Cold Shock Induces *qnrA* Expression in *Shewanella algae*[†]

Hong Bin Kim,^{1,2,3} Chi Hye Park,³ Mariah Gavin,³ George A. Jacoby,⁴ and David C. Hooper^{3*}

Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, Republic of Korea¹; Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Republic of Korea²; Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts³; and Lahey Clinic, Burlington, Massachusetts⁴

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Plasmid-carried quinolone resistance genes, like *qnrA*, are widespread in *Enterobacteriaceae*. To gain insight into its little-understood native functions, we studied the effect of environmental conditions on chromosomal *qnrA* expression in *Shewanella algae*. Among conditions of DNA damage, oxidative and osmotic stress, starvation, heat, and cold, only cold shock increased gene expression, as measured by quantitative reverse transcription-PCR (qRT-PCR). Induction was graded and occurred during growth arrest, suggesting that *qnrA* may contribute to the adaptation of *Shewanella* to low temperatures.

Plasmid-mediated quinolone resistance (PMQR) determinants have been increasingly recognized (11, 14, 19), and *qnr*-like genes have also been found on the chromosomes of both Gram-negative and Gram-positive bacteria (2, 11, 20, 21). In the case of *qnrA*, three alleles (*qnrA3* to *qnrA5*) have been found in strains of *Shewanella algae* (17), an aquatic organism that must adapt to various environmental conditions. *S. algae* may thus be a natural reservoir for *qnrA* (17), which in this organism seems unlikely to be present to confer resistance to synthetic quinolone antimicrobials. In order to determine the normal functions of *qnr* in *S. algae*, we measured the levels of expression of *qnrA* in response to environmental conditions that may be encountered by aquatic organisms.

***qnrA* in *S. algae*.** PCR amplification with previously described *qnrA* primers (12) was positive for *S. algae* BrY but not *Shewanella putrefaciens* strain CN-32. The sequence was identical to that of *qnrA2* (GenBank accession no. AY675584). For *S. algae*, the MIC of ciprofloxacin (0.125 µg/ml) was 4- to 8-fold higher than that for *S. putrefaciens* by Etest (AB Biodisk, Solna, Sweden). Using an inverse PCR strategy (23) with a series of outward-facing primers from both sides of the *qnrA2* gene (657 bp), we sequenced 2,603 bp upstream and 3,098 bp downstream (GenBank accession no. HQ449669). A consensus sequence binding site for the LexA protein (4), a key regulator of the SOS response, was not found upstream from *qnrA2*.

Exposure to ciprofloxacin and other DNA-damaging agents. The Luria-Bertani (LB) broth dilution MICs obtained for methyl methanesulfonate (MMS), mitomycin, and novobiocin (Sigma Chemical Co., St. Louis, MO) were 250 µg/ml, 16 µg/ml, and 250 µg/ml, respectively. We treated exponentially growing *S. algae* (optical density at 600 nm [OD₆₀₀] of ~0.5) for 30 min with 0.5 MICs of ciprofloxacin, MMS, and novobiocin. Because *S. algae* grew poorly at 0.5 MIC of mitomycin, we used 0.16 MIC (2.5 µg/ml), a concentration that is 12.5-fold higher than that which induced expression of *qnrB* in *Escherichia coli* (26). Cells were harvested, suspended in 0.45 ml

RLT buffer (RNeasy minikit; Qiagen, Germantown, MD), and disrupted by vigorous shaking in the presence of 0.1-mm zirconia-silica beads (BioSpec, Bartlesville, OK) for 1 min in an amalgamator, as previously described (3). Bacterial RNA was isolated using the RNeasy minikit, according to the manufacturer's protocol, and RNA samples were treated with DNase I (Turbo DNA-free; Ambion, Austin, TX). cDNA synthesis was performed using the Verso cDNA kit (ABgene, Epsom, Surrey, United Kingdom) with gene-specific reverse primers (Table 1), according to the manufacturer's instructions. Quantitative PCRs (qPCRs) used PTC-200 (Bio-Rad, Hercules, CA) and a qPCR SYBR green kit (ABgene, Thermo Scientific, Surrey, United Kingdom). The cycling parameters used were 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s. The primers used are shown in Table 1. The relative expression of *qnrA* was calculated by the threshold cycle ($\Delta\Delta C_T$) method and normalized to that of the *adh* housekeeping gene (1).

For exposure to UV, *S. algae* cells (OD₆₀₀ of 0.2 to 0.3 in LB broth) were divided between two petri dishes, with one exposed to UV-B (302 nm; 15-W bulb at 20 in [UVP, Upland, CA]) and the other exposed to ambient light. Samples were taken at 2, 5, and 10 min and treated immediately with RNeasy Protect (Qiagen, Germantown, MD); UV exposure resulted in a ~100-fold reduction in the number of CFU in 5 min. Expression of *recA* of *S. algae*, identified by homology to *Shewanella oneidensis* (18) and *E. coli* sequences, was a positive control for DNA damage.

The expression of *qnrA2* was unchanged by exposure to ciprofloxacin, mitomycin, and MMS (Table 2). In addition, exposure to UV-B irradiation resulted in decreases in *qnrA* transcript levels under conditions that resulted in the expected increases in transcript levels of *S. algae recA*.

Oxidative stress. *S. algae* cells (OD₆₀₀ of 0.5 in LB broth) were treated with H₂O₂ for 10 min at 37°C. A concentration of 0.1 mM was chosen as the highest concentration that did not affect cell viability. A similar exposure was shown to induce an oxidative stress response in *Pseudomonas aeruginosa* (15), but no significant change in *qnrA2* expression was seen (Table 2).

Heat and cold shock. *S. algae* was grown at 30°C to an OD₆₀₀ of 0.4, and aliquots were transferred to temperature-equili-

* Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114-2696. Phone: (617) 726-3812. Fax: (617) 726-7416. E-mail: dhooper@partners.org.

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TABLE 1. Primers used in this study

Gene	Primer	Sequence (5'-3')
<i>adk</i>	adk-F2	GGCATTCCACAGATCTCAACCG
	adk-R4	CCGGCGTCCATCACCTTTCT
<i>qnrA</i>	qnrA2-F	ACGCCAGGATTTGAGTGACAGCC
	qnrA2-R	GGCGCCGCTTTCAATGAACTGC
<i>recA</i>	recA-F3	GGTTCGCTTTCTCTGGACG
	recA-R3	CGGCAATCACTTCTAATGTCAG

brated flasks in water baths at $\leq 18^{\circ}\text{C}$, 30°C (control), and 42°C . Samples were taken at 30 min after transfer (5, 6). Upon the shift to cold (12° to 18°C), growth stopped immediately and resumed after a lag of about 1.5 to 2 h. In contrast, growth of *S. algae* accelerated at 42°C . *qnrA2* expression did not change upon the shift to 42°C , but progressive increases in *qnrA2* transcript levels occurred with shifts between 18°C and 12°C (Table 2). In a time-course experiment, upon the shift to 15°C , *qnrA2* expression increased by 3.2- and 2.3-fold at 30 and 60 min, respectively, but decreased to baseline by 90 to 120 min. We also tested plasmid-carried *qnrA* in *E. coli* strain 10-22, which carries *qnrA* on a high-molecular-weight plasmid (27). The shift from 30°C to 12°C resulted in a 2.2-fold increase in *qnrA* transcripts in *S. algae* but no change in *E. coli* 10-22. Thus, cold shock induction of *qnrA* appears to be selective for the *S. algae* background.

Nutrient limitation. *S. algae* cells grown at 37°C to an OD_{600} of 0.4 in LB broth were centrifuged and resuspended in pre-warmed phosphate-buffered saline (PBS). Growth stopped immediately and did not resume for over 90 min. At 30 min, the expression levels of both *adk* and *qnrA2* were decreased. Thus, we could not determine the relative level of *qnrA2* expression.

Elevated or decreased salt concentrations. *Shewanella* species inhabit diverse environments, including deep-sea and freshwater lake sediments. *S. algae* can grow in 6% (wt/vol) NaCl, in contrast to *S. putrefaciens* (10). Since seawater has an average salinity of 3.5%, we grew *S. algae* cells in LB broth with 3.5% NaCl as a control condition and resuspended cells in LB broth with 0.5% to 6% NaCl to generate osmotic stress. Under aerobic conditions, growth decreased in 0.5% to 1% NaCl, was drastically reduced without supplemental NaCl, and increased slightly with additional NaCl levels of up to 6%. Although *S. algae* is more tolerant to sodium salt stress than *S. oneidensis* MR-1 (13), we did not observe any substantial change in the expression of *qnrA2* after the shift to different concentrations of NaCl for 30 min (Table 2).

Whereas much information about gene transcription in *S. oneidensis* MR-1 is available (8), no such data exist for *S. algae*. Thus, to gain insight into the normal function of the *qnr* gene, we modified previous experimental approaches (5, 6, 13, 15, 26) and measured the expression of *qnrA2* after exposure to environmental stimuli that could be encountered in the native aquatic environments of *Shewanella* species.

As found previously for *qnrA1* (26), expression of *qnrA2* did not increase in response to ciprofloxacin, MMS, mitomycin C, or novobiocin. In contrast to *qnrB*, we found no LexA binding site in the upstream sequence of *qnrA2*, suggesting that *qnrA2* is not under SOS control following DNA damage. In addition,

TABLE 2. Expression of *qnrA2* in *S. algae* after exposure to environmental stimuli

Environmental stimulus	Mean relative expression ^a	SEM
DNA-damaging agents ^b		
Ciprofloxacin	1.00	0.80
MMS	0.93	0.04
Mitomycin C	1.28	0.61
Novobiocin	1.36	0.20
UV-B irradiation ^c	0.44	0.11
Oxidative stress ^b	1.28	0.62
Heat shock ^d	0.84	0.22
Cold shock composite ^{d,f}		
18°C	5.99	1.56
15°C	3.18	
12°C	4.73	1.50
	8.57	
Salt concn (NaCl) ^e		
0%	1.15	0.08
0.5%	1.15	0.07
1.0%	0.94	0.02
6.0%	1.17	0.31

^a Relative expression of *qnrA2* was normalized to expression of *adk*. Means and standard errors of the means (SEM) were calculated from the results from at least two independent experiments, except for those from experiments of cold shock at 18°C and 12°C , which were done once.

^b Expression compared to growth without treatment.

^c Exposure for 10 min, at which time there was a 2.1 ± 0.08 -fold increase in *recA* transcripts.

^d Expression compared to growth at 30°C .

^e Expression compared to growth in LB broth with 3.5% NaCl.

^f The composite indicates the means and SEM of results from cold-shift experiments at all three temperatures shown.

qnrA2 was induced neither directly by H_2O_2 nor via the SOS regulon by oxidant-dependent DNA damage. In addition, neither sodium salt stress nor heat shock affects its expression.

Temperature is an environmental factor that commonly varies, and virtually all organisms respond to an increase or decrease in temperature. When exponential-phase *E. coli* or *S. oneidensis* cells are shifted from 37°C to $<20^{\circ}\text{C}$, growth is arrested (6, 7), and a set of cold-induced proteins increase transiently to high levels (22). We observed a similar response for *qnrA2* in *S. algae*. A deleterious effect of cold shock is stabilization of the secondary structures of RNA and DNA, which may lead to a reduced efficiency of DNA replication, transcription, and translation (16). QnrA, as a pentapeptide repeat protein, is predicted to form a DNA-like structure similar to that of MfpA (9, 25), and thus, it might affect stabilization of secondary DNA and RNA structures. Notably, in *E. coli*, cold shock results in increased negative DNA supercoiling and *gyrA* expression (24), but in *S. oneidensis*, cold shock induces increased expression of *topB* encoding topoisomerase III but decreased or stable expression of *gyrA* (6). Both gyrase and topoisomerase III relax positive DNA supercoils, possibly accounting for the increased negative DNA supercoiling after cold shock. Thus, QnrA may play a role in modulating the function of or stabilizing DNA gyrase, topoisomerase III, or other DNA binding proteins that are important for bacterial adaptation to cold temperatures.

It remains possible that other environmental conditions not

studied here could also affect expression of *qnrA*, but since growth at a low temperature ($\sim 4^{\circ}\text{C}$) is a distinctive metabolic feature of the *Shewanella* genus, it seems likely that *qnrA* expression plays a role in this key adaptation. Further studies in which *S. algae* can be genetically modified to alter *qnrA* expression are needed to better understand the native function of QnrA in *S. algae*.

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