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NOTES

Regulation of *fur* Expression by RpoS and Fur in *Vibrio vulnificus*

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In a proteomic analysis of *rpoS*-deficient *Vibrio vulnificus* versus the wild type, one of the down-regulated proteins in the *rpoS* mutant strain was identified as a Fur protein, a ferric uptake regulator. The expression of a *fur*::*luxAB* fusion was significantly influenced by sigma factor S, the *rpoS* gene product, and positively regulated by Fur under iron-limited conditions.

Vibrio vulnificus, a causative agent of septicemia in humans, is a halophilic marine bacterium. Thus, it is thought that this microorganism has survived diverse environmental stresses and has developed efficient survival mechanisms, which involve sigma S (reviewed in reference 9). The biological functions of the *rpoS* gene were assessed by generating a knockout mutation of this gene in *V. vulnificus* (K.-J. Park et al., unpublished results). To identify the target genes regulated by this *rpoS* gene product, we compared the proteomic pattern of the *rpoS* knockout strain with that of the wild-type strain. Interestingly, one of proteins down-regulated in extracts of the *rpoS* mutant strain turned out to be Fur, which functions as a transcriptional repressor for the genes involved in iron acquisition and iron metabolism in many bacteria (4).

In nature, iron is present in an insoluble form, which is unavailable to microorganisms (16). Animal pathogens encounter an additional difficulty in obtaining iron because iron is present not in a free form but as complexes with iron-binding proteins in a mammalian host (1) as well as bound by the siderophore-mediated iron transport systems produced by normal-flora microbes. As a consequence, the synthesis of many toxins and virulence determinants is regulated by the intracellular iron concentration of the bacterial cells (10, 14). The correlation between the virulence of *V. vulnificus* and iron availability (19) suggests that iron is particularly important in the pathogenesis of *V. vulnificus* infections. The gene encoding Fur of *V. vulnificus* has been cloned (11), and it was later discovered that the synthesis of two outer membrane proteins—HupA, a heme uptake receptor, and VuuA, a ferric vulnibactin receptor—was regulated by iron concentration via the action of the Fur protein (12, 18).

In this study, the decreased level of Fur protein in *rpoS* mutant *V. vulnificus* was confirmed at a transcriptional level by using the *fur*::*luxAB* fusion, and the physiological connection between these two control systems in response to iron was investigated.

Reduced production of the Fur protein in an *rpoS* knockout mutant of *V. vulnificus*. Both the wild type and the *rpoS* mutant of *V. vulnificus* were cultivated in Luria-Bertani broth (1% tryptone, 0.5% yeast extract) supplemented with 2.5% (wt/vol) NaCl (LBS) to the stationary phase. Cell extracts were prepared in a 2-dimensional gel electrophoresis (2DGE) sample buffer (5 M urea, 2 M thiourea, 0.1% carrier ampholytes, 2% [wt/vol] sulfobetaine, 2 mM tributylphosphine), and then used for overnight rehydration of either pH 3 to 10 or pH 4 to 7 immobilized pH gradient (IPG) gel strips (13 cm; Amersham Pharmacia Biotech). A three-phase program was used for the isoelectric focusing: the first phase was set at 1,000 V for 1 h, the second phase was at 2,000 V for 2 h, and the third phase was a linear gradient from 2,000 to 8,000 V over 14 h. The second-dimension separation was carried out at room temperature on sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis gels (16 by 20 cm) without stacking gels. After electrophoresis at 60 mA gel^{-1} for 6 h, the proteins were visualized by silver staining.

According to 2DGE analysis using a pH 3 to 10 IPG strip, the majority of *V. vulnificus* proteins clustered between pH 4.0 and 7.0 and had molecular weights between 30,000 and 100,000 (data not shown). In another separation using a pH 4 to 7 IPG strip, the protein spot patterns of the two strains, i.e., the wild type and the *rpoS* knockout mutant, were compared by using the PDQuest program (Fig. 1). A high degree of conservation was observed between these two strains within the majority of the encoded amino acid sequences. Protein spots showing differential synthesis between the two strains were digested with trypsin (6, 17). Mass-spectrometric analyses were performed with a PerSeptive Biosystems (Framingham, Mass.) matrix-assisted laser desorption ionization–time of flight (MALDI/TOF) Voyager DE-RP mass spectrometer operated in delayed extraction and reflector mode. Peptide mixtures were analyzed by using a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile–0.1% trifluoroacetic acid (5). The PEPTIDENT pro-

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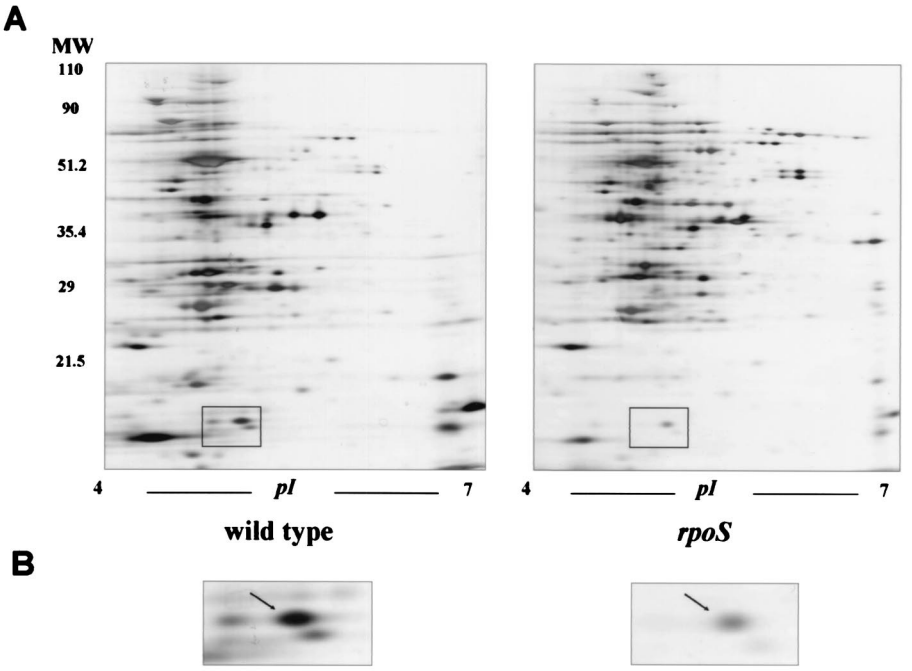

FIG. 1. (A) 2D protein patterns of wild-type *V. vulnificus* AR and of its isogenic *rpoS* knockout mutant. The box indicates a spot down-regulated in the *rpoS* mutant and identified as Fur by subsequent MALDI/TOF analysis. (B) Enlarged views of the areas on the 2D gels representing Fur.

gram of ExPASY was used for database searching. A protein spot (17 kDa, pI = 5.4) down-regulated in the *rpoS* mutant of *V. vulnificus* showed homology to the Fur proteins of gram-negative bacteria, including that of *Haemophilus ducreyi* (accession number P71333) (2) (Fig. 1). The Fur protein spot was clearly larger in extracts from the wild type than in extracts from the *rpoS* mutant (Fig. 1B). However, a low level of Fur was still detected in the mutant *V. vulnificus*, indicating that

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s) or nucleotide sequence	Reference or source
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Muλ*pir*, OriT of RP4 Km^r	Laboratory collection
V. vulnificus		
ATCC 29307	Clinical isolate	7
AR	ATCC 29307, Rif^r	Park et al., unpublished
KPR101	AR, Δ*rpoS*	Park et al., unpublished
MO6-24/O	Clinical isolate	19
MR	MO6-24/O, Rif^r	This study
HLM101	MO6-24/O, Δ*fur*	This study
Plasmids		
pRK415	Broad-host-range plasmid	8
pHK0011	pRK415, a promoterless *luxAB*, Tc^r	3
PHL01	pHK0011, *fur*::*luxAB*	This study
PHL02	pHK0011, *hupA*::*luxAB*	This study
pBluescript SK(+)	pUC19	New England Biolabs
pSKfurup	pBluescriptSK(+), 0.4-kb *fur* upstream DNA	This study
PSKfurup/down	pBluescriptSK(+), 0.9-kb Δ*fur*	This study
pDM4	oriR6K Mob RP4 Cm^r	15
pDMΔfur	pDM4, 0.9 kb Δ*fur* Used for construction of HLM101	This study
Primers		
furp-F	5′-CCCCGGTACCACTCCCGCCATACTGAG-3′	This study
furp-R	5′-ACCAGGATCCTTTAGCCGTTGGTTATTGTCT-3′	This study
furup-F	5′-TAAAGAGCTCATACTCCCGCCATACTGA G-3′	This study
furup-R	5′-ACCAGGATCCTTTAGCGCTTGGTTATTGTCT-3′	This study
furdown-F	5′-CATGGGATCCTAGTTTGTCTCGACTGCGG-3′	This study
furdown-R	5′-AAGCGGTACCTGAAAAAGGCTATCAGTTTGA-3′	This study
hupAp-F	5′-CGCGGGTACCAAGGTTATGGGGCTTTGCA-3′	This study
hupAp-R	5′-GGGCTCTAGAAGGAACGTTCTGTTGTACAT-3′	This study

significant amounts of Fur were present in this strain at the stationary phase, from which cellular extracts were prepared for 2DGE.

Decreased expression of the *fur*::*luxAB* fusion in *rpoS* knockout *V. vulnificus*. Based on the sequences published by Litwin and Calderwood (11) (GenBank database accession number, AAL67892), the oligonucleotides furp-F and furp-R (Table 1) were made and then used to amplify a 426-bp DNA fragment encoding the first 12 amino acids of the Fur protein and the 382-bp *fur* promoter region. A *Kpn*I site and a *Bam*HI site, located at either end of the resultant *fur* DNA, were used to clone this DNA into the corresponding sites of pHK0011, a *luxAB* reporter vector (3). The resultant *fur*::*luxAB* fusion, pHL01, was mobilized either to the wild-type *V. vulnificus* AR or to the *rpoS* mutant strain. The levels of expression of this fusion in both strains were monitored throughout the growth stage. Overnight cultures were diluted 100-fold with fresh LBS containing tetracycline (3 μg ml^{-1}) and grown with shaking at 30°C. Luciferase activities were determined with a luminometer by adding *n*-decyl aldehyde solution (0.006% in 20 mM Tween 80) to the cultures and expressed as specific bioluminescence by dividing the number of relative light units by the cell density (optical density at 595 nm [OD_{595}]) of the cultures. In wild-type *V. vulnificus*, the expression of *fur*::*luxAB* increased about ninefold as the cells entered the stationary phase (Fig. 2A). As expected from its proteomic pattern, the expression of *fur*::*luxAB* was significantly lower in *rpoS* knockout *V. vulnificus*; and less than 10% of the wild-type expression was observed in the *rpoS* mutant at all growth stages (Fig. 2B). Despite the lower expression, an increase of *fur*::*luxAB* expression at the stationary phase was also observed in the *rpoS* mutant.

The promoter region of the *fur* gene has been determined by primer extension (11), demonstrating a single transcript with a well-conserved −10 region but without any −35-like sequences. The observation that the expression of *fur*::*luxAB* increased as cells entered the stationary phase suggested that the *fur* gene may be transcribed by RNA polymerase containing sigma S under certain conditions. However, this growth-phase-dependent increase in the *fur*::*luxAB* expression was also observed in *V. vulnificus* without a functional RpoS. Thus, it is premature to speculate that RpoS is directly involved in the expression of the *fur* gene as a component of RNA polymerase at the present level of knowledge. However, RpoS was involved in the overall expression of the *fur* gene at all growth stages in an unidentified way. To understand the exact function of RpoS in *fur* expression, extensive analysis of the use of the *fur* promoter region should be performed in different strains of *V. vulnificus* during the exponential and stationary phases.

Effect of iron on the *fur*::*luxAB* expression. The effects of iron availability on the expression of the *fur* gene were examined by adding 2,2′-dipyridyl, an iron chelator, to cultures at the early exponential stage (OD_{595} of ~0.1). Upon the addition of 2,2′-dipyridyl, the cellular growth of both strains, the wild type and the *rpoS* mutant, was retarded (Fig. 3A and B). Five hours after the addition of the iron chelator, the activity of *fur*::*luxAB* in the wild type increased about twofold versus that in untreated *V. vulnificus*. Several concentrations of 2,2′-dipyridyl ranging from 0.01 to 1 mM were tested. It was found that the maximal expression of *fur*::*luxAB* occurred at 0.6 mM (data not shown). However, we chose to use 0.2 mM 2,2′-dipyridyl in the following experiments, because at this level, *fur*::*luxAB* expression was significantly induced and the toxic effect of 2,2′-dipyridyl of cellular growth was minimized (data not shown). Regardless of the presence of iron chelators, the expression of *fur*::*luxAB* was induced in the wild type as the cells entered the stationary phase. However, in the case of the *rpoS* mutant, the overall activity of *fur*::*luxAB* decreased in both the presence and absence of 2,2′-dipyridyl. Despite the lower level

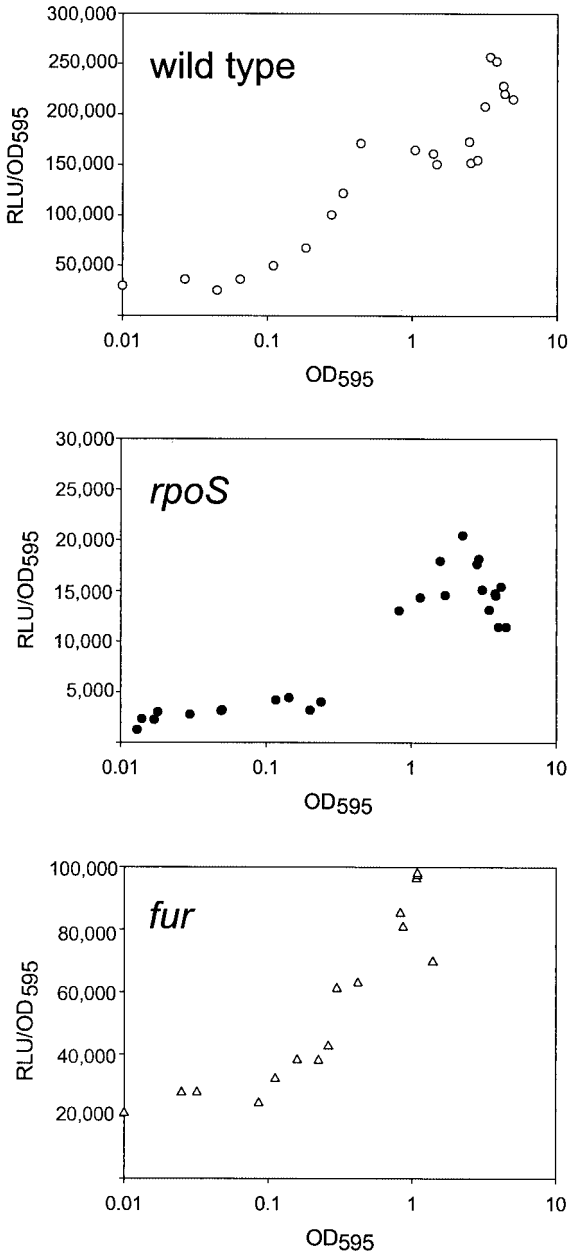

FIG. 2. Expression patterns of *fur*::*luxAB* (pHL01) in the wild type (AR), the *rpoS* knockout mutant (KPR101), and the *fur* knockout mutant (HLM101). *V. vulnificus fur*::*luxAB* activities were monitored throughout the growth stages and plotted against cell OD_{595}. Luciferase activities are expressed as normalized values. The results are from a single representative experiment from experiments performed in triplicate. RLU, relative light units.

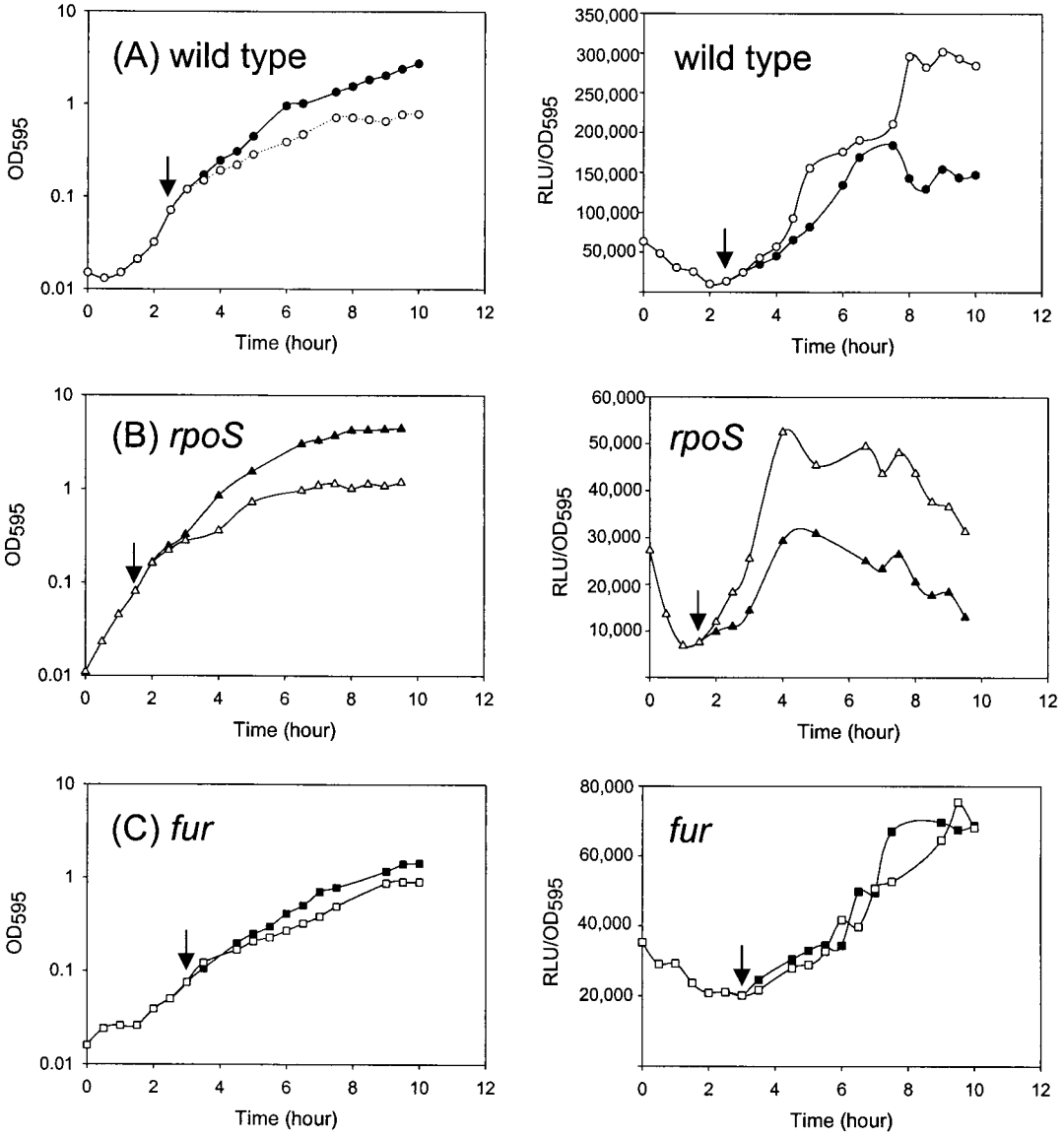

FIG. 3. Effects of iron availability on the expression of *fur*::*luxAB* (pHL01) in the wild type, the *rpoS* mutant, and the *fur* mutant. Iron in the medium was depleted by adding 0.2 mM 2,2′-dipyridyl to one of two cultures at the times indicated by the arrows. Culture OD_{595} and luciferase activity were determined in the absence (closed symbols) and presence (open symbols) of 2,2′-dipyridyl. Luciferase activities (right panels) are expressed as normalized values. The results are from a single representative experiment from experiments performed in triplicate.

of expression of *fur*::*luxAB*, the induction of its expression by the iron chelator was observed in the *rpoS* mutant strain.

To examine the role of Fur in the iron control of *fur* expression, a *fur* knockout *V. vulnificus* was made by three series of recombinations. A PCR product containing a 420-bp upstream sequence of the Fur open reading frame (PCR product obtained with the primers furup-F and furup-R) (Table 1) was cloned into the *Sac*I and *Bam*HI sites of pBluescript SK(+) to produce pSKfurup. In addition, a 528-bp downstream sequence of the *fur* gene, the PCR product of the primers furdown-F and furdown-R (Table 1), was cloned into the *Bam*HI and *Kpn*I sites of pSkfurup, resulting in pSKfurup/down, which contains a DNA fragment with regions adjacent to *fur* but with *fur* deleted. This DNA was cloned into the suicide vector pDM4 (15), yielding pDMΔfur, which was then mobilized from *Escherichia coli* SM10λ*pir* into a rifampin-resistant derivative of the wild-type strain *V. vulnificus* MO6-24/O. The resulting *fur* deletion mutant, HLM101, was then selected.

The *fur*::*luxAB* fusion was also transferred to the parental strain MO6-24/O, or HLM101, and its expression was monitored. The expression pattern of *fur*::*luxAB* in MO6-24/O was basically similar to that in the AR strain (data not shown). The cellular growth of the *V. vulnificus fur* mutant was slightly retarded in the presence of an iron chelator and impaired even under iron-supplemented conditions (i.e., in the absence of 2,2′-dipyridyl). The expression level of *fur*::*luxAB* in the *fur* mutant strain was reduced to 50% of that in the wild-type strain (Fig. 2C). The growth stage-dependent induction of *fur*::*luxAB* expression was, however, maintained in the *fur* mutant strain. The *fur*::*luxAB* activities in the *fur* mutant strain

remained consistently a half or a third of that of the wild type regardless of the presence of an iron chelator in the medium. Of note, no distinct induction of the expression of *fur*::*luxAB* was observed after the addition of 2,2′-dipyridyl to the *fur* mutant strain (Fig. 3C).

Therefore, we found that Fur directly or indirectly autoregulates its own expression in a rather unusual mode other than via RpoS-dependent *fur* expression. *fur* expression decreased significantly when *V. vulnificus* was deficient in Fur protein, though its effect was less than that of the *rpoS* knockout mutation. As expected, *fur* expression was induced by iron depletion in the wild type (Fig. 3), and its induction by iron depletion is retained in the *rpoS* mutant even though its overall expression in this strain is low under any conditions. Interestingly, the induction of *fur* expression under iron-depleted conditions was abolished in a *fur* mutant background (Fig. 3). In this case, Fur seems to serve as a transcriptional activator for its own expression under iron-depleted conditions, which is a different type of regulation from its well-characterized function as a repressor under iron-supplemented conditions. The definition of the nature of this autoregulation requires further investigation to determine whether Fur exerts an effect on its own expression by directly interacting with its own promoter or through interacting with other unidentified factors. A set of genes were previously shown to be positively regulated by Fur in *E. coli*. These included two ferritin genes (*ftnA* and *bfr*), a gene encoding superoxide dismutase, and some genes of the tricarboxylic acid cycle. This positive regulation by Fur was achieved indirectly, by repressing a small RNA, *ryhB*, in the presence of iron (13). Homology searches of the GenBank databases for the nucleotide sequence of the *ryhB* gene of *E. coli* (accession number AF480876) resulted in the identification of two candidate sequences of the *ryhB* gene of *V. vulnificus*. One of these was located in the noncoding region between the genes coding for DNA polymerase I and δ-aminolevulinic acid dehydratase on the chromosome I (accession number AE016800), whereas the other was located between the genes for a methyl-accepting chemotaxis protein and a periplasmic protein of the ABC-type phosphate transport system on chromosome II (accession number AE016813). Further study will be focused on the in vitro analysis of the *fur* promoter-Fur interaction as well as on the functional analysis of these *ryhB*-homologous genes.

Expression of the *hupA*::*luxAB* fusion in *V. vulnificus*. An important question that should be answered is that of the physiological significance of the connection between RpoS and Fur. Therefore, we examined whether RpoS is essential for the proper functioning of Fur with respect to iron control of the *hupA* gene. The *hupA* gene, encoding the heme uptake receptor in *V. vulnificus*, has been identified as a Fur-regulated gene, which is induced by the addition of an iron chelator (12). We constructed a transcriptional fusion between the promoter region of *hupA* and the *luxAB* gene and used this to examine the dependency of *hupA* expression on Fur and RpoS regulators. An upstream sequence of the *hupA* gene was cloned from the genomic DNA of *V. vulnificus* by using the primers hupAp-F and hupAp-R (Table 1) (12) (GenBank database accession number AF047484). A PCR-amplified *hupA* DNA product containing a 26-bp HupA coding region and a 254-bp promoter region was used to generate a *hupA*::*luxAB* fusion, pHL02. In wild-type *V. vulnificus*, the activities of *hupA*::*luxAB* were comparatively low in the absence of 2,2′-dipyridyl but were dramatically induced in the presence of the iron chelator (Fig. 4). The same pattern of induction of *hupA*::*luxAB* under iron-depleted conditions was also observed in the *rpoS* mutant strain. This result implies that even though less than 10% of Fur protein is present in the *rpoS* mutant cells relative to the wild type, this level of Fur protein is enough to properly repress the *hupA* gene under iron-supplemented conditions. In the *fur* mutant of *V. vulnificus*, the activities of *hupA*::*luxAB* were significantly increased (more than 100-fold) under iron-supplemented conditions (data not shown). Thus, the effect of iron on *hupA* expression is mediated by Fur, as was previously observed by using a different method (12).

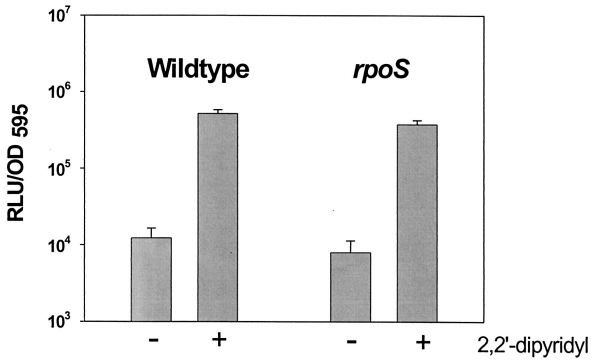

FIG. 4. Effect of iron on *hupA*::*luxAB* (pHL02) expression in the wild type (AR) and in the *rpoS* mutant. Iron in the medium was depleted by adding 0.2 mM 2,2′-dipyridyl. The *hupA*::*luxAB* activities were normalized by dividing the number of relative light units (RLU) by the OD_{595}. The *hupA*::*luxAB* activities of four independent cultures at the exponential phase (OD_{595} = ~0.5) were averaged and are indicated, with their standard deviations.

Microorganisms adapt to various stresses by modulating the expressions of a series of genes in appropriate amounts and in a timely fashion. RpoS and Fur are key transcription factors which play roles in survival during the stationary phase and in adaptation to limited iron levels, respectively. Extensive investigations have been performed on the molecular mechanisms of these regulatory proteins. However, no relationship between these two regulons has been reported. As a result of an attempt to identify target genes regulated by RpoS in *V. vulnificus*, Fur was discovered to be a member of the RpoS regulon. The dependency of Fur production on RpoS was confirmed by two independent methods, proteome and gene fusion analyses. In particular, the expression of *fur* was found to be significantly impaired when *V. vulnificus* was deficient in RpoS, implying that RpoS is involved in Fur synthesis in an unidentified way. Attempting to uncover the molecular relationship between RpoS and Fur regulons becomes complicated, since the expression of *rpoS* is also controlled by iron concentration (K.-J. Park and K.-H. Lee, unpublished results). Thus, the RpoS-dependent expression of the *fur* gene we observed in this study may be a net expression of the reciprocal actions of RpoS and Fur. In addition, Fur and RpoS may control the expression of *fur* gene in different manners which depend on the physiological conditions of the bacterial culture. For example, RpoS and Fur may function cooperatively in the *fur* regulation of *V. vulnificus* in the stationary phase, whereas Fur may be the sole modulator of *fur* expression under iron-depleted conditions.

In this study, RpoS was identified, for the first time, as a major factor in the expression of *fur* in *V. vulnificus*. However, little is understood at present regarding the physiological significance of RpoS-dependent *fur* expression. Future investigations should focus on defining this connection between iron control (Fur regulon) and survival control (RpoS regulon).

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