Rapid report

Construction of a deletion strain and expression vector for the *Escherichia coli* NADH:ubiquinone oxidoreductase (Complex I)

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Abstract

Complex I of *Escherichia coli* is encoded by 13 consecutive genes, called the *nuo* operon. A chromosomal deletion of all *nuo* genes has been achieved by homologous recombination. A vector that encodes all of the *nuo* genes has been constructed, and it expresses a functional enzyme. © 2006 Elsevier B.V. All rights reserved.

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Complex I is a multi-subunit, membrane-bound enzyme that functions in the electron transport chain as an NADH:quinone oxidoreductase. It is found in mitochondrial membranes of most eukaryotes, in the plasma membranes of many eubacteria, and possibly also in archaea. It typically serves 3 important roles: (1) It oxidizes NADH to NAD, which allows the tricarboxylic acid cycle to continue. (2) It reduces quinones so that they may function in electron transport chains. (3) It translocates protons, or possibly other cations, to generate the protonmotive force for ATP synthesis (for recent reviews of Complex I see Ref [1–3]).

Bacterial Complex I is encoded by 13 or 14 genes. In the case of *E. coli*, they are labeled *nuoA* through *nuoN*. *nuoCD* represents a genetic fusion of two genes that are separate in many other species. Seven of the genes, A, H, J, K, L, M, N, code for integral membrane proteins, and they are homologous to the seven mitochondrially-encoded genes of mammals. The others are homologous to nuclear genes in mammals. These subunits bind the Fe–S clusters and the flavin mononucleotide, and form a peripheral lobe of Complex I. This sub-complex from *Thermus thermophilus* has recently been crystallized and its structure solved at 3.3 Å resolution [4].

Complex I has been purified from *E. coli* by several labs [5–9], but no plasmid-derived expression system has yet been described. Four-fold over-expression was achieved in one case [5] by inserting a phage T7 RNA polymerase promoter upstream of the chromosomal *nuo* genes in *E. coli*. In addition to the possibility of over-production, the primary benefit of a plasmid expression system is the ability to construct mutations in all genes with ease, and to compare them in a common background. The characterization of such mutants would be facilitated by a strain with a chromosomal deletion of all *nuo* genes.

This report describes the construction of a strain of *E. coli* that is deleted of all *nuo* genes, and the construction of a vector that appears to express all 13 *nuo* genes. The method of Hamilton et al. [10], previously applied in this lab to make a small deletion in *nuoN* [11], was used. It utilizes the pMAK705 vector (5.6 kb), which has a temperature-sensitive origin of replication and provides resistance to chloramphenicol. This allows selection of cells with a plasmid integrated into the host chromosome, using chloramphenicol resistance at the non-permissive temperature of 44 °C. To facilitate recombination with the *nuo*-region of the chromosome, the pMAK705-derived plasmid has 714 bp of DNA from the nuoA upstream region and 1008 bp of DNA from the nuoN downstream region, as shown in Fig. 1. The nuoN region was constructed by PCR, in which one primer started at the *Kpn*I site 901 bp downstream from the stop codon of nuoN, and the other primer started within nuoN, 107 bp from the 3′ end, and introduced an *Xba*I site and a *Sac*II
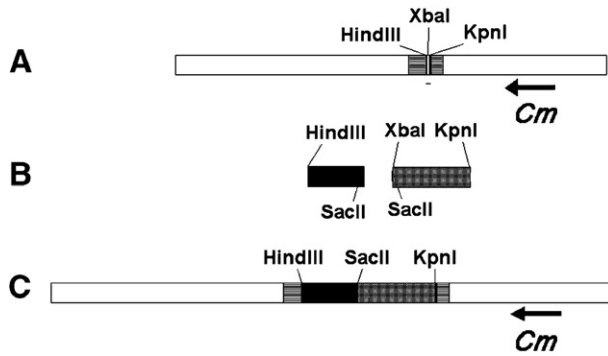

Fig. 1. Construction of the plasmid used to make the chromosomal *nuo* deletion. (A) A map of pMAK705 (5.6 kb) showing the multi-cloning site and gene for chloramphenicol resistance. (B) The black fragment, with *Hin*dIII and *Sac*II ends is the *nuoA*-upstream region (0.7 kb). The hatched fragment, with *Xba*I and *Kpn*I ends is the *nuoN*-downstream region (1.0 kb). Both were generated from chromosomal DNA by PCR. They were added stepwise to pMAK705. (C) The modified pMAK705 (7.3 kb) contains both *nuo* upstream and downstream regions.
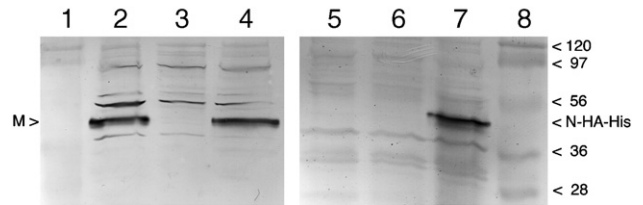

Fig. 3. Immunoblots showing the presence of subunits M and N from the expression system BA14/pBA4000. Lanes 1 and 8, pre-stained protein standards, with sizes in kilodaltons indicated at the right. Lanes 2 and 7, solubilized membrane fractions from BA14/pBA400. Lanes 3 and 6, solubilized membrane fractions from BA14. Lanes 4 and 5, solubilized membrane fractions from wild type strain 1100. Lanes 1–4 were probed with polyclonal antibody against the nuoM peptide, and lanes 5–8 were probed with monoclonal antibodies against the HA epitope. Both blots were probed with secondary antibodies conjugated with alkaline phosphatase. The migration of subunits M and N-HA-His is indicated.

site. The 1 kb fragment generated by digestion with *Xba*I and *Kpn*I was ligated to the pMAK705 vector following the same digestion. The upstream region of nuoA was introduced to the pMAK705 vector in a similar fashion. One PCR primer started at 1139 bp upstream of nuoA and introduced a *Hin*dIII site, and the other started 303 bp upstream of the nuoA start site and included a new *Sac*II site. Following digestion with *Hin*dIII and *Sac*II, the 0.7 kb PCR fragment was ligated to the modified pMAK705 vector to generate pMAK705-ΔnuoA-N.

1100 cells (*wild type*) were transformed with the deletion vector with selection on LB plates with chloramphenicol (34 mg/l) at 30 °C, and then a colony was used to inoculate LB broth, supplemented with 0.3% glycerol and chloramphenicol. After 2 days growth at 44 °C cells were plated on the same medium. Several colonies were used to inoculate 100 ml of the same medium, but now grown at 30 °C, to allow resolution of the vector from the chromosome. The next day, cells were plated on 1% tryptone and chloramphenicol at 30 °C, because it had been shown that Complex I mutants exhibited a growth phenotype on tryptone plates [12]. Thirty small colonies were selected. Analysis of plasmids from these thirty colonies indicated that one was very much larger in size than the others, all of which appeared to be about the same size as the starting

plasmid (7.3 kb). The candidate isolate was cured of its plasmid by restreaking it on chloramphenicol-free medium several times. Compared to its wild type parent 1100, it grows similarly on LB medium, but grows very poorly on minimal medium (M63)+acetate (0.2%). The nuo deletion strain was named BA14. The *nuo* deletion was confirmed by PCR using 2 of the primers used to construct the pMAK deletion vector. A 1.7 kb product was seen using BA14, but not in the wild type strain 1100 (results not shown). A 15 kb product would be predicted in 1100, but such a long product is unlikely to be made.

An expression vector was constructed starting with the 22.5 kb plasmid that was recovered from the original isolate. A *Hin*dIII digestion indicated that it appeared to contain the entire *nuo* operon. Therefore, we attempted to subclone the *nuo* genes into a low-medium copy plasmid, pACYC184 [13]. We had previously published a description of construction of a pUC19-derived vector that expressed nuoN along with a His tag and an HA epitope tag, called pBA200 [11]. This tagged nuoN gene was subcloned into pACYC184 using its *Hin*dIII site. The *Eco*RI site just downstream of the 3′ end of nuoN was made blunt by treatment with the Klenow fragment of DNA polymerase I and ligated to the similarly-treated *Bcl*I site in pACYC184, thus generating pBA300, as shown in Fig. 2. This plasmid was used to construct the *nuo* expression vector, by utilizing the *Asc*I site internal to nuoN, and the upstream *Hin*dIII site. An *Asc*I/*Afl*II fragment from the recovered plasmid was
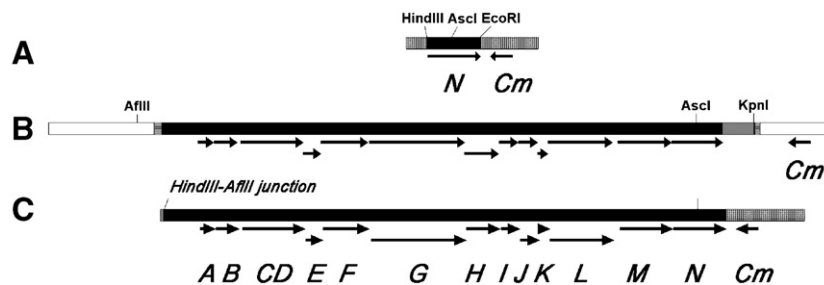

Fig. 2. Construction of the *nuo* expression vector. (A) The pACYC184-derived vector encodes *nuoN* with an HA epitope tag, followed by six histidine residues (3.8 kb). (B) A predicted map of the recovered plasmid (22.5 kb), which appeared to be pMAK705 with the entire *nuo* region. (C) A map of the expression vector pBA400 (18.5 kb), which encodes all of the *nuo* genes and the upstream control region. It carries the origin of replication and chloramphenicol resistance of pACYC184.

Table 1
Enzyme activities of membrane vesicles from the new strains

Source	Deamino-NADH oxidase activity [a] (μmol/min/mg)	Capsaicin sensitivity [b]	DeaminoNADH-Ferricyanide reductase activity [c] μmol/min/mg
1100 (*wild type*)	0.57 ± 0.11 (*n* = 8)	88% ± 5	2.1 ± 0.6 (*n* = 3)
BA14 (Δ *nuo*)	0.0	–	0.0
BA14/pBA400	0.21 ± 0.06 (*n* = 10)	78% ± 10	1.9 ± 0.2 (*n* = 3)

[a] Assay conditions: 50 mM MOPS (pH 7.3), 10 mM MgCl$_2$, 0.25 mM deamino-NADH, 1 μM FCCP, at 21 °C, using an extinction coefficient of 6.22 mM^{-1} at 340 nm.

[b] Capsaicin was added to the reaction medium for deamino-NADH oxidase assays at a concentration of 300 μM.

[c] Assay conditions: 10 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.15 mM deamino NADH, 1 mM K$_3$Fe(CN)$_6$, 10 mM KCN, at 21 °C, using an extinction coefficient of 1.0 mM^{-1} at 410 nm.

isolated (containing *nuo* genes), after the *Afl*II end was made blunt by treatment with the Klenow fragment of DNA polymerase I. This fragment was ligated to pBA300 that had been digested with *Asc*I/*Hin*dIII. The *Hin*dIII end was similarly treated to make it blunt. The resulting plasmid was designated pBA400. The junctions have been confirmed by sequencing, and it indicates that about 600 bp were lost from the *Afl*II end. Only 107 bp from pMAK705 remain in this vector. The *nuo* expression vector is about 18.5 kb and it provides chloramphenicol resistance. It contains all of the *nuo* genes and 1139 bp of the upstream control region [14]. It contains a 3′ fragment of the upstream *lrhA* gene. It is not known if this gene fragment is expressed. The nuoN gene encodes a His$_6$ tag and an HA epitope tag at its 3′ end, as described previously [11].

Membrane vesicles were prepared from the wild type strain 1100, BA14 and from BA14/pBA400, as described previously [11], to test for expression of the *nuo* genes by both immunoblotting and enzyme activity. It was important to grow the cultures to an optical density of no more than 1.4 A_{600}. Two types of antibodies were available to test for expression of *nuo* genes. Commercial polyclonal antibodies were raised against a peptide from nuoM (446-GKAKSQIAS-QELPGM-460) by Affinity BioReagents (Golden, CO, USA), and an anti-HA monoclonal antibody was purchased from Roche Diagnostics Corp. (Indianapolis, IN, USA) for detection of tagged-nuoN. Both subunits were readily detected in membrane vesicles, as shown in Fig. 3. After gel electrophoresis and blotting, a band corresponding to nuoM is visible from BA14/pBA400, and from 1100, but not from BA14, the deletion strain. Similarly, the HA antibody recognizes a band corresponding to nuoN in samples from BA14/pBA400, but not from BA14.

Membrane vesicles were also tested for enzyme activity, and the results are presented in Table 1. Assays were performed with deamino-NADH, since the alternative NADH dehydrogenase (NDH-2) is less able to oxidize this modified NADH [15]. The specificity of Complex I is also confirmed by measuring rates in the presence of capsaicin, which inhibits only Complex I [16]. Membranes from the BA14 strain show essentially no activity. Membranes from the complemented deletion strain, BA14/

pBA400, typically have about 40% of the deamino-NADH oxidase activity of the parent strain (1100). Capsaicin sensitivity is also somewhat reduced, from about 88% in the wild type to about 78% in the complemented strain. Assays with ferricyanide as the electron acceptor were also performed. In this case the complemented strain had about 90% of the wild type level.

In conclusion, we have constructed a strain deleted of the *nuo* operon in *E. coli*, and have constructed an expression vector containing all of these genes. Immunoblots indicate that at least one of the large membrane-bound subunits is expressed at normal levels. Enzyme assays have shown that a functional complex is assembled, but that the possibility for improvement in expression exists, probably through better growth conditions or alterations in the vector.

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