Jpn. J. Infect. Dis., 61, 402-406, 2008

Short Communication

Analysis of Gene Expression in Mouse Alveolar Macrophages Stimulated with Quorum-Sensing Mutants of *Vibrio vulnificus*

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(Received October 31, 2007. Accepted July 3, 2008)

SUMMARY: Bacterial pathogens manipulate host cells to promote pathogen survival and dissemination. In this study, microarray technology was used to identify the genes that are affected by the *Vibrio vulnificus* quorum-sensing genes, *luxS* and *smcR*. By comparing the expression profiles of mouse macrophage cell lines stimulated with either the parent strains or a *luxS smcR* mutant, differentially expressed genes were identified. The genes included those that affect host cell death, stress, signaling transduction, inflammation, and immune response. Macrophages stimulated with the *luxS smcR* mutant differentially expressed genes associated with removal of toxins, the complement pathway, regulation of cytokine expression, and antigen presentation, indicating that macrophages stimulated with the *luxS smcR* mutant induced an appropriate inflammation reaction and immune response for removal of bacteria. In summary, quorum-sensing in *V. vulnificus* could contribute to bacterial survival and increased pathogenesis by inducing a changed expression profile in macrophages.

Vibrio vulnificus is a Gram-negative estuarine bacterium, known as a significant human pathogen. A *V. vulnificus* infection, acquired via direct contact or the gastrointestinal route, is characterized by food-borne septicemia, skin infections with ulcer and edema in many clinical cases (1,2) and especially a high fatality rate in immunocompromised people with underlying conditions such as hemochromatosis, liver cirrhosis, and alcoholism (3-6). Our previous studies have provided in vitro and in vivo models of this infection (2,7,8).

V. vulnificus produces several virulence factors such as capsular polysaccharide, lipopolysaccharide (LPS), elastase, cytolysin, metalloprotease, siderophores, and phospholipase (9-13) and uses several genes for surviving or decreasing environmental stress (11,13-15). *V. vulnificus* infection proceeds through consecutive stages of infection, adaptation, survival, and septicemia. Because of the peracute process, *V. vulnificus* has to accomplish its purpose as soon as possible. For this reason, it was hypothesized that *V. vulnificus* could change the in vivo environment to a profitable condition for infection through a quorum-sensing system.

Quorum-sensing (QS) is an intercellular signaling mechanism in which bacteria monitor their own bacterial population density or that of other bacteria by recognizing the local concentration of chemical molecules, referred to as autoinducers, produced by the bacterial species (2). Many studies have sought to establish an association between QS and phenotypes in a number of bacterial species, and have revealed that QS controls various processes including bioluminescence, biofilm formation, antibiotic synthesis, and production of several virulence factors (16-19). *smcR* appears to play an important role in starvation adaptation and in the regulation of many stationary phase-regulated genes (12,20), and involvement of *luxS* with in vivo virulence and protease and hemolysin production has been proven (21).

The goal of this study was to investigate the effects of the *V. vulnificus* quorum-sensing system on host gene expression by comparing the transcript profile between macrophages exposed to the *V. vulnifiicus* parent strain and the *luxS smcR* double mutant.

Bacterial strains, VvAR, VvSRΔ*Z*, and VvSRΔ*ZSR*, were grown in Luria-Bertani media supplemented with 2.5% NaCl (LBS) with vigorous shaking at 37°C (2,7,8). VvAR is wild-type strain of *V. vulnificus* ATCC29307, VvSRΔ*Z* is a spontaneous *smcR* and *lacZ*-null mutant derived from VvAR, and VvSRΔ*ZSR* is a *luxS smcR* double mutant derived from VvSRΔ*Z* (2,7). Overnight cultures of the strains were inoculated in 20 ml of fresh LBS. After the fresh cultures were grown under the same conditions for 6 h, bacterial cells were separated from culture supernatants by centrifugation at 5,000 × g. The bacterial cells were washed with PBS twice, and resuspended in the same solution. The optical densities were measured at 600 nm, and the resuspension was diluted to 1.0×10^4 CFU/ml with RAW264.7 cell culture medium on the basis of a standard curve between bacterial cell number and optical density. The culture supernatants were collected by filtration with a 0.22 μ-pore-size filter, and diluted to the concentration of 100 μg/ml with RAW264.7 cell culture medium.

RAW264.7 was cultivated in complete Modified Eagle's minimal essential medium (DMEM) (Gibco, Gaithberg, Md., USA) supplemented with 10% fetal bovine serum (Gibco), 200 mM L-glutamine (Gibco), and 100 units/ml of penicillin at 37°C under 5% CO_2 atmosphere. 7.5×10^5 cells were added to each 75-cm^2 flask, and 4 flasks per group were prepared for microarray.

Prepared bacterial suspension and supernatants were inoculated onto monolayers of RAW264.7 cells in a 75-cm^2 flask. RAW264.7 cells were collected at 4 h or 3 h after stimulation with live bacteria or culture supernatants to extract total RNA, respectively. Total RNA was isolated from stimulated

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RAW264.7 cells using TRIzol reagent (Gibco), as described by the manufacturer, followed by extensive digestion with RNase-free DNase I (Promega, Madison, Wis., USA) at 37°C for 20 min. Extracted RNA was precipitated with isopropyl alcohol (Sigma, St. Louis, Mo., USA), washed with 75% ethanol, air-dried at room temperature for 5 - 10 min, and dissolved in RNA-free water (Gibco). The RNA concentration was determined by a spectrophotometer (Pharmacia Biotech, Cambridge, England). Prepared total RNA was stored at −70°C.

To prepare Cy3- or Cy5-labeled cDNA probes, 10 μl of total RNA, 1 μl of control mRNA, and 3 μl of oligo (dT) primer were mixed in 0.2 ml PCR tube. Each annealing reaction mixture was incubated at 70°C for 5 min, and chilled on ice immediately. New tubes were prepared to make the labeling mixture. The tube's contents were added to 18 μl of total volume comprised of the following reagents: 8 μl of 5 × AMV RT buffer, 4 μl of 10 × low dT dNTP mix, 3 μl of 1 mM Cy3(C) or Cy5-dUTP(T), 1 μl of RNase inhibitor (40 u/μl), and 2 μl of M-MLV reverse transcriptase (50 units). The annealing mixture was transferred into tubes containing the labeling reaction mixture, and incubated at 42°C for 1 h in a thermocycler. 2.5 μl of AMV RT was added into each tube, and incubated at 42°C again. After 1 h, the reaction was stopped by addition of 0.5M EDTA. One N NaOH was added into each tube and incubated at 37°C for 10 min. After 25 μl of 1M Tris·Cl was added, the mixtures were loaded into Chroma Spin column, and centrifuged at 1,300×g for 5 min. Three hundred microliters of 100% ethanol and 10 μl of 3M NaOAc were added into each tube for DNA precipitation, and stored at −70°C for 30 min. After being washed with 70% ethanol, the pellet was dried in air. The dried pellet was melted with ddH_2O, and centrifuged at 13,000×g for 2 min. Optical density was measured from 5 μl of both the control and test mixtures after 1/20 dilution. One new tube was added with the same quantity of control and test cDNA and 20 μl of 5 × fragmentation buffer, and increased in volume to 100 μl with ddH_2O. The tube was incubated 95°C for 15 min and chilled on ice immediately. The fragmented cDNA mixture was dried by Speed-vac.

For the hybridization, dried pellets of control and test probes were completely dissolved with hybridization buffer (6 × SSC, 0.2% SDS, 5 × Denhardt Solution, and 1 mg/ml Salmon sperm DNA). The mixture was boiled at 94°C for 3 min, and chilled immediately on ice for 1 min. After centrifugation, the probe was placed on mouse oligo 11K microarray chip (Macrogen Co., Seoul, Korea), and covered with cover glass. Under humified condition, the chip covered with aluminum foil was maintained at 62°C for 16 h. The chip was washed twice with 58°C washing buffer I (2 × SSC, and 0.2% SDS) with rolling in a 58°C hybridization oven for 30 min, and once with washing buffer II (0.05 × SSC) with shaking at room temperature for 5 min.

A hybridized slide was scanned with a GMS 418 scanner, and the image was analyzed with the Imagene 4.1 program. The data were Cy3- to Cy5-corrected with the global mean shifting method, and were expressed as '2log (Cy3 value/ Cy5 value)'. Data showing a 2-fold difference between Cy3 and Cy5 values were selected and confirmed for the intensity of Cy3 and Cy5. This experiment was carried out three times. The pattern analysis method employs a combination of a selection procedure for genes with either constant or non-reproducible up- or down-regulation in experimental repetitions. Genes were ranked according to their strongest differential expression based on the lowest absolute signal intensity ratios (22).

Microarrays were used to analyze gene expression profiles in the mouse alveolar macrophage cell-line stimulated with live bacteria or culture supernatants of wild-type or mutant *V. vulnificus*, VvAR, VvSRΔ*Z*, and VvSRΔ*ZSR*. Relative ratios of the regulation of gene expression in the cell line were analyzed. Twenty to more than 1,000 genes were differentially expressed depending on the mutation. In the case of the wild-type and *lacZ*-deleted strain, only 20 to 30 mRNAs of the 11,376 mRNAs on the array had significantly different levels of expression whereas many mRNAs, about 12.6% of the total mRNAs, were expressed differentially by the culture supernatant of the *luxS smcR* double mutant. This indicates that QS in *V. vulnificus* might control production of extracellular proteins related to interaction with macrophages. Most of the mRNAs elicited by *V. vulnificus* live bacteria encode genes whose products function in the immune response. These results agree well with a recent study that examined expression of the human monocytic U-937 cell line infected with *Salmonella enterica* subspecies *typhimurium* (23).

Data on the $luxS^{+}smcR^{+}$ strains- and $luxS^{-}smcR^{-}$ mutant-infected macrophages were compared to identify the effects of QS on host gene expression. The comparison of array data revealed that 938 or 477 genes in the culture supernatant-stimulated RAW264.7 cells were up- or down-regulated in the QS mutant compared to the $luxS^{+}smcR^{+}$ strains, while 3 or 26 genes were up- or down-regulated in the expression profiles of live *luxS smcR* double mutant-stimulated RAW264.7 cells, respectively. From these results, several genes considered to directly affect the response of the macrophage in bacteria-macrophage interactions were selected and are presented in Tables 1, 2 and 3.

Various genes associated with cell functions including cell

Table 1. The genes up or down-regulated in mouse macrophage cell line RAW264.7 stimulated with *luxS* and *smcR* mutated *Vibrio vulnificus* live bacteria

Regulation	Fold change	Accession no.	Protein or gene
Up-regulated	2.12	NM_023051	calsyntenin 1; cstn1-pending
	2.17	NM_009782	calcium channel, voltage-dependent, r type, alpha 1e subunit; cacna1e
	3.94	NM_009145	stromal cell derived factor receptor 1; sdfr1
Down- regulated	-3.89	NM_008350	interleukin 11; il11
	-3.83	NM_023755	transcription repressor crtr-1; crtr1-pending
	-3.32	NM_011329	small inducible cytokine a1; scya1
	-2.62	NM_010739	lymphocyte antigen 64; ly64
	-2.17	NM_008665	myelin transcription factor 1; myt1

$P < 0.05$.

Table 2. The major genes up-regulated in mouse macrophage cell line RAW264.7 stimulated with *Vibrio vulnificus luxS* and *smcR* mutated bacterial culture supernatant

Gene function	Accession no.	Fold increase	Protein or gene
Transcription	NM_053163	10.72414	mitochondrial ribosomal protein l36; mrpl36
	NM_010908	5.50966	nuclear factor of kappa light chain gene enhancer in b-cells inhibitor, beta; nfkbib
	NM_008394	3.48747	interferon dependent positive acting transcription factor 3 gamma; isgf3g
Cell cycle and apoptosis	AK008255	9.65161	homolog to arp2/3 complex 16 kda subunit (p16-arc)
	NM_007872	9.20346	dna methyltransferase 3a; dnmt3a
	NM_009807	6.69778	caspase 1; casp1
	AF067834	5.23744	caspase-8
	NM_022994	4.97342	death associated protein 3; dap3
	NM_023626	3.35385	inhibitor of growth family, member 3; ing3
Stress	NM_019814	5.19246	hypoxia induced gene 1; hig1-pending
	L43371	4.69833	hydrogen peroxide-inducible protein
	Y09085	2.92272	hypoxia-inducible factor one alpha; hif1a
	NM_011020	2.25227	osmotic stress protein 94 kda; osp94
	NM_010431	2.25173	hypoxia inducible factor 1, alpha subunit; hif1a
Chemokine	NM_011337	4.52320	small inducible cytokine a3; scya3
	NM_021274	3.45509	small inducible cytokine b subfamily (cys-x-cys), member 10; scyb10
	NM_031252	3.38213	interleukin 23, alpha subunit p19; il23a
	NM_010510	3.26011	interferon beta, fibroblast; ifnb
	NM_023065	3.18253	interferon gamma inducible protein 30; ifi30
	NM_013653	2.70617	small inducible cytokine a5; scya5
	NM_011338	2.63943	small inducible cytokine a9; scya9
	NM_053095	2.11615	interleukin 24; il24
Signal transduction	NM_009870	8.84442	cyclin-dependent kinase 4; cdk4
	NM_133211	7.69379	toll-like receptor 7; tlr7
	NM_011905	7.15822	toll-like receptor 2; tlr2
	NM_011949	6.24736	mitogen activated protein kinase 1; mapk1
	NM_011369	6.08891	shc sh2-domain binding protein 1; shcbp1
	NM_024290	4.31712	tumor necrosis factor receptor superfamily, member 23; tnfrsf23
	NM_009421	3.90105	tnf receptor-associated factor 1; traf1
	NM_021297	3.86671	toll-like receptor 4; tlr4
	NM_009164	3.78526	sh3-domain binding protein 1; sh3bp1
	NM_009806	3.61581	calcium/calmodulin-dependent serine protein kinase; cask
	BC003801	3.35940	similar to tnf receptor-associated factor 2
	NM_010559	3.30436	interleukin 6 receptor, alpha; il6ra
	NM_031167	3.25630	interleukin 1 receptor antagonist; il1rn
	AF115517	2.73016	survivin40
	NM_008372	2.60674	interleukin 7 receptor; il7r
Inflammation	X12905	8.39693	properdin (aa 5 - 441)
	NM_007824	8.19722	cytochrome p450, 7a1; cyp7a1
	NM_008959	7.63881	phosphatidylserine synthase 1; ptdss1
	NM_009945	7.53156	cytochrome c oxidase subunit viia 3; cox7a3
	AK020624	3.93393	superoxide dismutase 1, soluble
Other	NM_009983	2.90403	Cathepsin d; ctsd
	L38281	5.84413	immune-responsive gene 1; irg1
	NM_007654	3.98481	cd72 antigen; cd72
	NM_018770	3.61773	immunosuperfamily protein bl2; igsf4
	J00369	3.35289	complement component c3 prepropeptide, last
	NM_013671	3.20810	superoxide dismutase 2, mitochondrial; sod2
	NM_007969	3.10810	extracellular proteinase inhibitor; expi

$P < 0.05$.

division, apoptosis, inflammation, immune response, and signaling transduction were specifically identified in macrophages stimulated with the culture supernatant of the *luxS smcR* double mutant. Two inflammation-related genes play a role in the direct removal of bacterial exotoxins. Cytochrome P-450 oxidase helps to convert ingested toxins into harmless substances, and extracellular proteinase inhibitor inactivates extracellular toxins. This phenomena might be due to the differences in the production of exotoxins by the mutant strains (2,7,8). Cathepsin D affects antigen presentation as a lysosomal protease important for MHC peptide presentation (24,25). Two mRNAs related to the complement pathway were also found. Properdin expressed at such a high level as 8.4-fold is a positive regulator of the alternative pathway of complement activation, and C3 is the most important complement because it acts on both classical and alternative pathways. This suggests that QS could enhance bacterial survival by affecting the alternative complement pathway. This notion

Table 3. The major genes down-regulated in mouse macrophage cell line RAW264.7 stimulated with *Vibrio vulnificus luxS* and *smcR* mutated bacterial culture supernatant

Gene function	Accession no.	Fold decrease	Protein or gene
Immune system	NM_007648	-4.18401	cd3 antigen, epsilon polypeptide; cd3e
	NM_007640	-3.35249	cd1d2 antigen; cd1d2
	NM_009971	-3.32499	colony stimulating factor 3 (granulocyte); csf3
	AK010524	-3.39211	similar to h-2l(q) class i major histocompatability complex glycoprotein precursor (fragment)
	X91671	-2.32875	immunoglobulin kappa light chain
	AF401530	-2.18345	chemokine-like factor 2 variant 1; cklf2-1
	M13674	-2.15112	t-cell receptor beta-chain precursor (v9dj)
Signal transduction	AJ132271	-8.07142	phosphodiesterase 3b; pde3b
	M30880	-5.32898	t-cell receptor beta-chain v12-d1.1-j1.1-c0-c1 region
	NM_010703	-5.17452	lymphoid enhancer binding factor 1; lef1
	NM_008696	-2.72827	mitogen-activated protein kinase kinase kinase kinase 4;map4k4
	NM_008356	-2.24605	interleukin 13 receptor, alpha 2; il13ra2
Cell cycle and apoptosis	AF104984	-4.16684	tgf-b1-induced anti-apoptotic factor 1
	NM_008109	-2.92070	growth differentiation factor 5; gdf5
	NM_010175	-2.83736	fas (tnfrsf6)-associated via death domain; fadd
Inflammation	NM_008489	-3.77240	lipopolysaccharide binding protein; lbp
	NM_011435	-2.70545	Superoxide dismutase d, extraellular; sod3
	NM_013560	-2.65743	heat shock protein, 25 kda; hsp25
	NM_008713	-2.28190	nitric oxide synthase 3, endothelial cell; nos3
Adhesion	NM_013486	-3.13143	cd2 antigen; cd2
Other	NM_008479	-3.15849	lymphocyte-activation gene 3; lag3
	BC010546	-2.17377	similar to interferon activated gene 204

$P < 0.05$.

is supported by the data for serum sensitivity. Superoxide dismutases (SODs) are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism. There are three forms of SOD: cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD. The amount of SOD is crucial for the prevention of diseases linked to oxidative stress. In our data, expressions of SOD1 and SOD2 were increased. This means that macrophages confronted with the *luxS smcR* mutant could more efficiently prevent diseases linked to oxidative stress than parent strains.

The expression of a nitric oxide (NO) synthase-encoding gene was decreased in mouse macrophages by stimulation with *luxS*- and *smcR*-deficient bacterial culture supernatants. This is consistent with our previous experiment showing decrease of NO production from RAW264.7 cells stimulated with the *luxS smcR* double mutant in comparison with VvAR and VvSRΔZ (2). Table 3 also provides evidence that QS could be involved in septic shock. For example, the decrease of the LPS-binding protein is likely due to the fact that macrophages are less sensitive to septic shock-inducing factors. Phosphodiesterase can contribute to decreased production of TNF-α and NO. Cytokines or receptors related to TNF-α production were also regulated to repress cytokine production.

The experiments described in this study used microarrays to profile the transcriptional responses of macrophages infected with *V. vulnificus*. By comparing the expression profiles of *luxS*$^+$*smcR*$^+$ strains and the *luxS smcR* double mutant, we confirmed the relevance of the quorum-sensing system with host gene expression. The data suggested that QS in *V. vulnificus* could contribute to bacterial survival and increased pathogenesis by inducing a changed expression profile in host macrophages.

ACKNOWLEDGMENTS

This study was supported by KOSF, Brain Korea 21, KRF(KRF-2006-005-J02901) and the Research Institute for Veterinary Science, Seoul National University, Korea.

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