthold GmbH & Co. KG, Bad Wildbad, Germany) and was expressed as relative light units (RLU) divided by the optical density at 600 nm (OD_{600}) of the culture.

Construction of the *smcR* mutant. A deletion in *smcR* was introduced into the chromosome of *V. vulnificus* by in vivo allelic exchange. Plasmid pVR18 was first constructed by removing a fragment between the *Sac*II and *Sac*I sites in *smcR* (see Fig. 4) from pVR11. This resulted in a 445-bp deletion in *smcR*, which was confirmed by DNA sequence determination. The *Nhe*I-*Nhe*I fragment from pVR18 was cloned into the *Xho*I site in pCVD442 to create pVR19. Plasmid pCVD442 is a suicide vector containing the *sacB* gene, which allowed positive selection with sucrose for loss of the vector. Plasmid pVR19 was transferred from *E. coli* SM10λ*pir* to *V. vulnificus* by conjugation. The transconjugants, which had pVR19 integrated in the chromosome via homologous recombination, were selected by ampicillin and polymyxin B and tested for sensitivity to 10% sucrose. Such transconjugants were obtained at a rate of 1 per 10^8 recipients. One of the sucrose-sensitive transconjugants was grown in LB containing 10% sucrose at 37°C overnight and then spread onto a 10% sucrose-containing LB plate for selecting the sucrose-resistant clones. The resultant strains were further tested for ampicillin sensitivity. Of the 200 sucrose-resistant colonies tested, 34 were ampicillin sensitive.

Protease and cytolysin assays. The protease activity in the culture supernatant was determined as described by Kreger and Lockwood (12). The cytolysin activity in the culture supernatant was assayed as described previously (30) and was expressed as 100% × (OD_{545} of specimen/OD_{545} of complete hemolysis by Triton X-100).

Determination of bacterial concentration. The bacterial concentration in the broth culture was estimated either by determining the OD_{600} value of the culture with a spectrophotometer (U-2000 Spectrophotometer; Hitachi Ltd., Tokyo, Japan), or by measuring the culture turbidity (expressed as Klett arbitrary units [kau]) with a turbidometer (Klett-Summerson photoelectric colorimeter; Klett Mfg. Co., Inc.).

Preconditioned medium assay. Bacteria cultivated in LB overnight at 37°C were diluted 1:100 in fresh LB and grown for a given period. The culture supernatant was collected and sterilized by filtration through a 0.22-μm-pore-size membrane and was used as the preconditioned medium. The preconditioned media were prepared with an isogenic Δ*vvp* mutant (30) to exclude the protease activity in them. A solution of 20× LB was added to the preconditioned medium to a final concentration of 0.5× to correct the nutrients when necessary. To test the autoinducer activity in the preconditioned medium, an overnight bacterial culture was diluted 1:500 in the preconditioned medium and grown at 37°C, and the protease activity in the culture supernatant was assayed at intervals.

Virulence assay. Virulence of the mutant and parent strain were tested in either normal or iron-overloaded mice. C3H/HeN mice, 6 to 8 weeks old and purchased from the animal center of the College of Medicine, National Cheng-Kung University, were challenged by intraperitoneal (i.p.) injection of the bacterial suspension. Mice were made iron overloaded by injecting 25 mg of iron dextran (Sigma Chemical Co.) per mouse intramuscularly 2 h prior to challenge. A group of five mice was given 0.5 ml of a 10-fold serially diluted bacterial suspension in phosphate-buffered saline per mouse and mortality was recorded 48 h postinfection. The LD_{50} (the dose lethal to 50% of the mice) was calculated by the method of Reed and Muench (27).

Resistance to serum killing. Forty microliters of bacterial suspension in phosphate-buffered saline was mixed with 160 μl of pooled serum from healthy

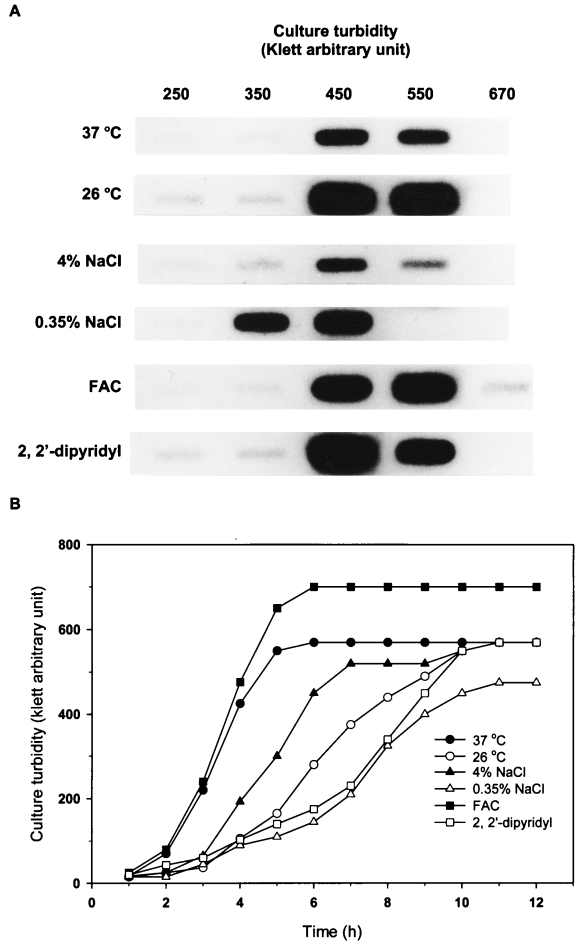

FIG. 1. (A) Transcriptional levels of *vvp* during growth under various culture conditions. Five micrograms of total RNA extracted from 10^9 bacteria of YJ016 was subjected to RNA slot blot hybridization with a ^{32}P-labeled probe derived from the *vvp* gene. FAC, ferric ammonium citrate. The concentrations of ferric ammonium citrate and 2,2′-dipyridyl in LB medium were 0.1 mg/ml and 0.15 mM, respectively. All cultures (except for that cultured at 26°C) were grown at 37°C. (B) Growth curves of YJ016 cultivated under various conditions. The culture conditions were the same as those in panel A.

volunteers, and the mixture was incubated with end-over-end rotation at 37°C for 30 min. The number of viable bacteria in the mixture was then determined by plate counts.

DNA database search. The National Center for Biotechnology Information service was used to consult the GenBank database with the BLAST algorithm for searching the homologous sequences.

RESULTS

Regulation of *vvp* transcription. The transcription levels of *vvp* during growth under various conditions were examined by RNA slot blot analysis. Bacteria grown aerobically usually reached a maximal turbidity of 550 kau, except for those grown at low salt concentrations and high iron levels, the maximal turbidity for which was 450 and 670 kau, respectively. Transcription of *vvp* was higher at 26°C than at 37°C, higher at low salt than at high salt concentrations, and higher at low iron than at high iron levels (Fig. 1A). In every condition studied, transcription of *vvp* was turned up dramatically when the culture was just about to enter the stationary phase (Fig. 1A and B). *V. vulnificus* grew poorly anaerobically and only reached 90 kau (data not shown), and the transcription of *vvp* was undetectable.

Detection of autoinducer activity in culture supernatant of *V. vulnificus* for protease expression. The kinetics of *vvp* transcription suggested that expression of this gene may be partly regulated by the quorum-sensing mechanism, in which the autoinducers secreted by the organism act as the signaling molecules. To test this, the culture supernatants collected from a 3-h (OD_{600} = 2.0) and a 6-h (OD_{600} = 6.6) culture were examined for autoinducer activity. *V. vulnificus* YJ016 grown in either preconditioned medium exhibited a similar growth rate as that of those grown in LB medium (data not shown). However, the bacteria grown in the preconditioned medium from a 6-h, but not a 3-h, culture expressed the protease activity at a lower bacterial cell density compared with those grown in LB (Fig. 2).

Cloning and nucleotide sequence determination of the *luxR* homologue in *V. vulnificus*. To identify the *V. harveyi luxR* homologue in *V. vulnificus*, a gene library (4) of a clinical strain, YJ016, was screened by colony hybridization. The probe used was amplified from a chromosome with a pair of degenerate primers derived from sequences that are conserved in various *luxR*-like genes. One clone thus obtained was further used to determine the nucleotide sequence of the insert in the recombinant plasmid. Two complete and one partial open reading frames (ORFs) were identified. Sequence comparison of the two complete ORFs with those in the database of GenBank showed that they were highly homologous to the gene of dihydrolipoamide dehydrogenase (Lpd) of *Vibrio parahaemolyticus* and LuxR of *V. harveyi*. The incomplete ORF shared sequence homology with the N terminal of the hypoxanthine ribosyltransferase (Htp) of *V. parahaemolyticus*. The deduced amino acid sequence of the cloned *luxR* homologue (GenBank accession no. AY007308) was identical to that of the *smcR* gene of *V. vulnificus* published recently by McDougald et al. (15). Therefore, the gene we cloned was *smcR*. The amino acid sequence of SmcR was 93, 93, and 78% identical to LuxR of *V. harveyi*, OpaR of *V. parahaemolyticus*, and HapR of *V. chol-*

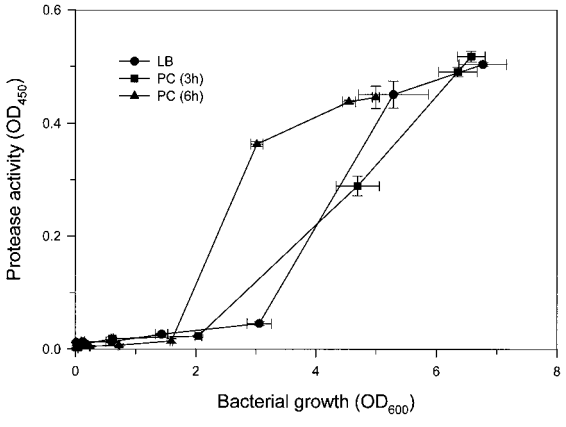

FIG. 2. Effects of preconditioned media (PC) on expression of protease activity in culture supernatant. An overnight culture of *V. vulnificus* YJ016 was diluted 1:500 in LB, 3-h preconditioned medium, and 6-h preconditioned medium with 0.5× LB. The bacteria were then grown at 37°C, and the OD_{600} of the culture as well as the protease activity in the culture supernatant was determined every hour.

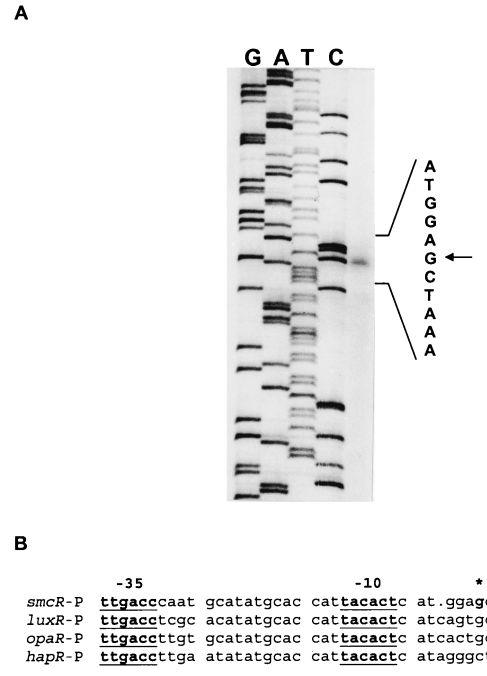

FIG. 3. (A) Determination of the tsp of *smcR*. Fifty micrograms of total RNA extracted from YJ016 was used in the primer extension reaction. Nucleotide sequences (GATC) of the noncoding strand up- and downstream of tsp obtained with the same oligoprimer are indicated. A G (indicated by an arrow) 87 bp upstream of the initiation codon was identified as the tsp. (B) Sequence alignment of the promoter regions of *smcR*, *luxR*, *opaR*, and *hapR*. The underlines indicate the promoter sequences (−10 and −35) and the asterisk denotes the tsp.

erae, respectively. The predicted molecular mass and isoelectric point of SmcR were 23.7 kDa and 5.81, respectively.

Determination of the tsp of *smcR*. The transcription start point (tsp) of *smcR* was determined by primer extension (Fig. 3A). A G located 87 bp upstream of the start codon was identified. The putative promoter sequence (13) determined according to the tsp was TTGACC for the −35 and TACACT for the −10 sequences, which were separated by 16 bp. Alignment of the promoter regions of the *luxR* family members revealed identical promoter sequences among them (Fig. 3B).

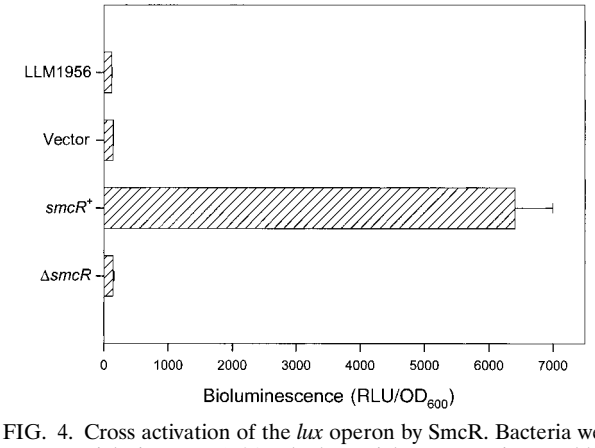

FIG. 4. Cross activation of the *lux* operon by SmcR. Bacteria were grown overnight in LB at 37°C. The bacterial concentrations and bioluminescence (relative light units [RLU]) of the cultures were then determined. LLM1956, the reporter strain; vector, LLM1956 with pJRD215 as a negative control; *smcR*$^+$, LLM1956 with pVR21 that carried the intact *smcR* gene; Δ*smcR*, LLM1956 with pVR22 that carried Δ*smcR*.

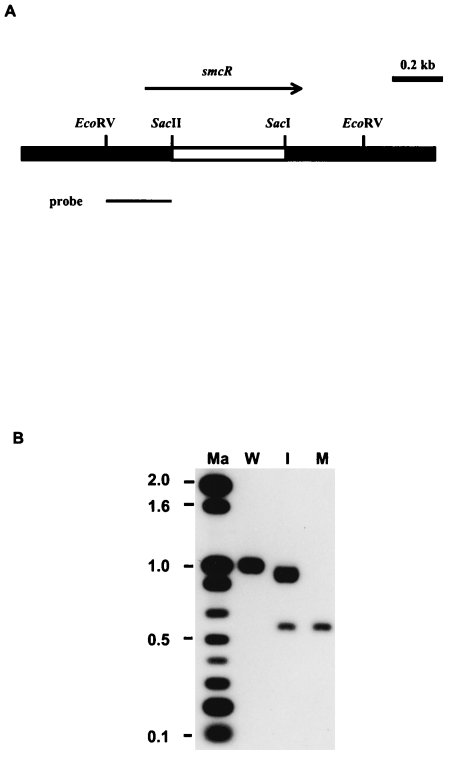

FIG. 5. (A) Restriction map of *smcR* and the flanking regions. The arrow indicates the direction of transcription. The extent of deletion (blank bar) in *vvpR* and the probe used in Southern hybridization are also depicted. (B) Detection of the deletion in *smcR* in the chromosome of *V. vulnificus*. Ten micrograms of genomic DNA was digested by *Eco*RV and then subjected to electrophoresis on a 1.2% agarose gel. Ma, molecular weight standards; W, *V. vulnificus* YJ016 (wild type); I, CP154 (YJ016 with pVR19 integrated in the chromosome); M, CP156 (Δ*smcR*).

Transactivation of *V. harveyi lux* operon by SmcR. An *E. coli* strain containing a functional but *luxR*-requiring *V. harveyi lux* operon in pRS205 was transformed with pJRD215, the vector; pVR21 that carried *smcR*; or pVR22 that carried Δ*smcR*. Bioluminescence produced by the transformants was then measured. As shown in Fig. 4, a high level of bioluminescence was detected in the presence, but not in the absence, of SmcR, indicating that LuxR can be replaced by SmcR for activating the *lux* operon.

Isolation of *V. vulnificus* SmcR-deficient mutant. To determine the roles of SmcR in the regulation of transcription of *vvp* and other genes in *V. vulnificus*, an isogenic Δ*smcR* mutant (RD mutant) was isolated from a clinical *V. vulnificus* isolate, YJ016. A suicide vector, pVR19, which carried the *smcR* gene with a 445-bp deletion (Fig. 5), was constructed and used to isolate the RD mutant by an allelic exchange technique. Twelve randomly chosen sucrose-resistant, ampicillin-sensitive colonies were examined by PCR using a pair of primers complementary to sequences flanking the deletion, and seven of them were shown to contain the deletion (data not shown). The presence of the *smcR* deletion in the chromosome of one

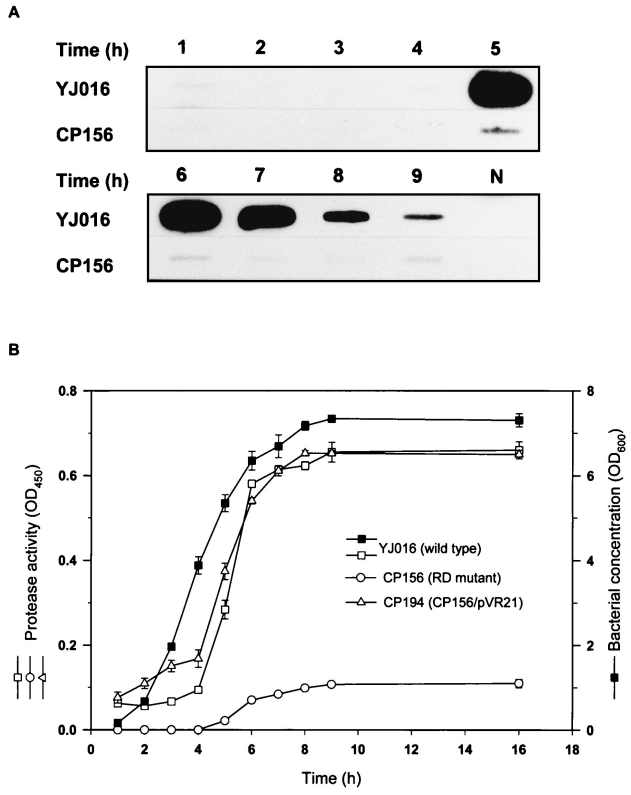

FIG. 6. Expression of Vvp by *V. vulnificus* strains. The bacteria were grown in LB at 37°C after a 1:100 dilution of an overnight culture. Total RNA (5 μg) was extracted from the bacteria harvested at intervals and examined for the transcriptional levels of the *vvp* gene by RNA slot blot hybridization (A). Bacterial cell density and the protease activity in the culture supernatant of each strain ($n = 3$) were determined at the same time (B). The growth curves of all strains were similar and only that of the wild-type strain is shown. N, *E. coli* total RNA used as a negative control.

of the deletion-containing colonies, CP156, was further confirmed by Southern hybridization (Fig. 5).

Expression of *V. vulnificus* metalloprotease and cytolysin in the RD mutant. When examined by RNA slot blot hybridization with the gene-specific probes, transcription of *vvp* and the cytolysin gene, *vvhA*, in the RD mutant was different from that in the parent strain (Fig. 6 and 7), whereas transcription of the nuclease gene, *vvn* (41), was not affected by disrupting *smcR* (data not shown). As shown in Fig. 6A, the level of *vvp* transcription in the wild-type strain rose rapidly at 5 h of growth from basal to a high level, which was maintained for a few hours afterwards. However, transcription of *vvp* was greatly reduced in the RD mutant. Coinciding with the transcriptional level, the protease activity in the culture supernatant of the RD mutant is dramatically reduced compared to that of the parent strain (Fig. 6B). Such a low level of protease activity was also found in the RD mutant cultured under any of the other conditions (listed in Fig. 1) that have been used to examine the regulation of *vvp* expression in the parent strain (data not shown). Protease activity in the culture supernatant was restored to the wild-type level when pVR21, which carried the intact *smcR*, was introduced into the RD mutant (Fig. 6B).

Transcription of *vvhA*, in contrast to that of *vvp*, was higher in the RD mutant than in the parent strain (Fig. 7A), particularly during the log phase of growth (2 to 6 h). The cytolysin activity in the culture supernatant not only was higher and detected earlier but also was sustained longer in the RD mutant than in the parent strain (Fig. 7B). Moreover, a biphasic expression was observed in the parent strain but not in the RD mutant for either the transcription of *vvhA* or the cytolysin activity in the culture supernatant. Introduction of pVR21 into the RD mutant resulted in greatly reduced cytolysin activity during the early log phase but not the late log phase and a slightly earlier decline of the cytolysin activity during the stationary phase (Fig. 7B).

Other phenotypes of the RD mutant. Opacity of the colonies of the RD mutant was between that of the opaque parent strain and a translucent mutant, YJ024 (data not shown). The virulence of the RD mutant, represented by the LD_{50} value, in either normal or iron-overloaded mice was comparable to that of the parent strain (Table 2). The RD mutant was also as resistant as its parent strain to the human serum killing effect ($P > 0.05$) (Table 2). The translucent mutant, which served as

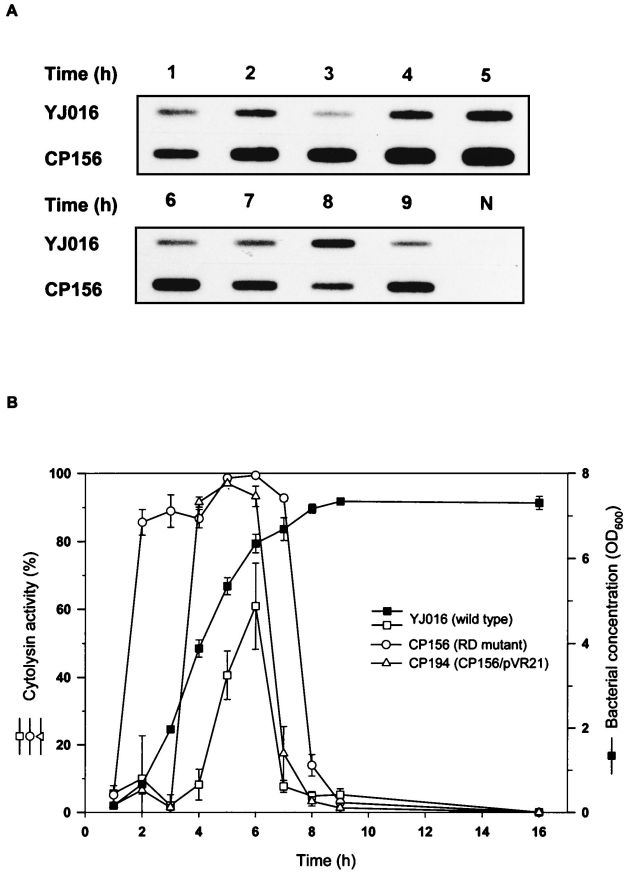

FIG. 7. Expression of the cytolysin by *V. vulnificus* strains. Bacteria were grown in LB at 37°C after a 1:100 dilution of an overnight culture. Total RNA (5 μg) was extracted from the bacteria harvested at intervals and examined for the transcriptional levels of the *vvhA* gene by RNA slot blot hybridization (A). Bacterial cell density and the cytolysin activity in the culture supernatant of each strain ($n = 3$) were determined at the same time (B). The growth curves of all strains were similar and only that of the wild-type strain is shown. N, *E. coli* total RNA used as a negative control.

TABLE 2. Virulence of *V. vulnificus* strains in mice and their resistance to human serum

Strain[a]	LD_{50} (CFU/mouse) for:		Bacterial resistance to human serum (%)[b]	
	Normal mouse	Iron-overloaded mouse	Untreated	Heat inactivated
YJ016	1.0×10^6	$<1.0 \times 10^1$	25.6 ± 11.7	35.9 ± 4.4
CP156	2.0×10^6	$<1.0 \times 10^1$	46.3 ± 11.6	38.9 ± 9.6
YJ024	3.2×10^7	9.5×10^5	0 ± 0	53.8 ± 3.8

[a] YJ016, wild-type strain; CP156, RD mutant; YJ024, translucent mutant of YJ016.

[b] Bacterial resistance to human serum, either untreated or heated at 56°C for 30 min to inactivate the complement, is expressed as 100% × (the number of viable bacteria after treatment/the number of viable bacteria before treatment). Values represent means ± standard deviations.

a control, was weakly virulent in mice and was very sensitive to human serum (Table 2).

DISCUSSION

Transcription of *vvp*, which encodes the metalloprotease of *V. vulnificus*, was shown to be affected by a variety of culture conditions, such as temperature, iron levels, and salt concentrations, indicating that multiple factors may be involved in the regulation of *vvp*. Nevertheless, in every case studied, transcription of *vvp* was turned up dramatically in the late log growth phase, like that of many other bacterial protease genes (10, 26). Some protease genes with such expression patterns have been shown to be regulated by the quorum-sensing, or cell density-dependent, regulatory systems (2).

Two different families of quorum-sensing regulatory systems, represented by those of *Vibrio fischeri* and *V. harveyi*, have been found widely distributed in the gram-negative bacteria. The quorum-sensing system in *V. fischeri* that regulates the genes involved in the production of bioluminescence is composed of a signaling molecule (the autoinducer) and a positive regulator, which is activated upon binding with the autoinducer (2, 33). The quorum-sensing system in *V. harveyi*, which is also involved in the regulation of the bioluminescence genes, is more complicated and is composed of two autoinducers (AI1 and AI2), the autoinducer receptors, a signal transducer, and a negative regulator (1, 2). Although the transcriptional activator, LuxR, of *V. harveyi* does not respond to stimulation by the autoinducers, it is required for the activation of transcription of the target genes (34).

Existence of a *V. harveyi* quorum-sensing system-like regulatory mechanism in *V. vulnificus* has been proposed based on the detection of an AI2-like activity in the culture supernatant (B. L. Bassler, personal communication) and the identification of a LuxR homologue (15). A LuxS-like AI2 synthase has also been identified recently (S. Y. Kim et al., Abstr. 100th Gen. Meet. Am. Soc. Microbiol. 2000, abstr. B-248, p. 97, 2000). We detected an autoinducer activity in a high-cell-density (OD_{600} = 6.6) culture supernatant, but not in that of a low-cell-density (OD_{600} = 2.0) culture, for the expression of protease at a lower bacterial cell density. This suggested that *vvp* may be partly regulated by the quorum-sensing mechanism. The LuxR homologue of *V. vulnificus*, SmcR, was shown in this study to complement a LuxR-deficient *E. coli* in activating the bioluminescence genes of *V. harveyi*. The functional similarity between LuxR and SmcR also implies that SmcR may be involved in a quorum-sensing regulatory system. We further demonstrated that SmcR is required for *vvp* expression because the transcription of *vvp* was greatly reduced in an SmcR-deficient *V. vulnificus* mutant grown under a variety of culture conditions.

The *V. harveyi luxR* family members identified so far, including *smcR*, are not only highly homologous in the encoding sequences but are also identical in the promoter regions (the −10 and −35 sequences). In addition, each member has been shown to be capable of transactivating the *lux* operon of *V. harveyi*, suggesting that they may employ a common mechanism in activating the target genes. Two LuxR-binding sites (LuxR boxes) have been identified in the promoter of *luxC*, a target gene of *luxR*, by footprint analysis (18, 35). However, the LuxR boxes were not found in the promoters of two putative target genes of the LuxR homologues: *hap* of *V. cholerae* (10) and *vvp* of *V. vulnificus* (4). Therefore, the LuxR homologues may recognize specific binding sequences that share a low level of homology with each other. Alternatively, the *hap* and *vvp* genes may each be regulated indirectly rather than directly by the LuxR homologue via another regulatory factor. A study of the interaction of SmcR and HapR with the promoter regions of *vvp* and *hap*, respectively, is required for distinguishing between the two possibilities.

Expression of the cytolysin of *V. vulnificus* has been shown previously to be regulated by Vvp at the posttranslational level (30). We further found in this study that transcription of the cytolysin gene, *vvhA*, was affected by disrupting *smcR*. Transcription of *vvhA* was increased during the log phase, suggesting that SmcR may be involved in negative regulation of *vvhA* expression. In the RD mutant, the production of Vvp is greatly reduced and the transcription of *vvhA* is increased. Consequently, the cytolysin activity in the culture supernatant is detected earlier and reaches a maximal activity higher than that of the parent strain, as demonstrated in this study. However, in contrast to the prolonged high cytolysin activity detected in the Vvp-deficient mutant (30), this high level of cytolysin activity was sustained for about 5 h and then declined to undetectable levels at 8 h of growth in the RD mutant. Decline of the cytolysin activity in the RD mutant may be caused by the low level of protease expressed.

The role of SmcR in the regulation of Vvp or cytolysin expression was confirmed by complementing the RD mutant with SmcR expressed from a plasmid. The pattern of Vvp expression in the reconstituted strain, as represented by the protease activity detected in the culture supernatant, was similar to that in the wild-type strain. However, the pattern of cytolysin expression in the reconstituted strain was different from that in the wild-type strain. The biphasic expression was restored by complementation with SmcR, but the maximal cytolysin activity in the reconstituted strain was much higher than that in the wild-type strain. We do not know currently what caused this partial repression of cytolysin expression in the reconstituted strain. Nevertheless, our results imply that the regulation of *vvhA* expression by SmcR may occur via a mechanism that is more complex than that involved in the regulation of *vvp* by SmcR.

Various colony morphological changes have been found to

be associated with the LuxR homologues in a number of vibrios. Disruption of *hapR* in *V. cholerae* resulted in a rugose phenotype (10), while expression of OpaR in a translucent strain of *V. parahaemolyticus* brought about opaque colonies (14). Morphological change of the colony was also observed in the RD mutant of *V. vulnificus*: the colonies of the RD mutant were less opaque than those of the parent strain. Such a morphological change could be caused by alterations of the bacterial cell surface compositions. Translucent variants are sometimes obtained from the opaque *V. vulnificus* strains, and the variation in opacity has been found to be accompanied by variation of capsular polysaccharide and bacterial virulence in mice (32). In contrast to the opaque strains, the translucent variants, including YJ024 used in this study, usually contain less or no capsular polysaccharide and are much less virulent in iron-overloaded mice (32). Although the RD mutant exhibited an intermediate phenotype between the parent strain and the translucent mutant in colonial opacity, it was as virulent as the parent strain in the iron-overloaded mice. Moreover, it showed a wild-type level of resistance to human serum killing activity. Therefore, disruption of *smcR* either did not affect the amount of the capsular polysaccharide or, if it had any effect on capsular synthesis, the effect was not sufficient to result in reduction of bacterial virulence in mice or resistance to human serum.

In conclusion, our data demonstrated that the *V. harveyi* LuxR homologue, SmcR, positively controls the transcription of the metalloprotease gene and may also be involved in the negative regulation of the cytolysin gene. The target genes of SmcR remain to be identified.

ACKNOWLEDGMENTS

This work was partly supported by grants DOH 88-HR-606 from the National Health Institute and NSC 89-2320-B-006-018 from the National Science Council, Taiwan, Republic of China.

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