Comprehensive analysis of iron-regulated genome expression in environmental bacterium

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Research aims

Iron is an essential element for the growth of almost all bacterial species, although most inhabit environments where the solubility of iron is very low. Bacteria have therefore evolved numerous mechanisms to efficiently acquire iron (and molecules containing iron) under limiting conditions. However, a high concentration of intracellular free iron is detrimental to bacteria because of the generation of highly toxic reactive oxygen and reactive nitrogen species (ROS and RNS). Bacteria have therefore evolved sophisticated systems to enzymatically remove ROS and RNS, as well as systems to maintain strict intracellular free iron homeostasis. *Burkholderia multivorans* ATCC 17616 is a soil-derived bacterial strain that has an extraordinary metabolic versatility and has been used to produce many industrially important enzymes[1)]. Our previous characterization of a *fur* ATCC 17616 deletant strain revealed that that the Fur protein is a global transcription regulator that is pleiotropically involved in free iron homeostasis. Consequently, it is also involved in the removal of ROS, tolerance to RNS, catabolism of TCA-cycle and aromatic carbon compounds, resistance to antibiotics, and survival in soil (Fig. 1)[2,3)]. To gain a greater insight into the pleiotropic role of the Fur protein of ATCC 17616, spontaneous suppressor mutants of the ATCC 17616 *fur* deletant that exhibit restored RNS tolerance were analyzed[4)]. RNS suppression led to the restoration of phenotypes almost identical to that of the wild-type strain (Table 1). One such suppressor mutant possessed a mutation in the *oxyR* gene, a transcriptional regulator that modulates the adaptive response to ROS/RNS stress. The *oxyR* mutation led to overproduction of ROS/RNS-removing enzymes in the *fur* deletant, explaining the suppression of ROS/RNS-sensitive phenotypes that are normally exhibited by *fur*

mutants. These results demonstrate the genetic interactions between Fur and OxyR function[4]. Since this previous study indicated that the analysis of suppressor mutations in the *fur* mutant is a viable strategy for demonstrating the transduction system of iron, we performed a study to identify and characterize additional gene(s) whose mutation(s) suppress the pleiotropic phenotypes of the *fur* deletant.

Methods

Molecular genetic analysis systems developed by our group, in concert with various well-established molecular biological and bacterial cultivation methods, were employed in this study [2-4]. Illumina re-sequencing was used to identify suppressor mutations and to quantitatively determine transcriptional start sites (TSS analysis) of the ATCC 17616 Δ*fur* and its derivative genomes. Homology analysis of DNA and protein sequences was performed using NCBI databases. More detailed analysis, when required, was performed using our custom computer software
(http://www.ige.tohoku.ac.jp/joho/labhome/tool.html).

Results

After examination, five suppressor mutants retaining the wild-type *oxyR* gene all possessed the same point mutations in a gene encoding a protein with 81 amino-acid residues. This protein showed 53% identity with a functionally unknown *Yersinia enterocolitica* protein, HemP, which is encoded by the first gene in a *hemPRSTUV* operon. Although the exact function of HemP is not known, the *hemPRSTUV* region contains genes responsible for the uptake and usage of heme (Fig. 2). Therefore, the identified ATCC 17616 gene was also designated *hemP*. We confirmed that the introduction of a *hemP* deletion in the Δ*fur* mutant gave rise to phenotypes indistinguishable from those of the original suppressor mutants. The ATCC 17616 *hemP* gene is located on the first chromosome, separated from the second chromosome-loaded *hemRSTUV* operon. This second region is also predicted to contain genes involved in the uptake and usage of heme. Transcription of *hemP* in ATCC 17616 was repressed and induced under free iron-replete and -depleted conditions, respectively. The transcription of *hemP* strain was higher in the Δ*fur* under both conditions. Genome-wide TSS analysis showed that *hemP* is one of the most transcribed 20 genes in the Δ*fur* genome. Upstream of the *hemP* promoter region is a putative Fur box, a consensus sequence that specifically binds the Fur protein. Our genetic analysis revealed that this putative box is

indeed able to specifically bind the Fur protein, demonstrating that *hemP* transcription is directly suppressed by the Fur-Fe^{2+} complex. Transcription of *hemP* was also induced by heme present in the culture medium. The *hemP* deletant of ACTT 17616 grew normally in Fe^{3+}-containing medium but was unable to grow in medium containing heme as the only source of iron (heme medium). This revealed the importance of the HemP protein for the use of heme as an iron source. Our biochemical analysis also indicated that HemP protein can bind heme and that HemP is located in the cytoplasm.
The ATCC 17616 *hemR* gene that putatively encodes an outer membrane receptor specific for heme also possesses the Fur box upstream of the promoter. Transcription of *hemR* was (i) repressed under iron-replete conditions by the direct binding of Fur-Fe^{2+} complex at the Fur box, and (ii) induced by heme in the culture medium. HemP was also required for the heme-dependent transcriptional induction of *hemR*. The growth rate of the ATCC 17616 *hemR* deletant was significantly lower in heme medium compared to Fe^{3+}-containing medium, indicating the importance of ATCC 17616 HemR for heme usage.

Conclusions

This study showed that (i) *B. multivorans* ATCC 17616 is capable of utilizing heme as a sole source of iron, (ii) *hemP* and *hemR* gene products are involved in heme usage, (iii) transcription of both genes under iron-replete conditions is directly repressed by Fur, and (iv) transcription of *hemR* requires *hemP*. Further transcriptional analysis of the two genes (i) indicated the preferential usage of free iron rather than heme, and (ii) led to the proposal of a working model in which heme in the culture medium induces *hemP* transcription. The resulting gene product either directly or indirectly induces *hemR* transcription. It remains unclear whether (i) HemP directly regulates *hemR* transcription by binding to specific sequence(s) around *hemR*, (ii) if HemP indirectly regulates *hemR* transcription by interacting with unidentified gene products or by regulating the transcription of such genes, or (iii) if Fur and HemP (or a further unidentified gene product) compete for binding to the *hemR*-containing region. These unresolved points will be clarified in future studies. For example, genome-wide SELEX analysis using the HemP protein and RNA-seq analysis with the *hemP* mutant. These analyses will also provide important information concerning the molecular mechanisms for how addition of *hemP* mutations to the Δ*fur* mutant leads to phenotypes very close to the wild-type strain. Clarification of such mechanisms will contribute to

understanding a wide variety of bacterial iron signal transduction systems.

References

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Table 1. Phenotypes of *B. multivorans* ATCC 17616 wild-type strain and its derivatives

Phenotype	Wild-type	*Δfur* deletant	Suppressor mutants	
			Δ*fur*Δ*oxyR*	Δ*fur* + *hemP* mutation
ROS sensitivity	Normal	Increased	Normal	Normal
RNS sensitivity	Normal	Increased	Normal	Normal
Fe^{3+} uptake	Normal	Increased	Increased	Increased
Intracellular free iron	Normal	Increased	Normal	Normal
Utilization of citrate	Normal	Decreased	Normal	Normal
Utilization of succinate	Normal	Decreased	Normal	Normal

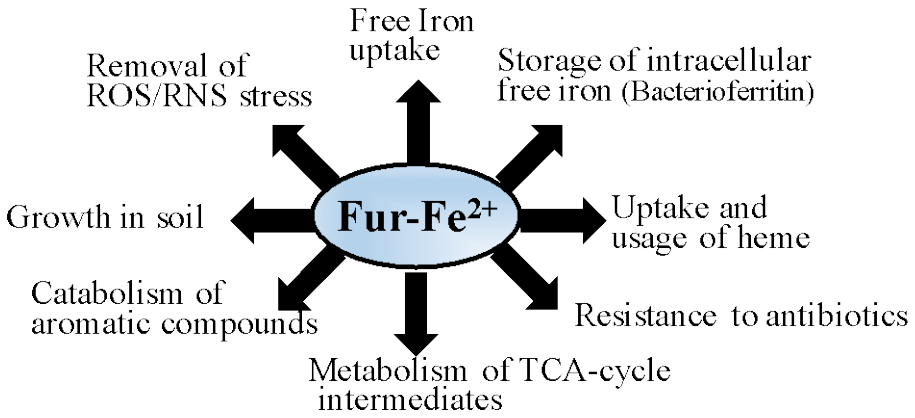

Fig. 1. Functional pleiotropy of the *B. multivorans* ATCC 17616 Fur protein

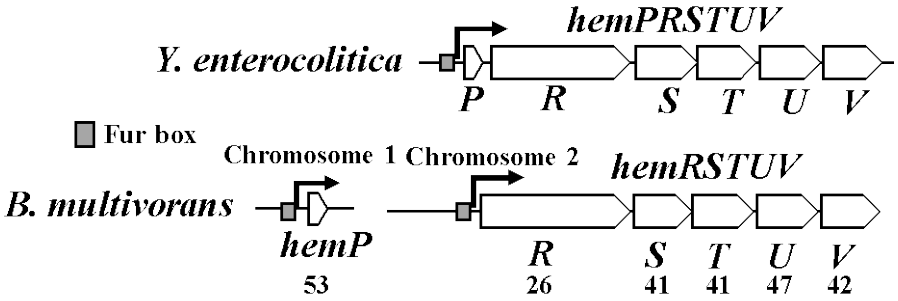

Fig. 2. Organization of genes for uptake and usage of heme. Arrow indicates the transcriptional start site and direction. Numerals show the percent identity of each gene product with the corresponding product of *Y. enterocolitica*. Function of *Y. enterocolitica* gene: P, unknown; R, outer-membrane heme receptor; S, heme monooxygenase; and T, U, and V, ABC transporter.