JOURNAL OF BACTERIOLOGY, May 2002, p. 2587–2594 Vol. 184, No. 10
0021-9193/02/$04.00+0 DOI: 10.1128/JB.184.10.2587–2594.2002

Proteome and Transcriptome Analysis of the Virulence Genes Regulated by the VirR/VirS System in *Clostridium perfringens*

Takeshi Shimizu,[1] Kensuke Shima,[1] Ken-ichi Yoshino,[2] Kazuyoshi Yonezawa,[2] Tohru Shimizu,[1] and Hideo Hayashi[1]*

Department of Microbiology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Ten-nohdai, Tsukuba 305-8575,[1] and Biosignal Research Center, Kobe University, 1-1 Rokkodai Nada, Kobe 657-8501,[2] Japan

Received 26 November 2001/Accepted 18 February 2002

The proteins under the control of the two-component system VirR/VirS in *Clostridium perfringens* were analyzed by using two-dimensional gel electrophoresis of the culture supernatant from the wild type and the *virR* mutant. Based on matrix-assisted laser desorption ionization-time of flight/mass spectrometry, seven positively regulated proteins and eight negatively regulated proteins were identified. Transcriptome analysis confirmed that 7 of the 15 proteins were regulated by the VirR/VirS system at the transcriptional level, but the remaining proteins were modified with a VirR/VirS-directed protease at the posttranslation and secretion levels. We purified and characterized the VirR/VirS-directed protease from the culture supernatant and identified it as a kind of clostripain. Because this proteolytic activity was strongly inhibited by leupeptin and antipain, it was concluded that this protease was a member of the family of cysteine proteases of *C. perfringens*.

Clostridium perfringens is the primary causative agent of clostridial myonecrosis, also known as gas gangrene (25). This anaerobic organism invades traumatized or ischemic tissue, and although the infection is relatively localized, the bacteria produce numerous extracellular toxins that are responsible for the extensive tissue destruction and necrosis seen in classical cases of gas gangrene (1, 9, 30).

Two-component regulatory systems, consisting of a membrane sensor (histidine kinase) and a cytoplasmic response regulator, enable bacteria to sense and respond to environmental conditions. In response to an appropriate signal, autophosphorylation occurs at a conserved histidine residue in the cytoplasmic domain of the sensor. The phosphate group is then transferred to an aspartate residue on the response regulator, which in turn stimulates or represses target genes at the transcriptional level. The importance of these two-component systems, in control of both metabolism and virulence factor regulation, has been demonstrated in a wide range of bacterial species (8).

Previous works have indicated that one of the two-component systems, VirR/VirS, of *C. perfringens* globally controls the production of the virulence factors alpha-toxin (phospholipase C), theta-toxin (perfringolysin O), kappa-toxin (collagenase), sialidase, protease, and hemagglutinin (18, 27). Studies on *virR* mutants have also disclosed that the VirR/VirS system regulates the expression of the genes *plc* (alpha-toxin gene), *pfoA* (theta-toxin gene), and *colA* (kappa-toxin gene) at the transcriptional level (5). Banu et al. (3) identified the other genes that were regulated either positively or negatively by the VirR/VirS system by means of a differential display method using the wild type and the *virR* mutant of *C. perfringens*.

Proteome analysis is an excellent tool for analyzing the final products of these regulated genes. The profiles of proteome and transcriptome should be different, based on differences in the posttranscriptional regulation that control the translation rate (12) and half-lives of proteins or mRNAs (33), their intracellular location, and their molecular association with other proteins (32).

In this study, we analyzed the secreted proteins that are regulated with the two-component system VirR/VirS by using two-dimensional gel electrophoresis. By comparing the proteome profile of the *C. perfringens* wild type with that of the *virR* mutant, 15 proteins were identified as members of the family of VirR/VirS-dependent proteins. One of the VirR/VirS-dependent proteins which showed proteolytic activity was purified and characterized.

MATERIALS AND METHODS

Bacterial strains and media. *C. perfringens* strain 13 (19) and the *virR* mutant TS133 (27) were grown in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) in all experiments. These strains have been shown to produce more extracellular proteins in this medium than in TSF medium (T. Shimizu, unpublished data).

Preparation of extracellular protein fraction. *C. perfringens* cells were grown in RPMI 1640 medium with and without protease inhibitors at 37°C under anaerobic conditions and harvested at the late exponential growth phase. The cells were harvested by centrifugation at 10,000 × *g* for 5 min at 4°C. The extracellular proteins in supernatant were precipitated with 10% (wt/vol) trichloroacetic acid overnight at 4°C and centrifuged at 10,000 × *g* for 5 min at 4°C. The resulting protein precipitate was washed with cold acetone and air-dried.

Two-dimensional electrophoresis. The proteins were treated with a mixture containing 9 M urea, 4% 3-[(3-cholamidylpropyl)-dimethylammoni]-1-propanesulfonate (CHAPS), 100 mM dithiothreitol, and 0.2% (wt/vol) Bio-Lytes 3/10 (Bio-Rad Laboratories, Hercules, Calif.) to obtain completely denatured and reduced proteins. The protein samples were separated using an immobilized pH gradients Ready Strip system (Bio-Rad Laboratories, Hercules, Calif.) in the pH range of 5 to 8 or 3 to 10. For database construction and the identification of proteins by mass spectrometry (MS), 70 to 80 μg of protein was applied. The proteins were silver stained using a Silver Stain II kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After the gels were scanned with an imaging system, analysis of the two-dimensional images was performed with the PDQuest software package (Bio-Rad Laboratories, Hercules, Calif.). More than three separate gels of each condition were analyzed, and only spots displaying the same pattern in all parallels were selected for further characterization.

* Corresponding author. Mailing address: Department of Microbiology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Ten-nohdai, Tsukuba, Japan 305-8575. Phone: 81-298-53-3354. Fax: 81-298-53-3354. E-mail: hihayash@md.tsukuba.ac.jp.

TABLE 1. Oligonucleotide primers used for PCR[a]

Primer	Sequence[a]	Location
1951-1	ATACTACCAATACCAGTTAAA	Internal CPE1951; forward primer
1951-2	TGGATCTCCTTGTACCCCTT	Internal CPE1951; reverse primer
1529-1	TCTACTGGTGCTGGAACAGA	Internal CPE1529; forward primer
1529-2	AAGAACCACTTCCAGTTGGT	Internal CPE1529; reverse primer
0846-1	AATTTCATCTTGGTACTTACCTTC	Internal CPE0846; forward primer
0846-2	CTATTATATCCACCAAGTGAAGTA	Internal CPE0846; reverse primer
1231-1	TGCTGCTAAAGCCTTTAACA	Internal CPE1231; forward primer
1231-2	TGATGCATACCATGGTGTTGT	Internal CPE1231; reverse primer
0220-1	AAGATGTGGTAGCAACGCCA	Internal CPE0220; forward primer
0220-2	TGGTGAACCAGTCTGTGGTA	Internal CPE0220; reverse primer
1428-1	AGCGGCACAATCATCAGGGGT	Internal CPE1428; forward primer
1428-2	TTCTTATCATAGCACCAGCT	Internal CPE1428; reverse primer
0202-1	AAGCAAGAGATATTAGGCGA	Internal CPE0202; forward primer
0202-2	TGAAGCCACCACCATGATCT	Internal CPE0202; reverse primer
1785-1	TGGACTACTCAGCAACTACA	Internal CPE1785; forward primer
1785-2	TCTTGACCACCACCGTGGA	Internal CPE1785; reverse primer
1632-1	TAGAGCCAACAGGAATGGCT	Internal CPE1632; forward primer
1632-2	AGCTATCTCCAGCAGCTGTA	Internal CPE1632; reverse primer
2297-1	TGGTGCAGGTACAATGGGT	Internal CPE2297; forward primer
2297-2	ATCCTGGTGCTTCAGCAACT	Internal CPE2297; reverse primer
1350-1	AGTATCAGAAGGTGCTGCT	Internal CPE1350; forward primer
1350-2	AGGGATTCCAGTACCACCGT	Internal CPE1350; reverse primer
2408-1	AGGCAGACAAGTACGGCGT	Internal CPE2408; forward primer
2408-2	TGAAGCTCACCCATACCAGCT	Internal CPE2408; reverse primer
0278-1	TCGCAACTCCACTTACAGAT	Internal CPE0278; forward primer
0278-2	TCTGCATCTACCTCTGGAGT	Internal CPE0278; reverse primer

[a] All primers are given in the 5′ to 3′ orientation and are based on nucleotide sequence data for the *C. perfringens* genome (AP003185 to AP003194 and AP003515).

Peptide mass fingerprinting. Peptide mass fingerprinting was performed by the method of Jensen et al. (14) with slight modification. The protein spots were excised with a scalpel and cut into pieces (1 mm by 1 mm). The gel pieces were placed in a microtube and washed in distilled water for 10 min. The gel pieces were washed twice in 25 mM ammonium bicarbonate–50% acetonitrile for 10 min and then once in acetonitrile for 5 min. A minimum volume of 100 mM ammonium bicarbonate was added to totally immerse the gel pieces, followed by incubation for 5 min. An equal volume of acetonitrile was added, incubated for 15 min, and discarded. The gel pieces were dried in a vacuum centrifuge for 30 min.

For enzyme digestion, 1 μg of lysylendopeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was dissolved in 100 μl of 100 mM Tris-HCl (pH 9.0) and gradually added to the dried gel pieces, followed by incubation for 45 min on ice. After swelling of the gel pieces, the supernatant was discarded, and a minimum volume of 100 mM Tris-HCl (pH 9.0) was added to immerse the gel pieces. Samples were incubated at 37°C for 20 h. After digestion, 40 to 60 μl of 25 mM ammonium bicarbonate was added and mixed for 15 min. Then an equal volume of acetonitrile was added, followed by mixing for 15 min and recovery of the supernatant. From 40 to 60 μl of 5% trifluoroacetic acid–50% acetonitrile was added, the solution was mixed for 15 min, and the supernatant was recovered twice. The extracts were combined, concentrated to about 20 μl by vacuum centrifugation, and supplemented with 80 μl of 0.1% trifluoroacetic acid. The extracts were then concentrated to about 20 μl by an additional vacuum centrifugation.

Before measuring the mass of the peptide mixture, the peptides were purified using a ZipTip C18 (Millipore, Bedford, Mass.) according to the manufacturer's instructions. Purified peptide solution (0.6 μl) was prepared with equal volumes of saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile–0.1% trifluoroacetic acid to create a sample template for matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) (Voyager Linear DE and Voyager DE RP; PE Biosystems, Foster City, Calif.). Peptide mass fingerprints were analyzed by using Mascot software (Matrix Science, Ltd., London, United Kingdom).

Northern hybridization. Northern hybridization was performed as described previously (5, 16). Chromosomal DNA from *C. perfringens* strain 13 was used as a template to amplify each DNA fragment by PCR with each primer set (Table 1), except the CPE0163 and CPE0178 genes, according to the DNA sequence data of the *C. perfringens* genome sequence (28). Plasmids pTS302 (29) and pKY3135 (20) were used as the templates to amplify each DNA fragment by PCR with a universal primer set in the CPE0163 and CPE0178 genes, respectively. Each DNA fragment was labeled with alkaline phosphatase using an AlkPhos Direct kit (Amersham Pharmacia Biotech United Kingdom, Ltd., Buckinghamshire, England) according to the manufacturer's instructions. The qualitative examination of the Northern hybridization analysis data was validated by scanning the blots with a densitometer and determining the relative amounts of each specific transcript with a Quantity One software package (Bio-Rad Laboratories, Hercules, Calif.).

Proteolytic activity assay. Proteolytic activities were determined by using the insoluble proteolytic substrate azocoll (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as previously described (10) with slight modification. The substrate (4 mg/ml) was suspended in 100 mM phosphate buffer (pH 7.0) containing 5 mM dithiothreitol. Then 500 μl of a sample was added to 500 μl of substrate suspension. The mixture was incubated for 2 h at 37°C with shaking. After incubation, the assay mixtures were centrifuged, and the absorbances at 520 nm were determined.

Proteases were distinguished through the use of different protease inhibitors. The samples were placed on ice, supplemented with the inhibitors, and left to react for 45 min before adding the proteolytic substrate azocoll. For determination of the proteolytic activity of purified protein, 0.1 mg of bovine serum albumin was added to the assay mixture. One unit of protease activity was arbitrarily defined as proteolysis that resulted in the release of 0.001 absorbance unit of dye for 2 h. Protease specific activities were expressed as units per milligram of protein. Protein concentrations were determined by the Bradford method (Bio-Rad Laboratories, Hercules, Calif.).

Purification of protease. *C. perfringens* wild-type strain 13 cells were grown in RPMI 1640 medium under anaerobic conditions and harvested at the late exponential growth phase. The cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. The supernatant was ultracentrifuged at 100,000 × *g* for 1 h at 4°C. Proteins in the supernatant were concentrated with an Amicon stirred-cell 8050 system and supplemented with a 1/10 volume of 1 M Tris-HCl (pH 7.5)–1.5 M NaCl. Concentrated supernatant solution was applied to a benzamidine-Sepharose 6B (Amersham Pharmacia Biotech United Kingdom, Ltd., Buckinghamshire, England) affinity column (bed volume, 2 ml) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl. The column was washed with 20 ml of the equilibration buffer and eluted with 0.1 M glycine-HCl (pH 3.0). Fractions of 1 ml were collected in a tube that contained 0.3 ml of 0.1 M Tris-HCl (pH 9.0), and the protease activity was assayed.

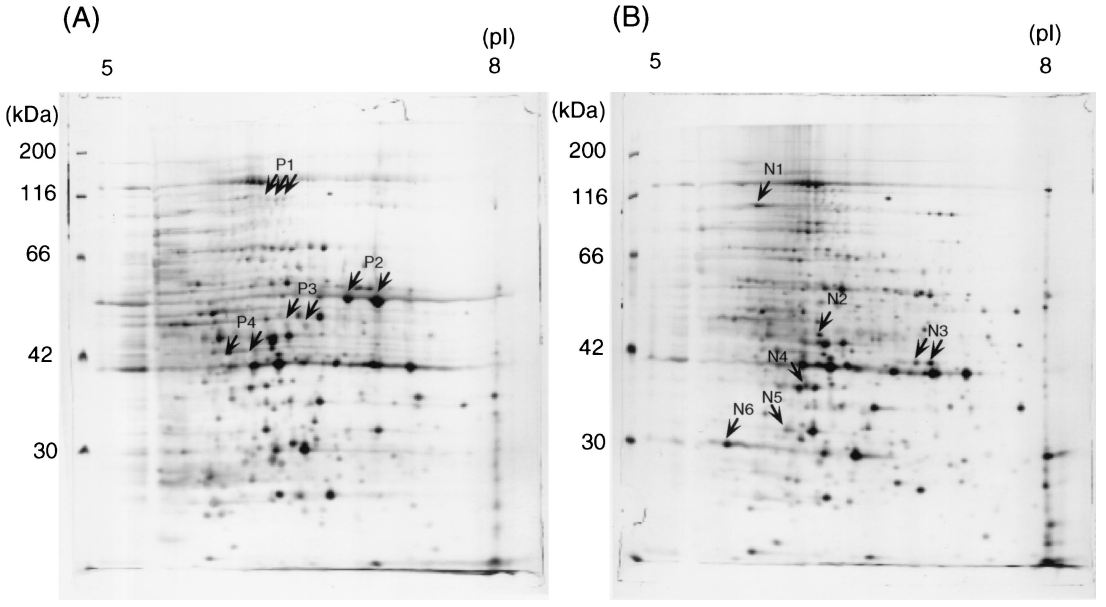

FIG. 1. Two-dimensional silver-stained gel of proteins in the culture supernatant *of C. perfringens* strain 13 (A) and the *virR* mutant TS133 (B). Proteins were separated in the first dimension by a pH 5 to 8 immobilized pH gradient gel and then in the second dimension by a 10% polyacrylamide gel. Reproducible differences in the density of protein spots were examined by at least three independent experiments. Spots were excised, and the corresponding proteins were identified by MALDI-TOF/MS and database searches. The spots are labeled on the gel according to the numbers presented in Table 2.

RESULTS

Analysis of culture supernatant proteins of wild type and *virR* mutant by two-dimensional gel electrophoresis. Because proteins normally have a wide range of isoelectric points, we used two gel systems: (i) pH 5 to pH 8 with 10% polyacrylamide and a pH 5 to 8 gel and (ii) pH 3 to pH 10 with 10% polyacrylamide and a pH 3 to 10 gel.

The culture supernatant of the wild-type strain 13 and the *virR* mutant TS133 were collected at the late exponential growth phase, and the proteins were separated by two-dimensional gel electrophoresis. The *virR* mutant expressed a pattern different from that of the wild type in the pH 5 to 8 gel, as shown in Fig. 1. The *virR* mutant showed six intensified and four reduced spots compared with the wild strain. This profile was reproducible. The density of the P2 spot was remarkably enhanced in the wild type but absent in the *virR* mutant. The P4 spot appeared transparent after silver staining in the wild type but was absent in the *virR* mutant. In the pH 3 to 10 gel, the *virR* mutant pattern showed three spots with reduced density and two enhanced spots (Fig. 2).

Those protein spots which were different between the two strains were subjected to in-gel digestion and MALDI-TOF/MS analysis. Table 2 summarizes the results. Seven protein spots were expressed exclusively in the wild type, while eight spots were restricted to the *virR* mutant. The proteins were further analyzed by peptide mass fingerprinting using MALDI-TOF/MS. The identified proteins were assigned the appropriate CPE gene numbers by reference to the *C. perfringens* strain 13 genome (28).

Transcription of VirR/VirS-regulated proteins. To investigate whether the expression of these proteins was directly regulated by the VirR/VirS system at the transcriptional level, Northern hybridization analysis was performed at the mid-exponential growth phase (Fig. 3). The results clearly demonstrated that the VirR/VirS system positively regulated CPE0163 (*pfoA*) and CPE0846 (Table 2 and Fig. 3). CPE1951, CPE1529, and CPE0173 were positively regulated by the VirR/VirS system at the transcriptional level (Table 2 and Fig. 3). However, CPE1231 and CPE0220 were not positively regulated by the VirR/VirS system at the transcriptional level (Table 2 and Fig. 3).

On the other hand, the products of CPE1428, CPE0202, CPE1785, CPE1632, CPE2297, CPE1350, CPE2408, and CPE0278 appeared to be negatively regulated by proteome analysis. However, six transcripts (CPE1428, CPE1785, CPE1632, CPE2297, CPE2408, and CPE0278) of these genes were not negatively regulated by the VirR/VirS system (Table 2 and Fig. 3). The transcription of CPE0202 and CPE1350 appeared to be negatively regulated at the transcriptional level (Table 2 and Fig. 3).

Analysis of VirR/VirS-regulated proteolytic activity of culture supernatant. Proteolytic activity of the culture supernatant of *C. perfringens* was reported to be positively regulated by the VirR/VirS system (18). In the present study, the proteolytic activity of the culture supernatant of the *C. perfringens* wild type and the *virR* mutant were determined at the late exponential growth phase. The activity of the culture supernatant of the wild type (2.8×10^5 U/mg) was much higher than that of the *virR* mutant (1.2×10^3 U/mg). The proteolytic activity of complemented strain TS133(pBT404) was 2.9×10^5 U/mg. In the former, the activity was inhibited by benzamidine, leupeptin, antipain, and EDTA, but it was not affected by phenylmethylsulfonyl fluoride (PMSF), pepstain, phosphoramidon, E-64, or soybean trypsin inhibitor (Table 3).

We suspected that the protease in the supernatant might affect the proteome. To examine the effect of the proteolytic

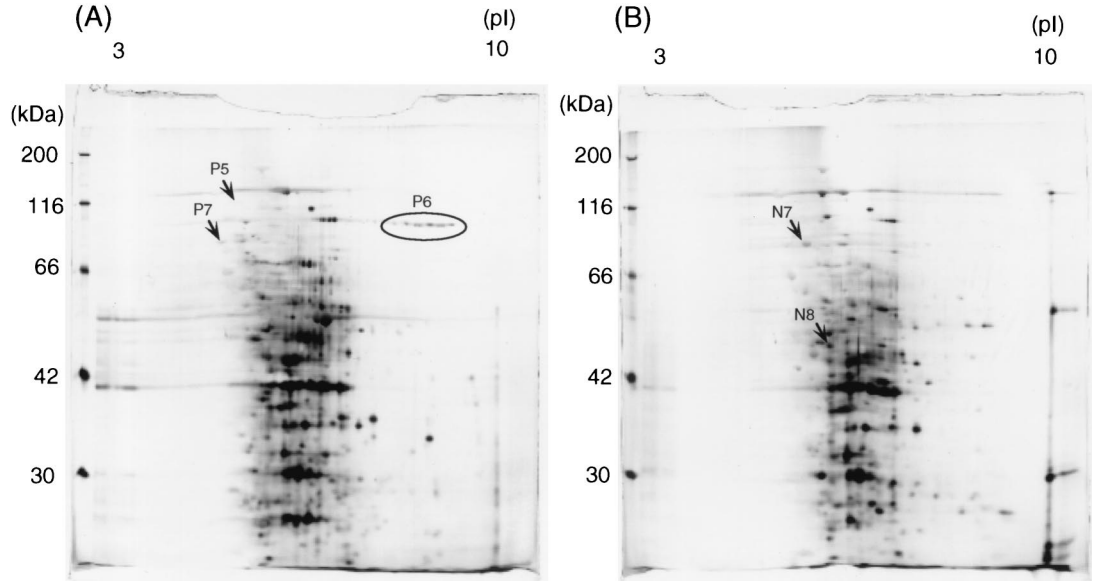

FIG. 2. Two-dimensional silver-stained gel of proteins in the culture supernatant *of C. perfringens* wild-type strain 13 (A) and the *virR* mutant TS133 (B). Proteins were separated in the first dimension by pH 3 to 10 immobilized pH gradients gel and then in the second dimension by a 10% polyacrylamide gel. Reproducible differences in the density of protein spots were examined by at least three independent experiments. Spots were excised, and the corresponding proteins were identified by MALDI-TOF/MS and database searches. The spots are labeled on the gel according to the numbers presented in Table 2.

activity on the two-dimensional pattern, the culture supernatants were examined after the addition of various kinds of protease inhibitors. The protein spots N1, N2, N3, N4, and N6 increased in intensity in the wild-type strain after the addition of benzamidine (5 mM), leupeptin (10 μM), and antipain (10 μM) (Fig. 4). However, the addition of PMSF (0.5 mM), pepstatin (1 μM), phosphoramidon (20 μM), or E-64 (0.7 μM) did not cause any change (data not shown). Protein spot P6 of the wild type disappeared after addition of benzamidine, leupeptin, and antipain (data not shown), while protein spots P2 and

TABLE 2. Summary of proteome analysis, Northern hybridization analysis, and effect of protease inhibitors on the two-dimensional pattern

Spot no.	CPE no.	Product (homologous protein) (reference)	Pattern[a]	Density[b]		Northern[c]	Effect of protease inhibitor[d]	
				Wild type	Mutant		L, A	B
P1	CPE1951	2′,3′-cyclic nucleotide 2′-phosphodiesterase	W	726	474	P(0.26)	+/−	+/−
P2	CPE0163	Perfringolysin O	W	22,167	732	P(0.22)	+/−	−
P3	CPE1529	Hypothetical protein (*ydaL* gene product from *Bacillus subtilis* [29.3%]) (17)	W	2,017	0	P(0.83)	+/−	+/−
P4	CPE0846	Cysteine protease (alpha-clostripain from *Clostridium histolyticum* [58.3%]) (7)	W	N.D.	N.D.	P(0.32)	+/−	−
P5	CPE0173	Collagenase	W	530	0	P(0.30)	+/−	+/−
P6	CPE1231	Probable surface protein (Aas surface protein from *Staphylococcus saprophyticus* [25.4%]) (13)	W	11,243	0	I(1.09)	−	−
P7	CPE0220	Hypothetical protein (chitinase A from *Clostridium paraputrificum* [38%]) (24)	W	1,158	0	I(1.04)	+/−	+/−
N1	CPE1428	ClpB protein (endopeptidase Clp ATP-binding chain B1 from *Synechocystis* sp. [55.8%]) (15)	M	0	4,780	I(1.00)	+	+
N2	CPE0202	Probable cell wall-binding protein (cell wall-binding protein from *Bacillus halodurans* [32.4%]) (31)	M	0	3,839	N(1.51)	+	+
N3	CPE1785	Iron-sulfur cofactor synthesis protein NifS	M	3,015	13,371	I(1.05)	+	+
N4	CPE1632	Ribokinase	M	0	5,352	I(1.08)	+	+
N5	CPE2297	β-Hydroxybutyryl-coenzyme A dehydrogenase, NAD dependent	M	0	6,403	I(1.00)	+/−	+/−
N6	CPE1350	Fructose-bisphosphate aldolase	M	0	10,109	N(1.46)	+	+
N7	CPE2408	Elongation factor G	M	1,792	3,525	I(0.95)	+/−	+/−
N8	CPE0278	Conserved hypothetical protein (p45 from *Listeria monocytogenes* [26%]) (26)	M	0	8,698	I(0.98)	+/−	+/−

[a] W, exclusive to the wild type, M, exclusive to the *virR* mutant.
[b] Values represent the ratio of spot density level (in ppm) to the total of all valid spots using PDQuest software (Bio-Rad). N.D., not determined.
[c] P, positively regulated; I, independently regulated; N, negatively regulated. Values represent the ratio of specific mRNA level in the mutant to that in the wild type.
[d] +/−, not altered; +, induced; −, reduced. L, leupeptin; A, antipain; B, benzamidine.

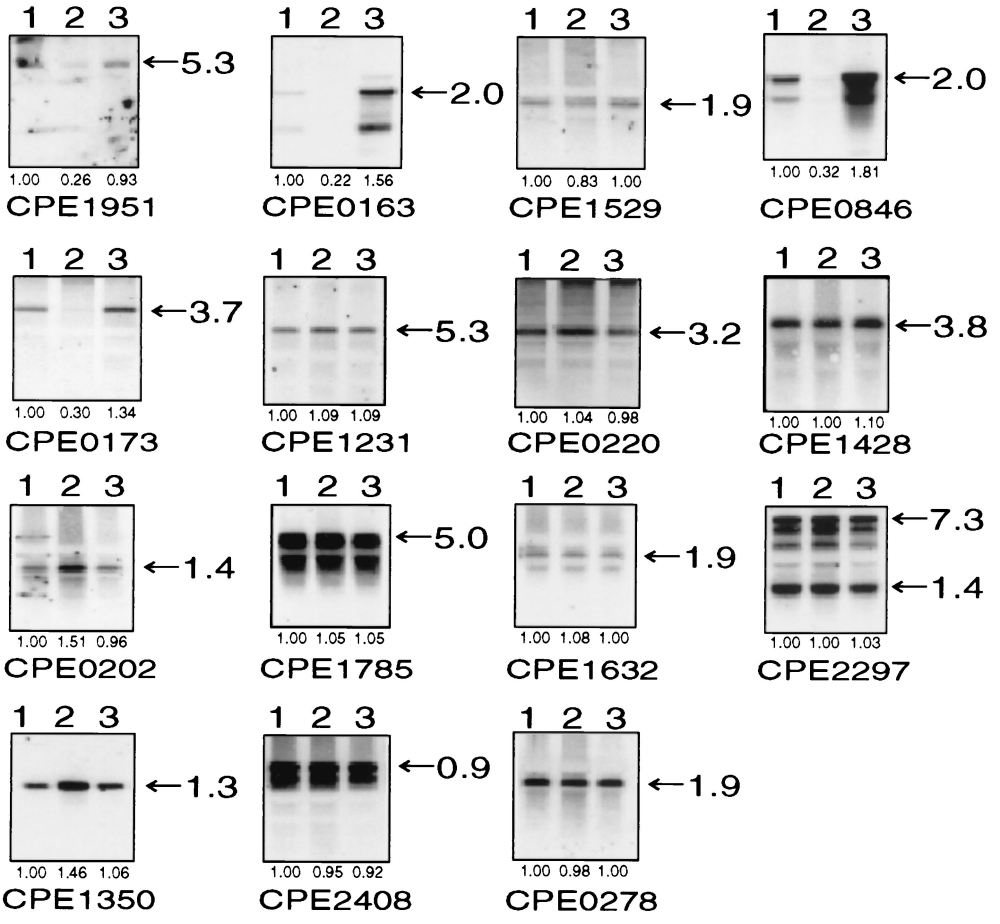

FIG. 3. Northern hybridization using various gene probes. The calculated sizes (in kilobases) of the mRNAs are shown at right. Values represent the ratios of specific mRNA levels to those in the wild type. These values are derived from three independent experiments. Lanes: 1, strain 13/pJIR418; 2, strain TS133/pJIR418; 3, strain TS133/pBT404 (*virR*$^+$) (27). These results are summarized in Table 2.

P4 were reduced by benzamidine (Fig. 4), PMSF, and phosphoramidon (data not shown). This result suggested that proteins N1, N3, N4, and P6 were not directly regulated by the VirR/VirS system at the transcriptional level, but rather were degraded by the VirR/VirS-directed protease(s) during culture, which activity was inhibited by benzamidine, leupeptin, and antipain. Proteins N2 and N6 were under the control of the VirR/VirS system at the transcriptional level and then also were degraded by the VirR/VirS-directed protease(s) during culture.

Purification and characterization of VirR/VirS-regulated protease. In order to characterize the VirR/VirS-directed protease(s) in the culture supernatant of *C. perfringens* strain 13, we purified the protein(s) from the culture supernatant at the late exponential growth phase using a benzamidine-Sepharose column. The 40-kDa single band was visualized by Coomassie brilliant blue R-250 staining on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Fig. 5). The 40-kDa band was cut out and subjected to in-gel digestion, followed by peptide mass fingerprinting using MALDI-TOF/MS. The protein was identified as a product of CPE0846 and shown to have 58.3% homology with clostripain of *C. histolyticum*, to be identical to the P4 protein, and to be positively regulated by the VirR/VirS system.

The specific activity of purified protease was 4.5×10^6 U/mg. Table 3 shows the effects of protease inhibitors on the activity of the purified protease. Leupeptin and antipain, which are cysteine protease inhibitors, strongly inhibited the proteolytic activity. However, E-64, which is also a cysteine protease inhibitor, did not inhibit the activity. PMSF, a serine protease inhibitor, and pepstatin, an aspartate protease inhibitor, did

TABLE 3. Effect of inhibitors on proteolytic activity[a]

Inhibitor	Concn	% Inhibition	
		Supernatant	Purified
PMSF	0.5 mM	1	21
Leupeptin	10 μM	84	100
Antipain	10 μM	82	100
E-64	0.7 μM	0	16
Pepstatin	1 μM	0	14
EDTA	10 mM	82	73
Benzamidine	5 mM	74	87
SBTI	0.1 mg/ml	0	17
Phosphoramidon	20 μM	0	16

[a] Proteolytic activities of the culture supernatant of wild-type and purified protease were determined with azocoll in the presence of inhibitors. Activities are expressed as percentages of the respective controls. E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; SBTI, soybean trypsin inhibitor.

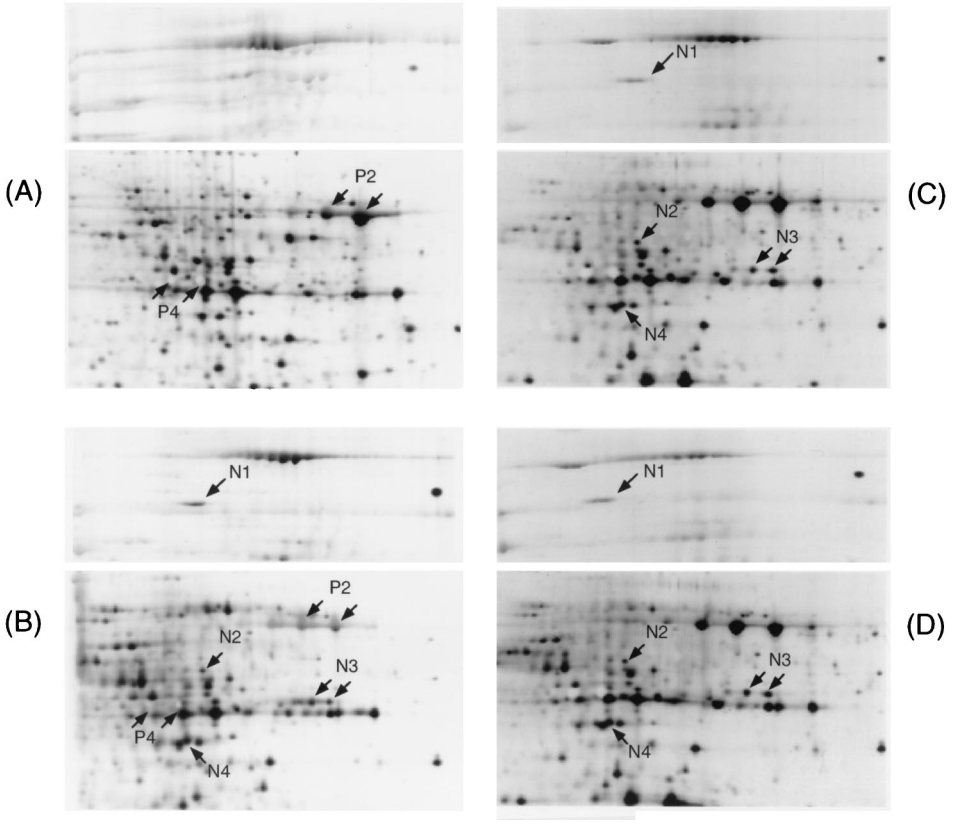

FIG. 4. Portion of the two-dimensional pattern of the culture supernatant proteins of the wild type (A), the wild type with benzamidine (5 mM) (B), the wild type with leupeptin (10 μM) (C), and the wild type with antipain (10 μM) (D). The spots are labeled on the gel according to the numbers presented in Table 2.

not inhibit the proteolytic activity. Benzamidine, a trypsin-like inhibitor, inhibited the proteolytic activity, but soybean trypsin inhibitor did not. EDTA, a metalloprotease inhibitor, partially inhibited the proteolytic activity. These results are consistent with the general properties of a cysteine protease except for the effects of E-64 and EDTA.

The activity of the purified protease was diminished during 1 week of storage in phosphate-buffered saline at −20°C. The autodigestive degradation of the peptide during storage was also confirmed by SDS-PAGE.

DISCUSSION

Approximately 200 protein spots were detectable in the culture supernatant of *C. perfringens* at the late exponential growth phase by two-dimensional gel electrophoresis and silver staining. Although these proteins were derived from the culture supernatant, the supernatant also contained intracellular proteins that had no putative signal sequences, such as ribokinase, β-hydroxybutyryl-coenzyme A dehydrogenase, fructose-bisphosphate aldolase, and elongation factor G (Table 2). Little difference was detected in the pattern according to the growth phases. This indicated that the growing cells in liquid medium undergo autolysis at a relatively high rate during growth.

Among the spots that were apparently different in two-dimensional gel electrophoresis of the wild type and the mutant, 15 spots were found to be the proteins regulated under the VirR/VirS system, and some spots were identified as fragments of the P2 and P5 proteins when analyzed by peptide mass fingerprinting using MALDI-TOF/MS. These fragments are probably derived from cleavage of P2 and P5 by a VirR/VirS-directed protease. The rest of the spots were not so consis-

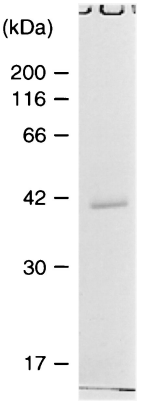

FIG. 5. SDS-PAGE analysis of purified protease. Concentrated culture supernatant was applied to a benzamidine-Sepharose 6B column (2 ml) in 50 mM Tris-HCl (pH 8.0)–0.5 M NaCl. The retained enzyme was eluted by applying 0.1 M glycine-HCl (pH 3.0). Fractions of 1 ml were collected and monitored for enzyme activity. Enzyme activity was determined by using azocoll as a substrate. Fractions showing proteolytic activity were pooled. Protein concentration was determined by the Bradford method, and this fraction was subjected to SDS-PAGE analysis under reducing conditions.

tently reproducible and therefore were not included in our analysis.

Among these 15 genes, CPE1951, CPE0163, CPE1529, CPE0846, and CPE0173 were positively regulated by the VirR/VirS system at the transcriptional level. The CPE0163 gene was found to be identical to the *pfoA* gene, and the CPE0846 gene had a consensus sequence that bound to VirR (6, 28). However, the CPE0173 gene, which was identified as the *colA* gene, and the CPE1951 and CPE1529 genes (28) did not have the consensus sequences to bind VirR. It is probable that the CPE0173, CPE1951, and CPE1529 genes are regulated by a secondary regulator, as in the case of the *hyp7* gene, whose expression has been shown to be regulated by the VirR/VirS system (4).

The CPE1231 gene was independent of the VirR/VirS system. The product of CPE1231 had homology with Aas, a surface protein of *Staphylococcus saprophyticus* (13). This product may be located on the cell surface of *C. perfringens* and could be released by the VirR/VirS-dependent protease (a product of CPE0846) into the culture supernatant. This may be supported by the following evidence: the P6 protein spot on the two-dimensional pattern disappeared when the culture supernatant was treated with benzamidine, leupeptin, or antipain (data not shown), and the molecular mass of the P6 protein (97 kDa), as estimated by two-dimensional gel electrophoresis, was smaller than that of the product of CPE1231 (122 kDa) (28).

Lyristis et al. (18) measured the proteolytic activity of the culture supernatants of *C. perfringens* using azocoll as the substrate and found that the activity was regulated by the VirR/VirS system. Award et al. (2) reported that a collagenase gene-defective mutant had very little azocoll activity compared with the wild type. These results suggested that the proteolytic activity of the culture supernatant of *C. perfringens* using azocoll as the substrate was mostly derived from the collagenase. However, in this study, leupeptin and antipain, which are cysteine protease inhibitors, inhibited 80% of the proteolytic activity in the culture supernatant and did not inhibit the collagenase activity. In the presence of benzamidine, leupeptin, and antipain, the two-dimensional pattern of the wild-type culture supernatant was similar to that of the *virR* mutant. These results indicated that the culture supernatant contained a VirR/VirS-regulated protease other than the collagenase. In this study, *C. perfringens* was grown in RPMI 1640, but the other studies used Gifu anaerobic medium (GAM) broth, so the difference is probably due to the use of different culture media. This inference was supported by the increased cysteine protease activity observed in the RPMI 1640 culture supernatant (data not shown).

We used a benzamidine-Sepharose column to purify this protease. The purified protein was homologous with clostripain of *C. histolyticum*, which is a cysteine protease. The clostripain is a trypsin-like cysteine protease that was specific for the cleavage of arginyl bonds and susceptible to various trypsin inhibitors (23). The purified protease from *C. perfringens* was also susceptible to trypsin inhibitors such as benzamidine but not to soybean trypsin inhibitor. The substrate specificity of clostripain was similar to that of trypsin; however, clostripain has been shown to preferentially cleave to the carboxyl-terminal side of arginine residues (21). E-64, which inactivates most cysteine proteases (4), did not affect the clostripain. The protease of *C. perfringens* was also not affected by E-64.

The seven amino acids (FDAC*L*MG) of the active site of the clostripain of *C. histolyticum* (11) were identical to those of the protease of *C. perfringens*. The clostripain of *C. histolyticum* requires calcium ions to enhance its proteolytic activity (22). Similar to this, the protease activity of *C. perfringens* was reduced with 10 mM EDTA. The presence of the protease in *C. perfringens* was predicted by a homology search study on the whole genome sequence of the wild strain, using the VirR binding motif as the target sequence (28). It was confirmed in the present study by purification of the clostripain-like protease in *C. perfringens.*

ACKNOWLEDGMENTS

We are grateful to William Ba-Thein for critical reading of the manuscript.

This work was supported by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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