High Yield Expression System of *Desulfovibrio vulgaris* Miyazaki F Cytochrome c_3

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The overexpression of a tetraheme cyt c_3 gene from a sulfate-reducing bacterium has been successful only in a homologus host, *D. desulfricans* G200.[1] In general, cultivation of a strict anaerobe is much more difficult than that of an aerobe. This was the serious drawback of the homologus cyt c_3 expression system. Recently, two distinct novel gene expression systems of the *Desulfovibrio* cyt c_3 have been reported.[2-4] The *D. Desulfovibrio* (*Dd*) tetraheme cyt c_3 gene was overexpressed aerobically in *E. coli* cotransformed with the *E. coli ccm* gene cluster, which encodes eight membrane proteins (CcmABCDEFGH).[2] In addition, the *D. vulgaris* Miyazaki F (*Dv*MF) tetraheme cyt c_3 was also expressed aerobically in *S. oneidensis*.[3] The expression system of *Shewanella* seems to be promising, because it has a variety of soluble and membrane-bound *c*-type cytochromes including multihemic ones.[3] But, the transformation process a little bit tedious. Here, we have shown for the first time that *S. oneidensis* can be electrotransformed directly by the pKF3-type universal vector for *E. coli* and have established a much more efficient gene expression system using it.

The PCR was used to amplify DNA segements of the gene encoding DvMF cyt c_3 (cyc). *Pst*I and *Eco*RI sites were introduced at the 5' and 3' ends of the *cyc* gene, respectively, by PCR amplification from the plasmid pMC3[4] using as primers 5'-AAACTGCAGGTTTACCCCTAACCCACCAG-AG-3' and 5'-GCGAATTCTTAGCTATGGCACTTGGAG-CCCTTGC-3'. For the construction of the expression vector, the *Pst*I-*Eco*RI-digested gene of *cyc* was subsequently ligated in the *Pst*I-*Eco*RI digested pKF3 vector to generate pKF3FPB. Then the *Aat* II digested PCR product of *ref*^{r} gene cassette was ligated into an *Aat* II site of plasmid pKF3FPB to yield pFPB. For electroporation, an E. coli Pulser apparatus (Bio-Rad) was used according to the directions for *E. coli* (2.50 kV for a 0.2-cm electrode gap cuvette or 1.8 kV for a 0.1-cm cuvette).

Shewanella belongs to the *r* domain of *Proteobacteria*, where *E. coli* is also allocated. If the vector for *E. coli* can be used in *Shewanella*, genetic engineering and gene overexpression should become easier and more efficient. Thus, transformation of *S. oneidensis* by a pKF3-type plasmid was examined. Surprisingly, direct electro-transformation of *S. oneidensis* by a pKF3-type vector, pFPB, was successful. The efficiency of *S. oneidensis* electrotransformation by pFPB vector was approximately 6×10^3 colonies/ug plasmid DNA on average. These values are much less than that of *E. coli* DH5a transformation (approximately 10^9 colonies/ug plasmid DNA), but enough to carry out overexpression experiments. By the minipreps of plasmids from 3 mL overnight LB (with 10 mg rifampicin/L) cultures of *S. oneidensis* transformants, the yield of pKF3-type plasmid found to be approximately 8 ug/3 mL culture (approximately 350 ug/g wet cells). This value did not change significantly after the growth in large-scale culture, suggesting that the plasmids are stable in *S. oneidensis* cells. The plasmid yield is close to that in *E. coli* DH5a (approximately 10 ug/3 mL culture or 500 ug/g wet cells). These results show that *S. oneidensis* can replicate and maintain the high copy number of a pKF3-type vector in spite of its low transformation efficiency.

S. oneidensis (pFPB) was aerobically grown at 30°C in LB (with 10 mg rifampicin and 100 mg kanamycin/L) for the best production of *Dv*MF cyt c_3. Recombinant *D. vulgaris* Miyazaki F cytochrome c_3 was purified from a supernatant obtained after centrifugation of the broken-cell supernatant treated with streptomycin sulfate (0.16 g per g of cells). Purification was carried out at 4 °C and pH 7.0. The recombinant cytochrome c_3 was purified in two steps. Fist, after dialysis against 10 mM sodium phosphate buffer, the supernatant was loaded onto an SP-Sepharose column (2.6 × by 10 cm) previously equilibrated with the same buffer. Under these conditions, *D. vulgaris* Miyazaki F cytochrome c_3 (pI = 10.6) binds to the ion-exchange resin, while endogenous *S. oneidensis* cytochrome c_3 (pI = 5.8) is eluted together with other proteins. A gredient of 0 to 500 mM NaCl in 10 mM sodium phosphate buffer was then used to remove the *D. vulgaris* Miyazaki F cytochrome c_3, which was eluted at 150 mM NaCl. Second, the eluted cytochrome c_3 fraction was futher purified by gel filtration on fast protein liquid chromatography system (Amersham Pharmacia Biotech) using a Hiload Superdex 75 column (2.6 by 60 cm) equilibrated with 50 mM NaCl-10 mM sodium phosphate buffer. Relative purity was confirmed by the absense of other bands after SDS-15% PAGE using CBB staining and a purity index (A_{552}RED/A_{280}OX) of 3.0.

The yield of the recombinant *Dv*MF cyt c_3 from *S. oneidensis* (pFPB) was 2.0 mg/g wet cells or 16.0 mg/L culture. The recombinant protein obtained from *S. oneidensis* (pFPB) was compared with the authentic *Dv*MF cyt c_3. The apparent

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**Abbreviations: PCR, polymerase chain reaction: SDA-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis: PMSF, phenylmethyl sulfonyl fluoride: NMR, nuclear magnetic resonance spectroscopy.

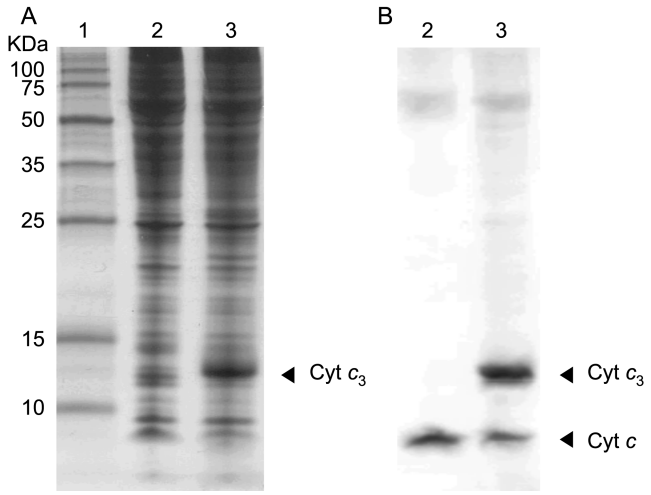

Figure 1. Fifteen percent SDS-PAGE analysis of the recombinant *Dv*MF cyt c_3 (A and B). The samples were analyzed by 15% SDS-PAGE and stained with Coomassie brilliant blue(A) or o-tolidine dihydrochloride (B). Lane 1, molecular weight marker; lane 2, cell lysate from *S. oneidensis*; lane 3, cell lysate from *S. oneidensis* (pKF3FPB).

molecular mass by SDS-polyacryamide gel electrophoresis (PAGE) analysis (Fig. 1) and UV-vis absorption spectrum of ferri- and ferro-forms (Fig. 2) were all same as those obtained for the authentic cyt c_3 from *Dv*MF. The NMR spectrum of the recombinant ferri-cyt c_3 was essentially identical with that of the authentic *Dv*MF cyt c_3 (Fig. 3).

There are two major important points in the expression system established in this work. In the first place, *S. oneidensis* has been transformed by a pKF3-type vector for the first time. This gives us major benefits. Because the DNA sequences of the pKF3-type vectors are completely known, and the vectors are stable and with high copy numbers, they are "user-friendly" vectors. The second point is that *S. oneidensis* has been directly transformed by a pKF3-type

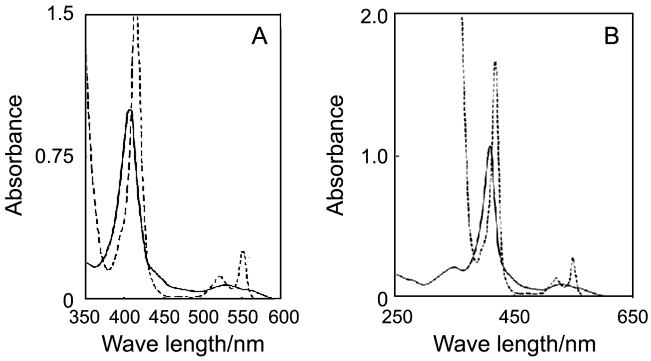

Figure 2. Absorption spectra of the oxidized (dark line) and dithionite-reduced (pale line) authentic (A) and recombinant (B) cyt c_3. The measurement was done in 10 mM sodium phosphate buffer, pH 7.0, at room temperature. Peaks, at 410 nm (ox) and at 552, 524, and 410 nm (red). The ferro-type spectra are masked by the absorption of dithionite in the region lower than 380 nm.

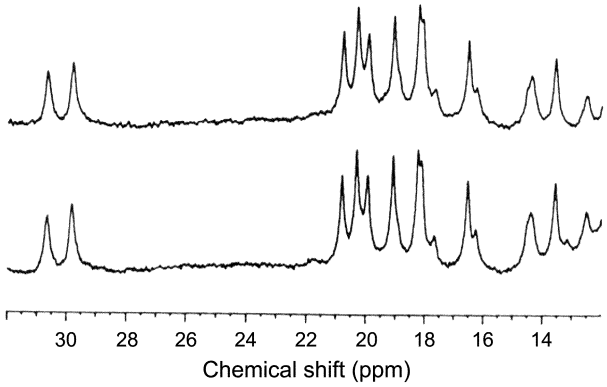

Figure 3. Five hundred MHz ^{1}H-NMR spectra of authentic (A) and recombinant (B) *Dv*MF ferricytochrome c_3 at 303 K. Only the fingerprient regions are presented. The protein was dissolved at 0.6 mM in a deuterated solution of 10 mM sodium phosphate buffer, in p^{2}H 7.0.

vector through electroporation. The transformation by electroporation is an absolutely simple and rapid method compared with the conjugal transfer from *E. coli*. Furthermore, the transformant can be grown aerobically.[3] These advantages enabled us to save time in making various mutants of a target protein. Namely, while the conjugal transfer method with a broad-host-range vector has taken us almost one month from the site-directed mutagenesis of a target gene to checking the expression of recombinant gene, the electroporation method with a pKF3 vector enables us to accomplish the same thing in a week.

The yield per liter of culture of the recombinant *Dv*MF cyt c_3 isolated from *S. oneidensis* became 40 times higher than that from *Dv*MF itself, and is about 2-fold of the recombinant *Dv*MF cyt c_3 from *S. oneidensis* (pMC3).[4]

In conclusion, a rapid simple, and highly efficient gene expression system of *c*-type multiheme cytochromes in a heterologous host has been established. This system would open a new horizon in various studies involving *c*-type multiheme cytochromes such as electron transfer mechanism, bioelectronics, and environmental chemistry.

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