The origin of the sodium-dependent NADH oxidation by the respiratory chain of *Klebsiella pneumoniae*

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Abstract Properties of *Klebsiella pneumoniae* respiratory chain enzymes catalyzing NADH oxidation have been studied. Using constructed *K. pneumoniae* mutant strains, it was shown that three enzymes belonging to different families of NADH:quinone oxidoreductases operate in this bacterium. The NDH-2-type enzyme is not coupled with energy conservation, the NDH-1-type enzyme is a primary proton pump, and the NQR-type enzyme is homologous to the sodium-motive NADH dehydrogenase of *Vibrio* and is shown to be a primary Na^+ pump. It is concluded that the NQR-type enzyme, not the NDH-1-type enzyme, catalyzes sodium-dependent NADH oxidation in *K. pneumoniae*.

Key words: Sodium translocation; Complex I; NADH dehydrogenase; *Klebsiella pneumoniae*; *Vibrio*

1. Introduction

NADH can be oxidized by the respiratory chain of bacteria via NADH:quinone oxidoreductases that belong to three different enzyme families without substantial homology between each other. These are enzymes of the NDH-1, NDH-2, and NQR types.

Bacterial NDH-1-type NADH:quinone oxidoreductases are homologous to mitochondrial complex I. They consist of 13–14 subunits and typically contain six to nine Fe–S clusters and non-covalently bound flavin mononucleotide (FMN) as cofactors [1–4]. The activity of these enzymes is coupled with proton translocation across the membrane [5,6].

The NDH-2-type enzymes are the simplest proteins among NADH:quinone oxidoreductases. They consist of only one subunit and contain only one cofactor (generally a non-covalently bound flavin adenine dinucleotide (FAD)) [1]. The activity of these enzymes is not coupled with energy conservation [1,5,7].

The NQR-type enzymes are sodium-motive NADH:quinone oxidoreductases [8]. They consist of six subunits [9–11] and contain the following set of cofactors: one non-covalently bound FAD [8], two covalently bound FMN residues [12,13], a 2Fe–2S cluster [14], and presumably one non-covalently bound riboflavin [15]. The NADH:quinone oxidoreductase activity of these enzymes is stimulated by sodium ions and is coupled with pumping of Na^+ [16–18], but not H^+ [13]. The NQR-type enzymes have been investigated in different bacteria of the genus *Vibrio*, such as *V. alginolyticus* [17,18], *V. harveyi* [14,19] and *V. cholerae* [15]. However, it has recently been shown that the operon closely homologous to *nqr* is present in various bacteria, including several pathogenic microorganisms [13].

The different types of NADH:quinone oxidoreductases have different substrate specificity with respect to pyridine dinucleotides. The NDH-1- and NQR-type enzymes oxidize NADH as well as its analog dNADH, while NDH-2-type enzymes generally oxidize only NADH, but not dNADH [1,7,13,20,21].

In 1989 it was demonstrated that NADH oxidation by subbacterial particles of *Klebsiella pneumoniae* is stimulated by Na^+ [22]. Later a Na^+-dependent NADH:quinone oxidoreductase was partially purified from this bacterium and characterized as an NDH-1-type enzyme [23,24]. In this preparation the quinone reductase activity was fully dependent on sodium ions and an incorporation of the protein mixture into liposomes resulted in NADH-dependent translocation of Na^+ (but not H^+) across the membrane. So it was suggested that it is an NDH-1-type enzyme operating as a primary sodium pump in *K. pneumoniae* [24,25]. These data overturned the majority of concepts concerning the mechanism of NDH-1 (complex I) functioning, because pumping of different ions (H^+ or Na^+) by very similar enzymes imposes significant limitations on such hypotheses [26]. However, the preparations of Na^+-dependent NADH:quinone oxidoreductase from *K. pneumoniae* were inhomogeneous and had low specific activity that could have been due to a small contamination with an NQR-type enzyme known to be a primary Na^+ pump. Thus, we tried to reinvestigate the nature of the Na^+-dependent NADH:quinone oxidoreductase of *K. pneumoniae*. Here we report data indicating that sodium-dependent NADH oxidation in the respiratory chain of *K. pneumoniae* is carried out by the NQR-type enzyme (an enzyme similar to the Na^+-motive NADH:quinone oxidoreductase of *Vibrio* species), not by the NDH-1-type enzyme.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; dNADH, reduced nicotinamide hypoxanthine dinucleotide; NDH-1, bacterial NADH:quinone oxidoreductase similar to mitochondrial complex I; NDH-2, non-coupled bacterial NADH:quinone oxidoreductase; NQR, bacterial NADH:quinone oxidoreductase similar to the sodium-motive NADH dehydrogenase of *Vibrio*; PCR, polymerase chain reaction

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2. Materials and methods

2.1. Bacterial strains, growth, and medium composition

The *K. pneumoniae* and *Escherichia coli* strains used in this study are listed in Table 1. The wild type *K. pneumoniae* strain 204 was obtained from collection of the L.A. Tarasevitch Institute of standardization and control of biomedical compounds. *E. coli* and *K. pneumoniae* strains were routinely grown in LB medium at 37°C. Antibiotic concentrations for *K. pneumoniae* were rifampicin at 100 μg/ml, ampicillin at 100 μg/ml, tetracycline at 3.3 μg/ml, kanamycin at 100 μg/ml, and chloramphenicol at 40 μg/ml. Antibiotic concentrations for *E. coli* were ampicillin at 100 μg/ml, tetracycline at 10 μg/ml, kanamycin at 50 μg/ml, and chloramphenicol at 20 μg/ml. All genetic manipulations in *E. coli* were carried out using the XL1-Blue strain.

2.2. Isolation of spontaneous Rf^R mutant of K. pneumoniae

K. pneumoniae (10^6 CFU) was spread on a plate containing 10 μg/ml rifampicin. The plates were incubated overnight at 37°C. Several colonies were picked up, suspended in LB medium containing 10 μg/ml rifampicin, and after 8 h cultivation the cells were spread on a plate containing 100 μg/ml rifampicin. A unique colony (named R150) was selected and its resistance characteristics were confirmed by restreaking onto agar plates containing 150 μg/ml rifampicin. All the consequent manipulations were performed with this *K. pneumoniae* R150 strain.

2.3. Construction of NDH-1-deficient K. pneumoniae strain

The *K. pneumoniae nuo*A–C fragment was amplified using polymerase chain reaction (PCR) with *Taq* polymerase and primers nuoA 5′-GAGGGTTTTGGCGCTACAC and nuoC 5′-GCCACCTTGAGCATGATGT. The amplified 1.6 kb fragment was cloned into a pGEM-T vector resulting in the pGnuo20 plasmid. A chloramphenicol resistance cassette was inserted into the pGnuo20 plasmid between two *Ehe*I sites of the *nuo*B gene, and Cm-containing plasmid (pGnuoCm2) bearing the *nuo* genes together with the unidirectionally transcribing chloramphenicol resistance cassette was selected. The *nuo*A–C::Cm fragment from pGnuoCm2 was subcloned into suicide vector pKNOCK-Tc [28] resulting in pKnuoCm405. *K. pneumoniae* R150 cells were transformed by the pKnuoCm405 plasmid using electroporation, and a Tc^S Cm^R phenotype clone (KNU210, *nuo*B::Cm) characteristic of a double-crossover introduced mutation was selected. Proper localization of the mutation in the *K. pneumoniae* chromosome was proved by PCR analysis.

2.4. Construction of NQR-deficient K. pneumoniae strains

The *K. pneumoniae nqr*AB fragment was amplified by PCR with *Taq* polymerase and primers nqrA 5′-CTGAGCAGCCCGGCAAATGAC and nqrB 5′-CGGCGTGTAGAAGATGGTCG. The amplified 1.9 kb fragment was cloned into pGEM-T vector resulting in the pGnqr1 plasmid. A kanamycin resistance cassette was inserted into the *Sma*I site of the *nqr*A gene in pGnqr1, and Km-containing plasmid (pGnqrKm1) bearing the *nqr* genes together with the unidirectionally transcribing kanamycin resistance cassette was selected. The *nqr*AB::Km fragment from pGnqrKm1 was subcloned into suicide vector pKNOCK-Tc resulting in pKnqrKm7. This plasmid was transferred into the *K. pneumoniae* R150 strain via conjugation using *E. coli* SM10λpir as the donor, and a Tc^S Km^R Rf^R phenotype clone (KNQ197, *nqr*A::Km) characteristic of a double-crossover introduced mutation was selected. The same procedure was also carried out with the *K. pneumoniae* KNU210 strain producing double *nuo*B::Cm *nqr*A::Km mutant strain KNN002. Localization of the mutations in the *K. pneumoniae* chromosome was proved by PCR analysis.

2.5. Cloning of the complete K. pneumoniae nqrABCDEF operon and construction of the Δnqr::Km nuoB::Cm double mutant strain of this bacterium

The *K. pneumoniae nqr*ABCDEF operon was amplified using PCR with 'Long Reading' polymerase (Sileks M) and primers nqrA (see above) and nqrF 5′-CAAAACCGAAGCAGCTGACCCG. The amplified 6.7 kb fragment was cloned into pGEM-T vector resulting in the pG53 plasmid. Internal deletion of the *nqr*A–F genes was achieved by restriction using the *Hin*dIII site in *nqr*A and the *Bam*HI site in *nqr*F and insertion of a kanamycin resistance cassette between these sites. A Km-containing plasmid (pDKm8) bearing the flanks of the *nqr* operon together with the unidirectionally transcribing kanamycin resistance cassette was selected. The Δ*nqr*::Km construct from pDKm8 was subcloned into suicide vector pKNOCK-Tc resulting in pKnDKm1. This plasmid was transferred into the *K. pneumoniae* KNU210 strain via conjugation using *E. coli* SM10λpir as the donor, and a Tc^S Km^R Cm^R Rf^R phenotype clone (KNN103) yielded by a double-crossover event was selected.

Transformation of *K. pneumoniae* cells by pG53 plasmid was achieved using electroporation.

2.6. Preparation of sub-bacterial particles from K. pneumoniae cells

The *K. pneumoniae* cells were grown aerobically using a shaker (250 rpm, 37°C) in M9 medium containing 23 mM sodium citrate as a carbon source. The cells were harvested by centrifugation (10 000×*g*, 10 min) and washed twice with medium 1 (100 mM KCl, 10 mM Tris–HCl and 5 mM $MgSO_4$, pH 7.75). The cell pellet was suspended in medium 2 (10 mM HEPES–Tris, 5 mM $MgSO_4$, 50 mM KCl, 1 mM dithiothreitol and 0.5 mM EDTA, pH 7.75) and the suspension was passed through a French press (16 000 psi). Undamaged cells and cell debris were removed by centrifugation at 22 500×*g* (10 min) and the supernatant was further centrifuged at 180 000×*g*, 60 min. The membrane pellet was suspended in medium 2 (at 20–30 mg protein/ml) and immediately used for measurements of activities.

2.7. The rates of NADH and dNADH oxidation by K. pneumoniae sub-bacterial particles

The rates of NADH and dNADH oxidation by *K. pneumoniae* sub-bacterial particles were measured in medium 2 at 30°C using a Hitachi-557 spectrophotometer as described previously [29]. The reduced pyridine dinucleotides were used at final concentrations of 120 μM. To determine sodium stimulation, medium 2 was supplemented with 20 mM NaCl. It is noteworthy that dNADH is commercially available only in the form of the sodium salt, therefore in our experiments the sodium salts of NADH and dNADH were used. Thus, as measured by flame photometry, the background sodium concentration in the reaction media without added NaCl was about 300 μM.

2.8. Inhibition of the K. pneumoniae terminal oxidases by HQNO

Respiration of *K. pneumoniae* sub-bacterial particles was measured in the presence of different HQNO concentrations by a Clark-type oxygen electrode using 50 μM Q_1 and 2.5 mM dithiothreitol as electron donors.

2.9. Proton translocation by K. pneumoniae cells

Proton translocation by *K. pneumoniae* cells was measured as described previously [6]. *K. pneumoniae* cells were grown anaerobically at 37°C in M9 medium containing 50 mM MES, 0.4% (v/v) glycerol, 60 mM dimethylsulfoxide (DMSO) and 1 μM ammonium molybdate. Cells were harvested by centrifugation for 10 min at 7000×*g*, then washed twice with 150 mM KCl and resuspended in reaction medium (100 mM KCl, 25 mM KSCN, 10 mM glycerol, 0.5 mM MES–KOH, pH 6.5). This suspension was immediately injected into a 2-ml vessel equipped with a pH electrode. After bubbling with argon, the vessel was sealed and the pH was adjusted to 6.5 with KOH. During O_2 or DMSO pulses, 15 nmol O_2 or 50–100 nmol of DMSO were injected into the vessel. Calibration was carried out with 50 nmol HCl additions from a stock solution saturated with argon. All measurements were performed within the pH range 6.0–6.5.

2.10. Benzyl viologen:DMSO oxidoreductase activity

Benzyl viologen:DMSO oxidoreductase activity in *K. pneumoniae* cells was measured by alkalinization of the reaction medium following the benzyl viologen:DMSO reductase reaction as described in [6].

2.11. Sodium translocation by K. pneumoniae cells

Sodium translocation by *K. pneumoniae* cells was monitored as described previously [30] with some modifications. *K. pneumoniae* cells were grown aerobically at 37°C in M9 medium containing 0.4% (v/v) glycerol. The cells were washed twice with 150 mM NaCl and resuspended in reaction medium (150 mM NaCl, 10 mM glycerol and 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)). This suspension was immediately injected into a 2-ml vessel equipped with a pH electrode. After bubbling with argon, the vessel was sealed and the pH was adjusted to 7.5 with NaOH. O_2 (5–15 nmol) was injected into the vessel and pH shifts were measured. Calibration was carried out with 25 nmol HCl additions from a stock solution saturated with argon. All measurements were performed within the pH range 7.2–7.5.

Table 1
Bacterial strains and plasmids used in this study

Strains and plasmids	Genotype	References
Strains		
K. pneumoniae 204	wild type	
K. pneumoniae R150	204 Rf^R	This study
K. pneumoniae KNU210	R150 *nuo*B::Cm, Rf^R Cm^R	This study
K. pneumoniae KNQ197	R150 *nqr*A::Km, Rf^R Km^R	This study
K. pneumoniae KNN002	R150 *nuo*B::Cm *nqr*A::Km, Rf^R Cm^R Km^R	This study
K. pneumoniae KNN103	R150 *nuo*B::Cm Δ*nqr*::Km, Rf^R Cm^R Km^R	This study
E. coli Sm10λpir	*thi thr leu ton*A *lac*Y *sup*E *rec*A::RP4-2-Tc::Mu, Km^R	[27]
E. coli XL1-Blue	*Rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F′ *pro*AB *lac*I^qZM15 Tn*10*], Tc^R	Stratagene
Plasmids		
pGEM-T Easy	PCR product cloning vector, Ap^R	Promega
pG53	pGEM with *nqr*ABCDEF, Ap^R	This study
pKNOCK-Tc	mobilizable suicide vector, Tc^R	[28]

2.12. Protein concentration

Protein concentration was measured by the bicinchoninic acid method with bovine serum albumin (Serva, type V) as standard.

3. Results and discussion

3.1. Analysis of the unfinished K. pneumoniae genome and construction of mutant strains

BLAST search in the unfinished *K. pneumoniae* genome (http://genome.wustl.edu/projects/bacterial/kpneumoniae) shows that this bacterium contains a gene encoding NADH:quinone oxidoreductase of the NDH-2 type (97% similarity with respect to *E. coli* NDH-2), 13 genes for all the subunits of NDH-1 (94–98% similarity to corresponding subunits of *E. coli* NDH-1), and six genes for all the subunits of NQR (76–94% similarity to corresponding subunits of *V. alginolyticus* NQR). Using site-directed mutagenesis, *K. pneumoniae* strains with inactivated genes encoding NDH-1, NQR, and both these enzymes have been constructed (Table 1).

3.2. Sodium stimulation of (d)NADH oxidation by sub-bacterial particles from K. pneumoniae

Sub-bacterial particles from *K. pneumoniae* catalyze oxidation of NADH as well as dNADH ([23], Table 2). Both these activities were slightly activated in the presence of sodium ions. The low level of Na^+ stimulation indicates that only one of the *K. pneumoniae* NADH:quinone oxidoreductases is sodium-dependent. As shown previously [23], Na^+ stimulation of dNADH oxidase activity is higher with respect to NADH oxidase activity, because both the Na^+-dependent enzyme and Na^+-independent NDH-2 participate in the latter process. It was postulated that all Na^+ stimulation of NADH oxidation in *K. pneumoniae* membranes is due to activity of NDH-1, which operates as a primary Na^+ pump [23–26]. If this is correct, sub-bacterial particles from the mutant *K. pneumoniae* strain with inactivated *nuo* genes (KNU210) must lose the sodium-dependent dNADH oxidase activity. However, as can be seen from Table 2, (d)NADH oxidation in this strain not only remained sodium-dependent, but its Na^+ stimulation became significantly higher with respect to the wild type strain. At the same time, (d)NADH oxidation by the *K. pneumoniae* strain with inactivated *nqr* genes was completely sodium-independent. The double *K. pneumoniae* strains with both NDH-1 and NQR inactivated (KNN002 and KNN103) were fully unable to oxidize dNADH. Sub-bacterial particles from these strains oxidized only NADH, and this reaction (as well as NADH oxidation in NQR-deficient stain KNQ197) was not stimulated by Na^+. Transformation of the double KNN002 strain by the pG53 plasmid bearing the cloned *nqr* operon led to recovery of the Na^+-dependent dNADH oxidase activity (the same data were obtained on KNN103 strain with deleted *nqr* operon). Thus, the inability of *K. pneumoniae* strains with inactivated *nqr* operon to catalyze Na^+-dependent (d)NADH oxidation is not caused by a polar effect of the mutations but is connected with the NQR disruption. Summarizing the data presented in Table 2, we conclude that there are three NADH:quinone oxidoreductases in the respiratory chain of *K. pneumoniae*. NDH-1 oxidizes NADH as well as dNADH, and both these activities are Na^+-independent. NDH-2 oxidizes only NADH (but not dNADH), and this NADH oxidation is also sodium-independent. NQR oxidizes both NADH and dNADH in a sodium-

Table 2
NADH and dNADH oxidase activities of sub-bacterial particles from different *K. pneumoniae* strains

Strain of *K. pneumoniae*	NADH		Na^+ stimulation (%)	dNADH		Na^+ stimulation (%)
	$-Na^+$	$+Na^+$		$-Na^+$	$+Na^+$	
R150 (wild type)	365	400	10	138	180	30
KNU210 (Δ*nuo*)	230	290	26	38	100	163
KNQ197 (Δ*nqr*)	180	170	−6	52	51	−2
KNN002 (Δ*nuo* Δ*nqr*)	110	105	−5	0	0	–
KNN103 (Δ*nuo* Δ*nqr*)	130	125	−4	0	0	–
KNN002 (Δ*nuo* Δ*nqr*)/pG53	200	280	40	56	140	150
KNN103 (Δ*nuo* Δ*nqr*)/pG53	210	275	31	45	115	156

Results of a typical experiment are presented, and activities are given in nmol of (d)NADH oxidized/min/mg protein.

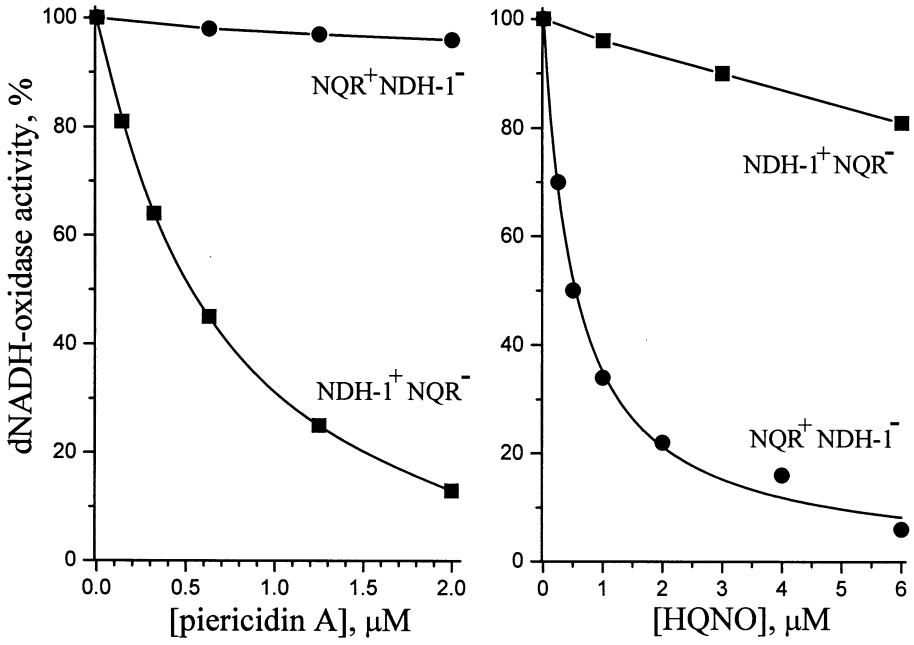

Fig. 1. Effects of piericidin A (left panel) and HQNO (right panel) on the dNADH oxidase activities of sub-bacterial particles from *K. pneumoniae* strains KNU210 (circles) and KNQ197 (squares). Activities were measured at a protein concentration of 0.1–0.2 mg/ml in medium 2 containing 20 mM NaCl. Activity without the inhibitors was 110 nmol of dNADH oxidized/min/mg protein for vesicles from the KNU210 strain and 60 nmol/min/mg protein for vesicles from the KNQ197 strain.

dependent manner, and the sodium stimulation of NADH oxidase activity in *K. pneumoniae* is due to only NQR operation.

3.3. *Inhibition of dNADH oxidation by* K. pneumoniae *sub-bacterial particles in the presence of piericidin A and HQNO*

As mentioned above, dNADH oxidation by sub-bacterial particles from strain KNQ197 is carried out solely by NDH-1, whereas in strain KNU210 it is oxidized exclusively by NQR. This makes sub-bacterial particles from these strains a useful tool for inhibitory analysis of NDH-1 and NQR. It is well known that all studied NDH-1-type enzymes are sensitive to piericidin A [31,32], while NQR-type enzymes are inhibited by low concentrations of HQNO [13,18]. In accordance with this, activity of *K. pneumoniae* NDH-1 was sensitive to piericidin (I_{50} = 0.48 μM), but *K. pneumoniae* NQR activity was resistant to this inhibitor (Fig. 1). At the same time, low HQNO concentrations inhibited activity of *K. pneumoniae* NQR (I_{50} = 0.54 μM), but had practically no effect on *K. pneumoniae* NDH-1 (the terminal oxidases of *K. pneumoniae* were inhibited only at relatively high HQNO concentrations with I_{50} = 7.2 μM). Using these data the effect of HQNO and piericidin A on sodium stimulation of dNADH oxidase activity of sub-bacterial particles from wild type *K. pneumoniae* strain was studied. As shown in Table 3, piericidin treatment led to an increase in the Na^+ stimulation, while dNADH oxidation in the presence of HQNO was completely sodium-independent. These data confirm the conclusion that Na^+ stimulation of (d)NADH oxidation in the *K. pneumoniae* respiratory chain is connected with operation of the NQR-type enzyme, but not with NDH-1.

3.4. *Proton translocation by NADH:quinone oxidoreductases of* K. pneumoniae

It was concluded previously that NDH-1 from *K. pneumoniae* is unable to pump protons [25]. We tried to check this hypothesis using the constructed mutant strains of this bacterium. A method to study H^+ translocation by NDH-1 on whole bacterial cells was previously developed for *E. coli* [6]. This technique is based on registration of pH shifts in anaerobic suspension of cells containing penetrating ions upon additions of DMSO. The respiratory chain of anaerobically grown *E. coli* cells contains quinol:DMSO oxidoreductase [33], whose activity is not coupled with proton translocation [6]. Thus, ΔpH generation in response to a DMSO pulse can be caused only by a NADH:quinone oxidoreductase (for detailed description of this method see [6]). The *K. pneumoniae* genome contains genes that are highly homologous to the *E. coli dms*ABC operon encoding the DMSO reductase, and *K. pneumoniae* cells are capable of anaerobic respiration on DMSO (data not shown). This makes it possible to use the DMSO pulse technique for this bacterium. As seen in Fig. 2,

Table 3
Na^+ stimulation of dNADH oxidase activities of sub-bacterial particles from wild type *K. pneumoniae* R150 strain measured in the presence of piericidin A (2 μM) or HQNO (6 μM)

Inhibitor	dNADH oxidase activity		Na^+ stimulation (%)
	$-Na^+$	$+Na^+$	
None	97	128	32
+piericidin A	28	70	150
+HQNO	64	62	−3

Activities are given in nmol of dNADH oxidized/min/mg protein.

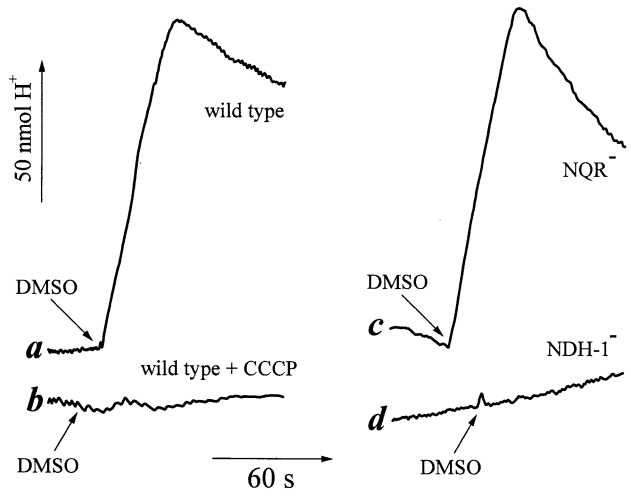

Fig. 2. Proton translocation in whole cells of wild type *K. pneumoniae* R150 (a,b) or of NQR-deficient strain KNQ197 (c) or of NDH-1-deficient strain KNU210 (d). In the case of curve b, the reaction medium was supplemented with CCCP (50 μM). When indicated by arrows, 100 nmol of DMSO was added to the anaerobic cell suspension.

addition of DMSO to anaerobic cell suspension of wild type *K. pneumoniae* strain resulted in transient acidification of the reaction medium. This acidification was completely sensitive to the uncoupler CCCP which indicates transmembrane translocation of protons. NQR inactivation (KNQ197 strain) did not lead to significant changes in this response. However, ΔpH formation after DMSO pulse was sensitive to the NDH-1 inhibitor capsaicin (data not shown) and was completely lacking in *K. pneumoniae* strain KNU210 with disrupted NDH-1 (see Fig. 2d). The inability of strain KNU210 to generate ΔpH in response to DMSO addition cannot be explained by low DMSO reductase activity (in strain KNU210 the DMSO reductase activity was the same as in wild type strain) or by high membrane permeability to H^+ (because ΔpH generation caused by activity of terminal oxidases in strain KNU210 was the same as in wild type strain). From these data we conclude that the activity of NDH-1 from *K. pneumoniae* is coupled with transmembrane translocation of protons.

3.5. Na^+ pumping by NADH:quinone oxidoreductases of K. pneumoniae

A simple method to study the Na^+ translocation by NQR in whole *V. alginolyticus* cells was previously developed [17,30]. It is based on a technique where H^+ translocation is measured in the presence of protonophorous uncoupler and in the absence of penetrating ions other than H^+. Under these conditions, the H^+ influx discharges membrane potential generated by the Na^+ pump so the Na^+ and H^+ fluxes become equal and oppositely directed. An operation of primary sodium pumps in the presence of CCCP should cause an inverted, so-called alkaline pulse of protons (for detailed description of this method see [30]). As shown in Fig. 3, addition of O_2 to anaerobic suspension of wild type *K. pneumoniae* resulted in transient alkalinization of the reaction medium. This pH shift was completely sensitive to the penetrating anion SCN^-. Inactivation of NDH-1 (KNU210 strain) did not lead to significant alterations in the response. The 'alkaline' pulse was sensitive to the NQR inhibitor HQNO (data not shown) and was completely lacking in *K. pneumoniae* strain KNQ97 with disrupted NQR (Fig. 3d). These data prove that NQR (but not NDH-1) activity is coupled with Na^+ pumping in *K. pneumoniae*.

The cells of the *K. pneumoniae* strain with both NQR and NDH-1 inactivated (KNN002) demonstrated neither H^+ efflux after DMSO addition nor CCCP-dependent H^+ influx after O_2 addition (data not shown). This indicates that activity of NDH-2 from *K. pneumoniae* (as well as activity of homologous enzymes from other bacteria) is not coupled with sodium- or proton-motive force generation.

3.6. Conclusion

Here we report data demonstrating that three different NADH:quinone oxidoreductases operate in the respiratory chain of *K. pneumoniae*. The NDH-2-type enzyme is not coupled with energy conservation, the NDH-1-type enzyme is a primary H^+ pump, and the NQR-type enzyme is a primary Na^+ pump. The previous conclusion that it is NDH-1 that pumps Na^+ in *K. pneumoniae* [23–26] seems to be incorrect. Apparently, that conclusion was made erroneously due to contamination of NDH-1 preparations by the NQR-type enzyme.

Simultaneous operation of NADH:quinone oxidoreductases of all three types in *K. pneumoniae* makes this bacterium an interesting object for investigation of their physiological roles in the bacterial cell. It is also noteworthy that *K. pneumoniae* is closely related to *E. coli*, thus this bacterium looks like a very attractive organism for NQR study by genetic methods because the majority of genetic vectors and techniques developed for *E. coli* are also useful in the case of *K. pneumoniae*.

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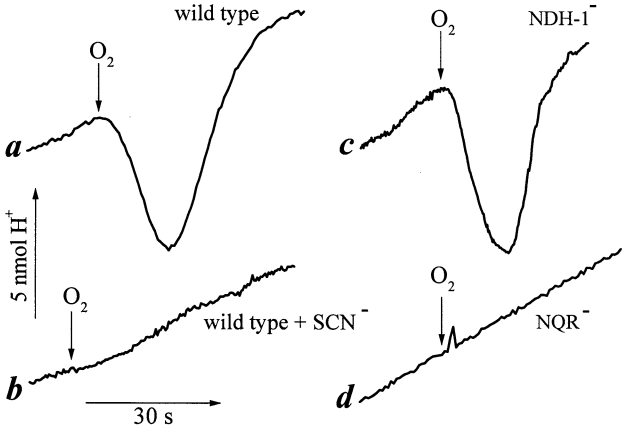

Fig. 3. CCCP-mediated proton influx in whole cells of wild type *K. pneumoniae* R150 (a,b) or of NDH-1-deficient strain KNU210 (c) or of NQR-deficient strain KNQ197 (d). In the case of curve b, the reaction medium was supplemented with KSCN (25 mM). When indicated by arrows, 15 nmol of O_2 was added to the anaerobic cell suspension. The acidification before addition of oxygen is a reflection of fermentative activity of cells under anaerobic conditions.

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