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Original Article

A phosphoporin homolog plays a critical role in the dissimilatory iron-respiration linked to iron (III) reduction by a cold-adapted bacterium, *Shewanella livingstonensis* Ac10

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Summary

Under anaerobic conditions, some bacteria use various metal compounds as terminal electron acceptors, and can thus obtain energy for their growth by respiration in the absence of oxygen. Molecular mechanism of anaerobic metal reduction by the mesophilic *Shewanella* and *Geobacter* strains, model organisms of bacterial dissimilatory metal reduction, has been well studied so far. In this study, we focused on the iron respiration by an Antarctic cold-adapted bacterium, *Shewanella livingstonensis* Ac10, which can grow at a temperature range of 0℃–25℃, and has a potential application in the biological reduction of metal contaminants under cold environments. In the presence of iron (III) citrate, *S. livingstonensis* Ac10 inducibly produced an outer membrane phosphoporin homolog, PhoE, but not in the fumarate-containing medium. By western blotting and real-time RT-PCR analyses, it was confirmed that the production of PhoE was dependent on the presence of iron (III) citrate, and the expression of *phoE* was regulated at the transcriptional level. When the gene coding for PhoE in *S. livingstonensis* Ac10 was disrupted, the cell proliferation and iron (II) ion (Fe^{2+}) production by the mutant strain grown in the presence of iron (III) citrate was significantly reduced than that by the wild-type strain. Introduction of an expression vector for *phoE* into the *phoE*-disrupted mutant restored the growth defect of the mutant, revealing that a phosphoporin homolog plays a critical role in the dissimilatory iron reduction by *S. livingstonensis* Ac10.

Introduction

Microbial metal reduction is a key reaction for the bioconversion of soluble and insoluble minerals. Respiration-linked bioreduction of metal compounds is useful for bacterial leaching and bacterial mining, and is also gaining attention as an efficient method for the conversion of toxic metal contaminants to non-toxic compounds[1]. Recent studies on the metal-reducing microorganisms revealed that several bacteria are capable of dissimilatory metal reduction and respiratory growth with various metals such as iron, copper, manganese, cobalt, chromium, uranium, selenium, and technetium as the terminal electron acceptor. Two such microorganisms that have received much attention are the metal-reducing bacteria, *Shewanella oneidensis*[2] and *Geobacter sulfurreducens*[3]. Both these strains are classified as mesophilic bacteria, for which the optimum growth temperatures are reported to be around 30℃.

Shewanella species have evolved mechanisms for utilizing insoluble metals as electron acceptors, and can survive in various aerobic and anaerobic habitats, such as deep sea, intestinal tract of fish, freshwater, and coastal sediments. Regarding anaerobic metal reduction by *S. oneidensis* MR-1, a well-studied model for bacterial metal respiration, three possible mechanisms are suggested: (i) direct electron transfer following contact between minerals and multihaem *c*-type cytochromes on the cell surface[4–6] or extracellular nanowires[7], (ii) delivery of the reducing equivalents from the cytoplasmic membrane to the extracellular matrix by the electron shuttles such as flavin[8, 9], (iii) solubilization of metal oxides with metal chelators[10, 11] such as menaquinone so that they can contact with the membrane-associated electron transport components. Although these electron transport mechanisms are supposed to contribute to the survivability of *Shewanella* species at various anaerobic environments, the exact mechanism for respiration-linked metal reduction at cold environments widely distributed across the Earth is not well understood.

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Cold-adapted bacteria belonging to the *Shewanella* genus have been isolated from various cold environments. For example, *Shewanella livingstonensis* Ac10 was isolated from the Antarctic seawater[12]. Cold-adapted *Shewanella* species could be important for understanding the biogeochemical processes occurring in cold environments that are widely distributed in nature[13]. At low temperatures, the diffusion rate of hydrophilic nutrients and respiratory substrates is decreased as the result of the low diffusion coefficient and high water viscosity, and the efficiency of enzymatic reaction and membrane fluidity are also decreased. Under such unfavorable conditions, the bacteria, which evolve to adapt to cold environments, are required to acquire unique systems for their anaerobic growth. To elucidate the molecular mechanism of anaerobic respiration-linked iron reduction by *S. livingstonensis* Ac10 at cold environments, we attempted to identify proteins inducibly produced in the presence of iron (III) citrate in this study. By the construction of a gene-disrupted mutant of an iron-inducible protein and characterization of the anaerobic growth of the mutant, we found that a homolog of the phosphate-selective outer-membrane porin, PhoE, was an iron (III)-inducible protein and played a critical role in the anaerobic respiration of *S. livingstonensis* Ac10.

Materials and methods

Bacterial strains, plasmids, and growth conditions

S. livingstonensis Ac10 isolated from Antarctic seawater was used as the parent strain for gene disruption[14]. *S. livingstonensis* Ac10 and its derivatives were cultured in Luria–Bertani (LB) medium supplemented with rifampin (50 μg/mL) and kanamycin (30 μg/mL) when necessary. *Escherichia coli* S17-1/λ_{pir}[15] was used as a donor cell for conjugative transformation and construction of a *phoE*-deletion plasmid, and was grown in LB medium supplemented with kanamycin (30 μg/mL) at 37℃.

For cultivation under anaerobic conditions, defined medium (DM) was used. The basal part of DM contained the following ingredients (per liter): 0.68 g NaCl, 0.285 g $MgCl_2 \cdot 6H_2O$, 56.6 mg $CaCl_2 \cdot 2H_2O$, 0.398 g Na_2SO_4, 0.15 g NH_4Cl, 12.5 mg $Na_2HPO_4 \cdot 12H_2O$, 0.3 g KCl, 20 mg L-glutamate, 20 mg L-serine, 3.5 g 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, and 20 mg L-arginine HCl (Wako Pure Chemical Industries, Osaka, Japan). All the chemicals except L-arginine HCl were purchased from Nacalai Tesque (Kyoto, Japan).

Cells were grown in DM with the following supplements (per liter): 10 mL trace minerals, 0.2 g yeast extract, 0.1 g peptone (Difco, Sparks, MD, USA), and 5.67 mL sodium D, L-lactate (Wako Pure Chemical Industries) as the carbon source and electron donor. The trace mineral solution contained 1 mM NaCl, 0.054 mM $FeSO_4$, 0.5 mM $CoSO_4$, 0.5 mM $Ni(NH_4)_2(SO_4)_2$, 0.39 mM Na_2MoO_4, 0.15 mM Na_2SeO_4, 1.3 mM $MnSO_4$, 0.1 mM $ZnSO_4$, and 0.02 mM $CuSO_4$. As electron acceptors, 15 mM iron (III) citrate (Sigma, St. Louis, MO, USA) and 15 mM fumarate were used for the anaerobic growth of *S. livingstonensis* Ac10. The pH of the medium was adjusted to 7.5. All the chemicals, whose manufacturers are not specified above, were purchased from Nacalai Tesque.

The cells were inoculated into 5 mL LB and grown at 18℃ for 24 hours on a reciprocal shaker at 180 rpm (Taitec, Koshigaya, Japan, BR-33FL). Fifty microliters of the resultant culture were inoculated into 5 mL LB, and the cells were cultivated at 18℃ for 12 hours at 180 rpm until the absorbance at 600 nm reached to about 0.6. Then, the cells were washed twice with DM without the electron accepter, and were suspended in 5 mL DM without the electron acceptor. Cell suspensions were then incubated at 18℃ for 12 hours to acclimatize the cells to the DM. Then, the suspensions were transferred (0.01% inoculation) to 40 mL of DM containing one of the electron acceptors, as indicated above in an anaerobic glove chamber, model MDB-1KP-KKHSR (Miwa MFG Co. Ltd., Osaka, Japan). The bacterial strains and plasmids used in this study are listed in Table 1.

Construction of *phoE*-disrupted mutant, *phoE*-complementation plasmid, and *phoE*-expression plasmid

The bacterial strains, plasmids, and primers used in this study are listed in Table 1 and Table 2. The *phoE* gene was disrupted by the integration of a gene-deletion plasmid, pKNOCK-Km^r, as previously reported[15]. Briefly, an internal fragment of *phoE* was amplified from *S. livingstonensis* Ac10 genomic DNA by PCR with the primers, dphoE F and dphoE R, which are listed in Table 2. The PCR product was digested with PstI/KpnI and cloned into identical restriction sites of pKNOCK-Km^r to generate a plasmid for knockout gene transfer as described previously[14]. A single crossover recombinant was selected from the LB plates containing kanamycin (30 μg/mL) and rifampin (50 μg/mL). The insertion of the plasmid into the chromosomal DNA was confirmed by sequencing analysis.

The construction of the *phoE*-complementation vector, pNat*phoE*, was carried out as follows: A linear fragment of pJRD215-Cm^r[16] was prepared by PCR with the primers, pJRD Cm^r F and pJRD Cm^r R. The *phoE* gene with its 5' upstream region including the predicted promoter was

Table 1 Strains and plasmids used in this study

Strains	Descriptions	Ref.
Escherichia coli		
S17-1/λ_{pir}	S17-1 derivative, host for *pir*-dependent plasmids	15
BL21(DE3)	Host for expression of target protein	
Shewanella livingstonensis Ac10		
Ac10-Rif^r	Parent strain, rifampin-resistant mutant of Ac10	14
Δ*phoE*	Rif^r *phoE*::pKNOCK-Km^r	This work
Δ*phoE*/pJRD-Cm^r	Rif^r *phoE*::pKNOCK-Km^r, containing pJRD214-Cm^r	This work
Δ*phoE*/*phoE* pJRD-Cm^r	Rif^r *phoE*::pKNOCK-Km^r, containing pJRD215-Cm^r, native promoter and *phoE*	This work
Plasmids		
pKNOCK-Km^r	RP4 *oriT* and R6K *γ-ori*; Km^r	15
pJRD215-Cm^r	A broad-host-range vector	16
pNat*phoE*	pJRD215-Cm^r harboring *phoE* and its native promoter	This work
pET21a_phoE^{His}	pET21a harboring *phoE*-hexa-His	This work

Table 2 Primers used in this study

Primer	Sequence (5'→ 3')	Target gene and plasmid
Used for real-time RT-PCR		
phoE F	CGGCGCGGTAAACGGTGTC	*phoE*
phoE R	GCCGACGTTGAACTTGTTGCCC	
16S F	GGCAGGCCTAACACATGCAA	16S rDNA
16S R	GGCAGATCCCTAGGCATTACT	
Used for gene disruption and gene complementation		
dphoE F	AAAACTGCAGAAAGCCCGTAACCAGTTTGT	*phoE*
dphoE R	CGGGGTACCCGTGCTTAATACGAAGCCATC	
Used for gene complementation		
pJRD215 Cm^r F	TAGTATAGTCTATAGTCCGTGG	pJRD215 Cm^r
pJRD215 Cm^r R	CGTAATCCATGGATCAAGAG	
NatphoE F	CTCTTGATCCATGGATTACGGTGACACAGAGTAAGTGAC	*phoE*
NatphoE R	ACGGACTATAGACTATACTATTAAAATTTGTGCTCTAAACCTAC	
Used for expression in *E. coli*		
pET phoE F	CCGGAATTCATGTACGGCAAGTTAAATGTAACAG	*phoE*
pET phoE his R	CCGCTCGAGTTAATGATGATGATGATGATGAAATTTGTGCTCTAAACCTACACC	

Underlined bases indicate the recognition sites for restriction enzymes

amplified by PCR with the primers, NatphoE F and NatphoE R, and introduced into pJRD215-Cm^r plasmid by using the NEBuilder HiFi DNA Assembly Cloning System (New England Biolabs, Japan, Inc., Tokyo, Japan) kit to generate pNat*pho*E. The purified plasmid was then introduced into *E. coli* S17-1/λ_{pir} cells for conjugation and replication. The transformant of *S. livingstonensis* Ac10 harboring the *phoE*-complementation plasmid was selected from a chloramphenicol (30 μg/mL) LB plate.

The gene coding for His-tagged *phoE* was amplified from *S. livingstonensis* Ac10 genomic DNA with the primers, pET phoE F and pET phoE his R. PCR fragments and the vector pET21a were digested with EcoRI (Takara Bio Inc., Kusatsu, Japan) and XhoI (Takara Bio Inc). The digested products were ligated by using Ligation high Ver. 2 (TOYOBO, Osaka, Japan) kit. Then the ligated DNA was introduced into the *E. coli* BL21 (DE3) plasmid. Transformants harboring the *phoE*-expression plasmid (pET21a_phoE^{His}) were selected from an ampicillin (100 μg/mL) LB plate.

Expression and purification of His-tagged PhoE

His-tagged PhoE was purified from the *E. coli* BL21 (DE3) carrying plasmid, pET21a_phoE^{His} as the inclusion body. The inclusion body was dissolved in 50 mM KPB (pH 8.0), 300 mM NaCl, and 8 M urea. Solubilized PhoE was purified by using Ni-NTA resin. Purified protein fractions were resolved on a 12.5% SDS-polyacrylamide

gel (PAGE) and stained with Coomassie Brilliant Blue R250 dye. The band of PhoE (34 kD) was excised from the gel, put into a dialysis membrane, and extracted by using SDS-PAGE electrophoresis tank. The buffer solution was changed to phosphate-buffered saline, PBS (pH 7.4). Protein solution was concentrated by using Amicon Ultra filters (Merck Millipore Co., Darmstadt, Germany) with a 10 kD molecular weight cut-off marker.

Production of an antibody raised against PhoE

A monoclonal antibody raised against His-tagged PhoE of *S. livingstonensis* Ac10 was prepared by using the rat hybridoma technology[17]. The pellet of the mixture of the rat lymph node cells and P3X63Ag8 cells[18] was suspended in a 50 mL hybridoma selection medium consisting of RPMI 1640 (Nacalai Tesque) supplemented with 2% HAT supplement (Thermo Fisher Scientific, San Jose, CA, USA), 10% fetal bovine serum (Serum Source International, Charlotte, North Carolina, USA), 0.1 mg/mL gentamicin (Nacalai Tesque), and 1% Glutamax I (Thermo Fisher Scientific, San Jose, CA, USA) and plated on 96-well tissue culture plates (100 μL/well). After cell fusion, the culture supernatants were collected and screened by an enzyme-linked immunosorbent assay (ELISA). The hybridomas that gave a positive signal against the purified recombinant PhoE were cloned, and the culture supernatants were collected as an anti-PhoE antibody.

Western blotting analysis

Samples for western blotting analysis were prepared as follows: *S. livingstonensis* Ac10 was cultivated anaerobically in DM containing 15 mM iron (III) citrate or fumarate at 18℃ for 5 days. Whole cells were disrupted by an ultrasonic homogenizer (Digital Sonifier, Branson, CT, USA). For positive control, the purified insoluble His-tagged PhoE was used. His-tagged PhoE was detected by the anti-PhoE-His monoclonal antibody as the primary antibody and the peroxidase-conjugated goat anti-rat antibody (Sigma) as the secondary antibody. Chemi-lumi One Super (Nacalai Tesque) system was used for visualization of the bands. The signals were captured by C-Digit Blot Scanner (LI-COR Biosciences, Lincoln, NB, USA).

Analysis of transcriptional level of *phoE*

S. livingstonensis Ac10 was grown in DM with 15 mM iron (III) citrate or fumarate at 18℃ for 72 hours. The total RNA was extracted by using the RNeasy kit (Qiagen, Valencia, CA, USA). The concentration of the transcripts of *phoE* was assessed by using the primers, phoE F and phoE R, and the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA).

Growth curve and determination of ferrous concentration

During 14 or 15 days, cells grown under anaerobic conditions were collected, and stained with DAPI (4', 6-diamidino-2-phenylindole). The number of DAPI-stained cells was determined by using a bacterial counter (ERMA, Tokyo, Japan) and fluorescence microscopy (E6F-RFL equipped with 100X oil objective; Plan Fluor, Nikon, Tokyo, Japan).

The ferrous concentration was measured by using 1,10-phenanthroline as previously described by Herrera, L., *et al*[19]. The complex formation between iron (II) and 1,10-phenanthroline was quantitated by measuring its absorption at 510 nm by a SpectraMax 190 Microplate Reader, (Molecular Devices, Sunnyvale, CA, USA). Briefly, one hundred microliters of the culture were mixed with 4.9 mL of 0.5 N HCl. After 5 minutes of incubation, the mixture was centrifuged at 20,000×*g* for 1 minute, and 100 μL of clear supernatant was collected as a sample. Ferrous concentrations were determined based on a standard curve generated from the known concentrations.

Results

Iron-dependent production of a homolog of a phosphate-selective porin, PhoE

To elucidate the iron-respiration mechanism of the Antarctic cold-adapted bacterium, *S. livingstonensis* Ac10, we performed the proteomic analysis of this strain grown in the presence of different electron acceptors, fumarate and iron (III) citrate, and identified an outer membrane porin homolog, PhoE, as one of the iron-inducible proteins under anaerobic conditions (data not shown). In this study, we focused on the expression and physiological function of PhoE in the iron-respiration of *S. livingstonensis* Ac10. To confirm the iron-dependent production of PhoE by *S. livingstonensis* Ac10, western blotting was carried out with an anti-PhoE monoclonal antibody. The wild-type strain grown anaerobically in the presence of fumarate or iron (III) citrate for 5 days at 18℃, the optimum temperature of this strain, was obtained. In the presence of iron (III) citrate, a higher intensity band for PhoE was detected than that grown with 15 mM fumarate (Fig. 1A).

To check whether the expression of PhoE was induced by iron (III) citrate at the transcriptional level in *S. livingstonensis* Ac10, the amount of *phoE* mRNA in the cells grown anaerobically in the presence of fumarate and iron (III) citrate was determined by real-time RT-PCR. In the presence of iron (III) citrate, the amount of *phoE*

mRNA was 3.3 fold higher than that of the cells grown with fumarate, indicating that the expression of *phoE* was regulated at the transcriptional level depending on the presence of iron (III) citrate (Fig. 1B).

Anaerobic growth of *phoE*-disrupted mutant of *S. livingstonensis* Ac10

To elucidate the physiological function of PhoE in the iron respiration by *S. livingstonensis* Ac10, a *phoE*-disrupted strain (Δ*phoE*) was constructed by integration of a knock out plasmid into the *phoE* gene. Wild-type *S. livingstonensis* Ac10 and Δ*phoE* were cultivated anaerobically in DM containing yeast extract, peptone, trace mineral solutions, sodium D, L-lactate, and electron acceptors, fumarate or iron (III) citrate at a concentration of 15 mM. Δ*phoE* normally grew in the presence of fumarate (Fig. 2A). When grown in the presence of iron (III) citrate, Δ*phoE* showed growth retardation (Fig. 2B), and the cell number of this mutant was about 10% to that of the wild-type strain after the incubation period. Under the same conditions, the concentration of Fe^{2+} produced by the wild-type strain and Δ*phoE* was measured by using a chelating reagent for the bivalent cations, 1,10-phenanthroline (Fig. 2C). The wild-type strain produced Fe^{2+} as the cells increased in number, and after 15 days of anaerobic incubation, the concentration of Fe^{2+} reached to the maximum at about 15 mM. During the tested period, the production of Fe^{2+} by Δ*phoE* was lower than that by the wild-type strain, and was less than 5 mM after 15 days of incubation.

Change in phosphate concentration does not affect the anaerobic growth of Δ*phoE*

In *E. coli* and *Pseudomonas aeruginosa*, phosphoporin is inducibly produced under phosphate starvation conditions and enhances the uptake of phosphate ion ($H_2PO_4^{2-}$, HPO_4^{2-}, PO_4^{3-}) selectively[20, 21]. It is considered that iron (III) ions and phosphate form an insoluble iron (III) phosphate metal complex in the presence of both 15 mM iron (III) citrate and 0.03 mM phosphate. Therefore, our results raise concerns that during the anaerobic growth of

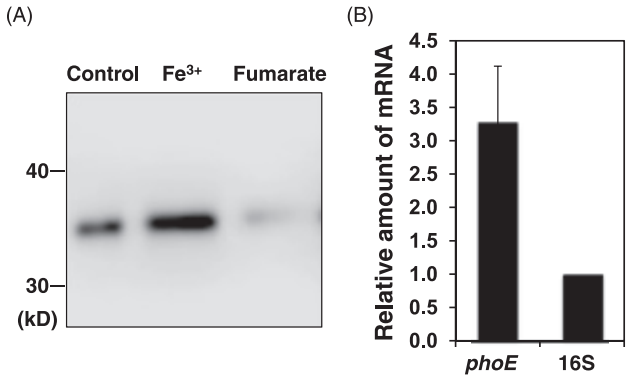

Fig. 1 Iron-dependent production of PhoE
(A) Western blotting analysis of PhoE of *S. livingstonensis* Ac10. The cells were grown anaerobically with an electron acceptor, 15 mM fumarate or 15 mM iron (III) citrate. Whole cell samples were resolved on an SDS-PAGE, and PhoE was detected by using an anti-PhoE antibody. Purified PhoE-His was used as a positive control. (B) Transcriptional levels of the gene coding for *phoE*. The ratio of the amounts of mRNA of the cells grown in DM containing 15 mM fumarate or 15 mM iron (III) citrate is shown. The value was normalized with the ratio of the amounts of 16S rRNA in the cells grown at the same conditions. Error bar represents the standard deviation (SD) of three replicates.

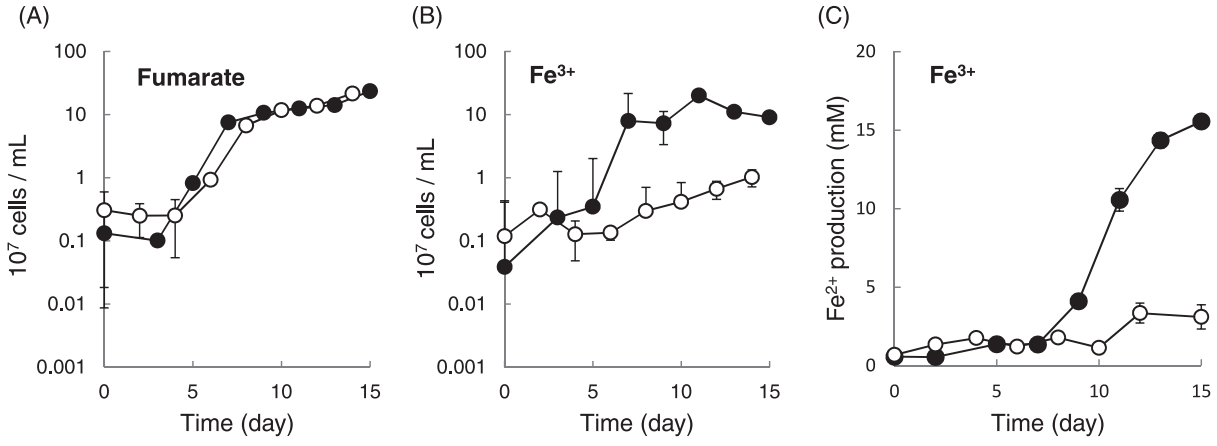

Fig. 2 Growth characterization of the *phoE*-disrupted mutant
The cell number of the wild-type strain (filled circle) and Δ*phoE* (open circle) grown anaerobically with fumarate (A) or iron (III) citrate (B) was measured. Iron-reducing activity of the wild-type strain (filled circle) and Δ*phoE* (open circle) grown anaerobically with ferric citrate was determined by using a chelating reagent, 1,10-phenanthroline (C). Error bar represents the SD of three replicates.

S. livingstonensis Ac10, phosphate starvation caused by the formation of iron (III) phosphate from iron (III) citrate and phosphate induces the expression of *phoE*. To clearly reveal the physiological role of PhoE in the iron respiration of *S. livingstonensis* Ac10, the wild-type strain and Δ*phoE* were cultivated anaerobically with excess amounts of inorganic phosphate. When fumarate was used as an electron acceptor, the excess amount of phosphate (15 mM and 150 mM) did not affect the growth of the wild-type strain and Δ*phoE*, and these strains grew similarly (Fig. 3A and 3D). When iron (III) citrate was used as an electron acceptor, Δ*phoE* showed significant growth retardation compared with that of the wild-type strain, even in the presence of high concentration of phosphate (15 mM and 150 mM) (Fig. 3B and 3E), indicating that the growth retardation of Δ*phoE* was not caused by phosphate starvation, although the effects of phosphate concentration on the growth of these strains should be examined in more detail statistically in future studies. The concentration of Fe^{2+} produced by the wild-type strain reached to the maximum of about 15 mM in about 10 days of incubation in the presence of 15 mM and 150 mM phosphate (Fig. 3C and 3F). The rate of Fe^{2+} production was similar to that observed in the presence of 0.03 mM phosphate (Fig. 2C). The production of Fe^{2+} by Δ*phoE* was lower than that by the wild-type strain, and the amount of Fe^{2+} was less than 1 mM after 15 days of incubation in the presence of 15 mM and 150 mM phosphate (Fig. 3C and 3F). The result was similar to that obtained in the presence of 0.03 mM phosphate (Fig. 2C).

Functional complementation of Δ*phoE*

In various bacteria, the phosphate-responsive gene cluster is conserved, in which genes coding for proteins involved in phosphate uptake, PhoE, and signal transduction during phosphate starvation, PhoB and PhoR, are included[22]. Also in *S. livingstonensis* Ac10, a gene coding for PhoE was found in a gene cluster containing PhoB and PhoR, which is supposed to be a phosphate-responsive PHO cluster conserved in various Gram-negative bacteria. Target gene disruption occasionally affects the expression efficiency of the neighboring genes and the function of

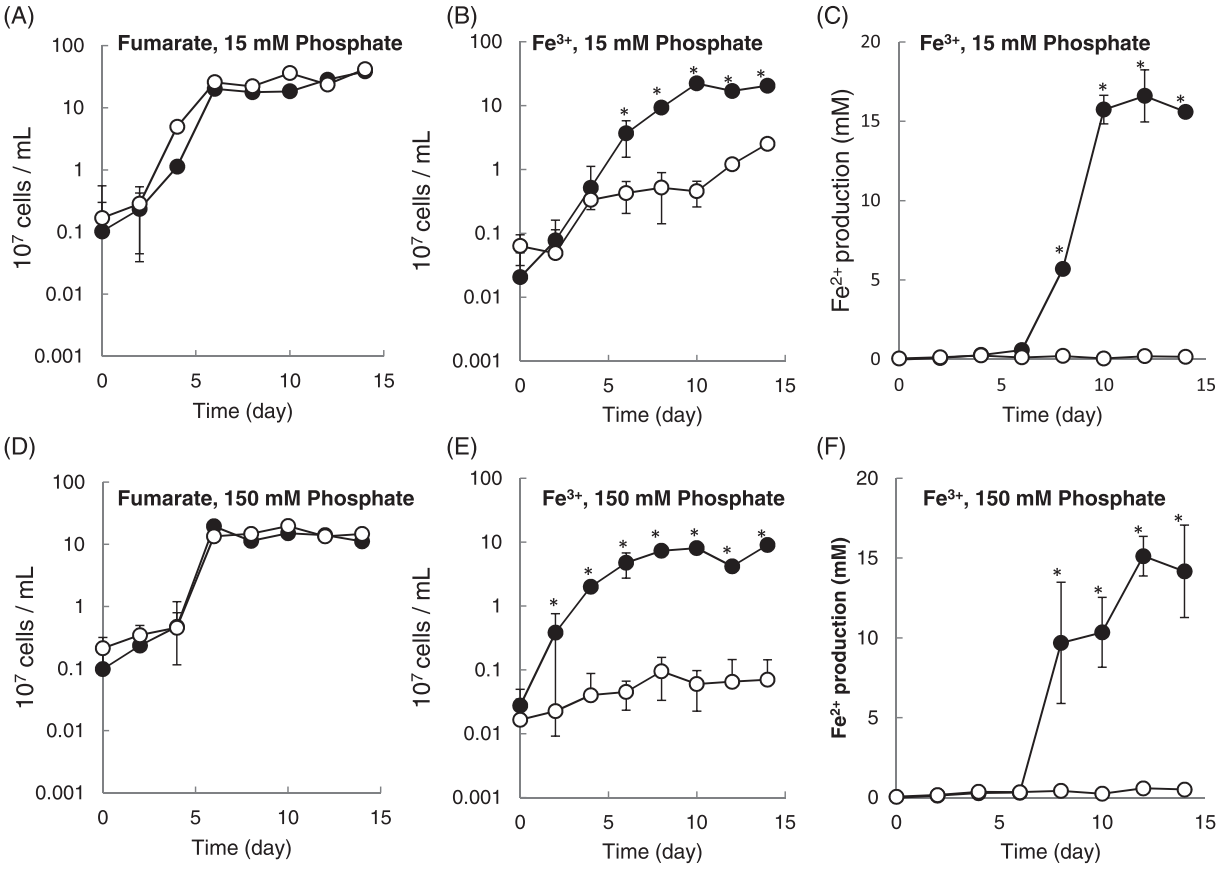

Fig. 3 Effects of phosphate concentration on the anaerobic iron-respiration by Δ*phoE*
Growth of the wild-type strain (filled circle) and Δ*phoE* (open circle) grown anaerobically in DM containing 15 mM and 150 mM of phosphate and fumarate (A and D) or iron (III) citrate (B and E). Iron-reducing activity of the wild-type strain (filled circle) and Δ*phoE* (open circle) grown anaerobically in the presence of iron (III) citrate was determined by using a chelating reagent, 1,10-phenanthroline (C and F). Error bar represents the SD of three replicates. The differences were statistically analyzed by Student's *t*-test. Significant differences at $p < 0.05$ are indicated by asterisks (*).

their gene products. To confirm that the phenotype observed in Δ*phoE* was caused by the loss of PhoE and not by any defect of other genes, a plasmid harboring *phoE* was introduced into Δ*phoE*. A *phoE*-expression plasmid under the control of a *phoE* promoter of *S. livingstonensis* Ac10 was constructed and introduced into Δ*phoE*. Δ*phoE* and the complemented strain were cultivated in DM containing electron acceptors, fumarate, and iron (III) citrate, and different concentrations of phosphate. In the presence of iron (III) citrate, growth retardation and the defect in Fe^{2+} production by Δ*phoE* harboring a control plasmid were observed (Fig. 4A-F). In the presence of iron (III) citrate, the complemented strain grew faster than Δ*phoE*, and similar to the wild-type strain (Fig. 2 and 3), the cell number of the complemented strain reached to the maximum in 5–10 days of incubation. Under the same conditions, Fe^{2+} production was restored by the introduction of a *phoE*-expression vector, and the concentration of Fe^{2+} reached to 10–15 mM during the incubation period (Fig. 4G-I). Changes in the phosphate concentrations of DM did not affect the growth and iron-reducing activity of all the tested strains.

Discussion

S. livingstonensis Ac10, isolated from the Antarctic seawater, is able to grow aerobically at a temperatures range from 4℃ to 25℃. Under anaerobic conditions, this bacterium can grow by reducing iron (III) citrate at 4℃ (data not shown) and 18℃. Therefore, *S. livingstonensis* Ac10 has

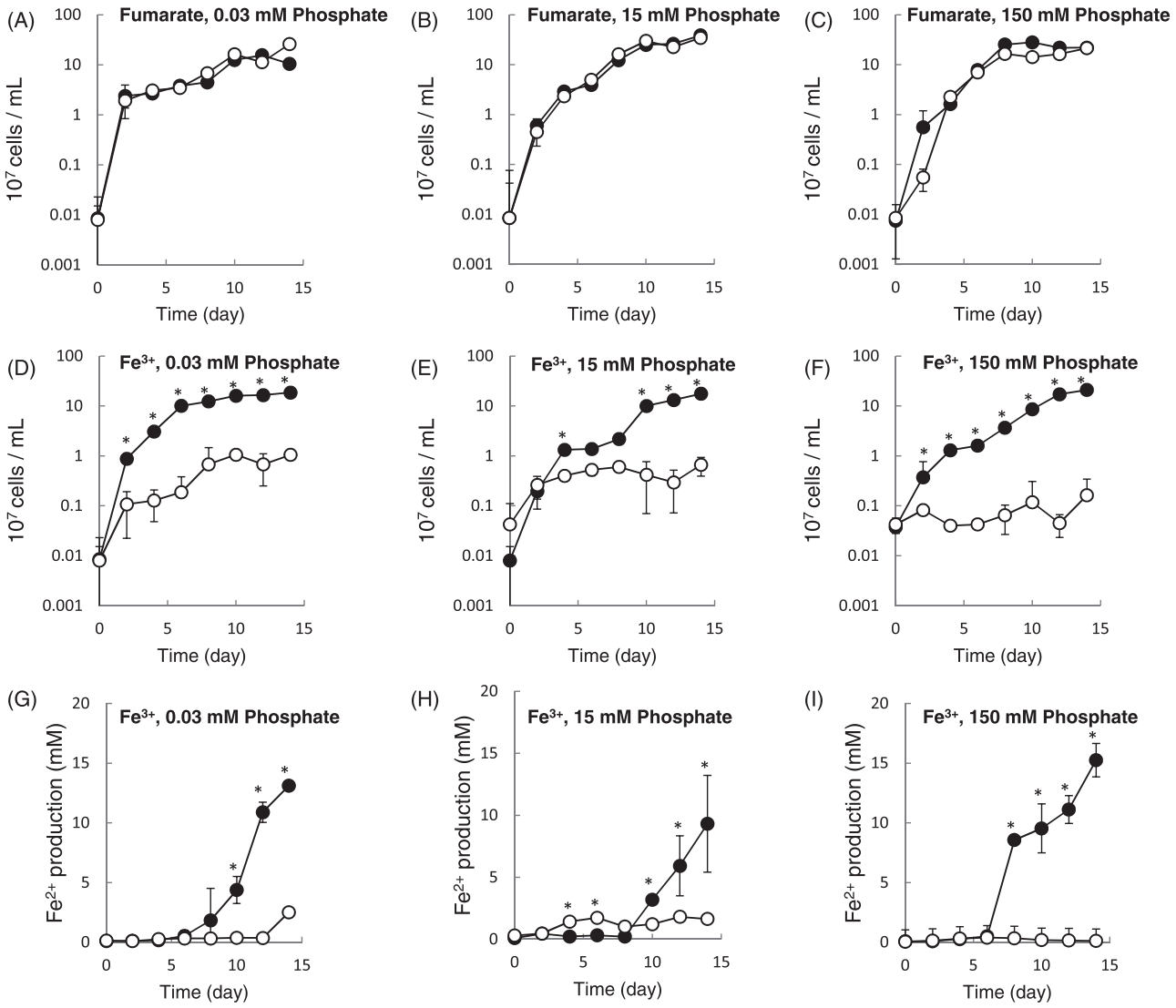

Fig. 4 Growth and iron respiration of the *phoE*-complemented strain
Δ*phoE* harboring an empty plasmid (open circle) and the *phoE*-expression vector (filled circle) were grown anaerobically in DM containing 0.03 mM, 15 mM, and 150 mM of phosphate, and fumarate (A-C) or iron (III) citrate (D-F), respectively. Fe^{2+} production of Δ*phoE* harboring an empty plasmid (open circle) and the *phoE*-expression vector (filled circle) grown anaerobically in the presence of iron (III) citrate is shown in Fig. 4G-I. Error bar represents the SD of three replicates. The differences were statistically analyzed by Student's *t*-test. Significant differences at $p < 0.05$ are indicated by asterisks (*).

potential applications towards biological decontamination of heavy metals in the polluted cold environments. By proteomic study of this strain grown in the presence of iron (III) citrate, a homolog of an outer membrane phosphoporin, PhoE, was identified as one of the iron-inducible proteins (data not shown). PhoE of *S. livingstonensis* Ac10 has 19.9% sequence identity with *E. coli* PhoE (GenBank accession number P02932) and is predicted to be a porin family protein, which forms a transmembrane β-barrel structure at the outer membrane of the Gram-negative bacteria, and functions as an outer membrane passage for hydrophilic solutes[23]. β-barrel porin proteins play essential roles in the nutrient transport and membrane biogenesis[24]. In *E. coli* and other bacteria, PhoE homologs form diffusion channels for hydrophilic substrates during inorganic phosphate starvations and enhance the selective uptake of phosphate ion and other anionic solutes. On the other hand, the involvement of PhoE in bacterial anaerobic growth has not been reported. In this study, we found the iron (III)-dependent production of PhoE by the cold-adapted bacterium, *S. livingstonensis* Ac10, grown at anaerobic conditions, and the expression of PhoE was regulated at the transcriptional level in the presence of iron (III) citrate. These results suggested that PhoE was involved in the iron-respiration linked to iron (III) reduction pathway of *S. livingstonensis* Ac10.

Regarding the bacterial iron uptake, it is well known that many bacteria secrete small iron (III)-binding molecules called siderophores, and the uptake of iron (III)-siderophore complexes is mediated by an outer membrane siderophore transport protein, TonB-dependent receptor. FecA, a homolog of TonB-dependent receptor, has been identified as a citrate-dependent iron transporter, and serves as an iron (III) transporter across the outer membrane, through which iron (III) citrate is provided to the periplasmic and cytoplasmic regions[25]. On the other hand, the involvement of a homolog of phosphoporin in the bacterial iron uptake and the dissimilatory iron reduction at anaerobic conditions has not been reported so far. To demonstrate the physiological role of a phosphoporin homolog, PhoE, in the respiration-linked metal reduction, the PhoE-deleted strain (Δ*phoE*) was generated. Under anaerobic conditions, Δ*phoE* could grow normally in the fumarate-containing media, but not in the presence of iron (III) citrate. Moreover, Δ*phoE* demonstrated marked defect in Fe^{2+} production. The defects in the anaerobic growth and Fe^{2+} production, observed when iron (III) citrate was used, were restored by an expression vector of *phoE*, indicating that the anaerobic metal reduction by this strain requires the proper functioning of PhoE.

In *E. coli,* PhoE has been identified as a phosphate selective channel that is induced when inorganic phosphate concentration decreases. PhoE enhances the incorporation of phosphate ion through the cationic interface of hydrophilic passage of this protein[26]. In this study, we examined the effect of phosphate concentration on the iron respiration by Δ*phoE*. Even in the presence of excess amounts of phosphate, Δ*phoE* showed growth retardation and defect in Fe^{2+} production when iron (III) citrate was used as an electron acceptor, indicating that, in *S. livingstonensis* Ac10, PhoE played a crucial role in the anaerobic respiration-linked iron (III) reduction. Similar to phosphate uptake by phosphoporins, *S. livingstonensis* Ac10 might enhance the uptake of iron (III), possibly as a complex with anionic mediators such as iron (III) citrate, by the function of PhoE. The production of PhoE might be a counter action against the decreased diffusion rate of the respiratory substrates, iron (III)-siderophore complexes such as iron (III) citrate, at low temperatures. Moreover, our results suggested that in *S. livingstonensis* Ac10, the anaerobic respiration-linked iron (III) reduction occurred at the periplasmic or the cytoplasmic space, which was different from the extracellular iron-reduction systems of *S. oneidensis* MR-1[27]. Although further study is needed to understand the detail of the uptake of respiratory substrates by PhoE, iron (III)-dependent expression mechanism of *phoE*, and the presence of the dissimilatory iron-reducing mechanism at the periplasmic or cytoplasmic region of this strain, this is the first evidence revealing the physiological function of a phosphoporin homolog in the dissimilatory iron reduction by the *Shewanella* strain at low temperatures.

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