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Study of Cytochrome *bo* Function in *Vitreoscilla* Using a *cyo*⁻ Knockout Mutant

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The bacterium, *Vitreoscilla*, produces a $\Delta\mu_{Na^+}$ across its membrane during respiration. A key enzyme for this function is the cytochrome *bo* terminal oxidase which, when incorporated into synthetic proteoliposomes, pumps Na^+ across the membrane upon the addition of a substrate. A *Vitreoscilla* cytochrome *bo* knock out (*cyo*⁻) mutant was isolated by transposon mutagenesis using pUT-mini-Tn5*Cm*. The membranes of this mutant lacked the characteristic 416 nm peak and 432 nm trough in CO difference spectra, which are clearly visible in spectra of the *Vitreoscilla* wild-type, but peaks at 627, 560, and 530 nm in reduced minus oxidized difference spectra indicate that cytochrome *bd* is still present. The specific NADH oxidase and ubiquinol-1 oxidase activities of the cyo⁻ mutant membranes were less than those of *Vitreoscilla* wild-type and *Escherichia coli* membranes, and the stimulation of these activities of the mutant and *E. coli* membranes by 75 mM NaCl was approximately 50% less than that of *Vitreoscilla* wild-type membranes. The ubiquinol-1 oxidase activity of the cyo⁻ mutant membranes was inhibited by 10 mM KCN to a lesser degree than that of the *Vitreoscilla* wild-type and *E. coli* membranes (50, 80, and 85%, respectively). This result is also consistent with the cyo⁻ mutant membrane fragments containing only the cytochrome *bd* terminal oxidase, which is known to be less sensitive to KCN. Although the maximum respiration and growth of the cyo⁻ mutant were less than those of the wild-type, this mutant is still capable of growing with cytochrome *bd* alone.

Key words: cytochrome *bd*, NADH-oxidase, Na^+ transport, ubiquinol oxidase.

Although most aerobic bacteria generate a H^+ gradient ($\Delta\mu_{H^+}$) across the cell membrane during respiration, there are exceptions (*1*, *2*). For example, under alkaline conditions *Vibrio alginolyticus* has a Na^+ pumping NADH-quinone oxidoreductase (NQR) which generates a Na^+ gradient ($\Delta\mu_{Na^+}$) across the membrane (*3–5*). Another exception is the Na^+ pumping cytochrome *bo* from *Vitreoscilla* (*6*, *7*). This terminal oxidase exhibits some structural similarity to the H^+ pumping cytochrome *bo* of *Escherichia coli* (*8*). Both organisms also contain cytochrome *bd* terminal oxidases (*9–11*). When either of the two terminal oxidases was deleted in *E. coli* there was little effect on aerobic growth (*12*, *13*). There is evidence that cytochrome *bo* functions primarily when the oxygen level is high, and exhibits both scalar and vectorial proton pumping, whereas cytochrome *bd*, which has higher affinity for oxygen, functions primarily under hypoxic conditions and can generate a H^+ gradient solely through a scalar mechanism (*14–16*). Whether these two oxidases have similar functions in *Vitreoscilla* is not known.

One approach for answering this question is to look at the effects of inactivation of one of these terminal oxidases. This was accomplished accidently: we were screening for *Vitreoscilla* mutants of the hemoglobin gene (*vgb*), which had been inactivated by a transposon, and found one mutant in which cytochrome *bo* was lacking. Consequently, this paper reports the inactivation of the cytochrome *bo* gene (*cyo*) in *Vitreoscilla* with a transposon and the effects on certain respiratory parameters, including the response of the oxidase activities to added Na^+.

MATERIALS AND METHODS

Bacterial Strains and Transposon Mutagenesis—*E. coli* strain cc118 was used as a host for conjugation of transposable element pUT-mini-Tn5*Cm* (*17*, *18*) into *Vitreoscilla* sp. strain C1 to create the *Vitreoscilla cyo*⁻ mutant. *E. coli* strain JM103 was used as the *E. coli* control for the biochemical assays. The mating experiment to transfer pUT-mini-Tn5*Cm* from *E. coli* strain cc118 to *Vitreoscilla* was performed by a modification of the procedure described by Herrero *et al.* (*17*). *E. coli* cc118 bearing pUT-mini-Tn5*Cm* was grown overnight with shaking at 37°C in 2.5 ml of LB medium containing 100 μg/ml of ampicillin and 30 μg/ml of chloramphenicol. The recipient strain was cultured for 24 h with shaking at room temperature in 2.5 ml of PY medium (1% peptone and 1% yeast extract, pH 7.8) without antibiotics, and 0.7 ml of each culture was transferred to a microfuge tube and centrifuged. The supernatants were removed and the pellets were combined and resuspended in a total of 25 μl of PY. The mixture was filtered (Millipore

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membrane, 25 mm, 0.45 μm pore size), and then the filter was suspended in 1 ml of PY. After serial dilution (10^{-1}, 10^{-2}, and 10^{-3}), 100 μl aliquots were plated on selective medium (PY containing 30 μg/ml chloramphenicol, 23.75 μg/ml sulfamethoxazole, and 1.25 μg/ml trimethoprim) on which only *Vitreoscilla* exconjugants bearing mini-Tn*5Cm* could grow (drug sensitivities were determined in preliminary experiments). Two duplicate sets of eight master plates, each plate containing 65 colonies, were made by picking colonies from the 10^{-2} dilution plates after four days growth. One set of master plates was grown under normal atmospheric conditions, and the other set was grown in a desiccator under 2% oxygen. After four days, 13 colonies were selected that appeared to be paler and/or to grow more poorly under hypoxic conditions. The normal/hypoxic selection process was repeated, and eleven colonies were confirmed to exhibit poorer growth under hypoxic conditions. The CO difference spectrum of cells (*11*) grown from each of these colonies was examined, and three *vgb*$^-$ mutants and one *cyo*$^-$ mutant were found (the latter was lacking the cytochrome *bo* oxidase peak at 416 nm and the trough at 432 nm).

Southern Hybridization—Vitreoscilla wild-type and *Vitreoscilla cyo*$^-$ total DNA were isolated by Marmur's method (*19*). Small-scale purification of pUT-mini-Tn*5Cm* and the plasmid (pUT-KJS) from the *Vitreoscilla cyo*$^-$ mutant were performed with a Wizard™ Minipreps DNA purification system (Promega). The DNA was digested with several restriction enzymes, and then analyzed by electrophoresis on 1% agarose gels and subsequently stained with ethidium bromide.

DNA fragments were transferred from agarose gels to nylon membranes and then hybridization was performed with labeled pUT-mini-Tn*5Cm* as a probe using the Enhanced Chemiluminescence (ECL) direct nucleic acid labeling and detection system (Amersham Pharmacia) according to the manufacturer's procedure.

Antibiotic Resistance Test—pUT-KJS was transformed into *E. coli* cc118 using a published procedure (*20*). *E. coli* cc118 wild-type, *E. coli* cc118 bearing pUT-mini-Tn*5Cm*, and *E. coli* cc118 bearing pUT-KJS were plated onto LB, LB-ampicillin (50 μg/ml), LB-chloramphenicol (50 μg/ml), and LB containing both antibiotics. *Vitreoscilla* wild-type and *cyo*$^-$ mutant strains were also plated onto PY, PY-ampicillin (50 μg/ml), PY-chloramphenicol (50 μg/ml), and PY containing both antibiotics.

Growth Curves—Single colonies of the *Vitreoscilla* wild-type or *Vitreoscilla cyo*$^-$ mutant from PY plates were inoculated into 50 ml of PY liquid medium in 250 ml Erlenmeyer flasks and then grown at 30°C and 200 rpm for 22–24 h. 100 μl of 0.1 A_{600} units from each of these initial cultures was then used to inoculate 100 ml of liquid PY in a 500 ml Erlenmeyer flask. Samples were taken at intervals and A_{600} was measured with a Bausch and Lomb Spectronic 21 spectrophotometer. Samples were diluted with fresh medium as needed to keep the measured A_{600} below 0.5.

Preparation of Membranes and Assays for Oxidase Activities—Cells for these experiments were grown in 2.8 liter Fernbach flasks containing 1.5 liters of PY medium at 30°C and 200 rpm for 24 h (early stationary phase), and harvested by low speed centrifugation. Spheroplasts and membrane preparations were obtained using published procedures (*11*) with the single modification that 0.05 M Tris-HCl buffer, pH 7.5, replaced the pH 7.2 potassium phosphate buffer. For the NADH oxidase assay the total volume of the reaction medium was 2 ml and contained 10 mM Tris-HCl, pH 7.6, 1.0–1.5 mg of membrane protein, and varying concentrations of NaCl; NADH to 150 μM was added to start the reaction. The decrease in A_{340} was monitored for 5 min at room temperature with a Cary Model 300 Scan spectrophotometer and the initial velocity was used to calculate the activity. For the ubiquinol-1 oxidase activity the assay medium contained 60 mM Tris-HCl, pH 7.7, 300–400 μg of membrane protein, 10 mM DTT, and varying concentrations of NaCl, in a final volume of 5.0 ml. Following one-minute pre-incubation at 32°C, ubiquinol-1 was added to 16 μM to start the reaction, which was monitored polarographically at 32°C for O_2 uptake using a YSI model 53 oxygen meter. The O_2 uptake in the absence of membranes was similarly measured at every salt concentration and used to correct for autoxidation of ubiquinol-1. The inhibition by KCN was examined at 10 mM. Protein was estimated by A_{280} measurement of dilute samples.

Whole Cell Difference Spectra and Respiration—Cells for these experiments were grown in 500 ml flasks containing 200 ml PY medium at 30°C and 200 rpm for 24 h (early stationary phase), and harvested by low speed centrifugation. Reduced minus oxidized and CO difference spectra were obtained with a Varian Cary 300 Scan spectrophotometer as previously described (*13*). The respiration of intact cells was determined polarographically with a YSI model 53 oxygen meter in a final volume of 5 ml at 25°C. The assay medium contained 60 mM Tris-HCl (pH 7.7) and 0.5–0.6 A_{600} units of whole cells. Both endogenous and succinate-stimulated respiration were determined. Succinate was used at 20 mM and KCN, when used, at 10 mM.

RESULTS

The *Vitreoscilla* cytochrome *bo* terminal oxidase-knockout (*cyo*$^-$) mutant was produced by conjugation between *E. coli* strain cc118 bearing pUT-mini-Tn*5Cm* and the *Vitreoscilla* wild-type. pUT itself contains the ampicillin resistance gene and is a suicide vector that cannot be replicated in strains which do not have π factor Mini-Tn*5Cm* is a transposable element that is integrated randomly into the chromosome and contains the chloramphenicol resistance gene. After initial selection on medium containing chloramphenicol (to select against wild-type *Vitreoscilla*) and sulfamethoxazole and trimethoprim (to select against *E. coli* strain cc118), screening was performed with CO difference spectra; in these spectra, *Vitreoscilla* cytochrome *bo* terminal oxidase exhibits a peak at 416 nm and a trough at 432 nm. The absence of this peak and trough for the *cyo*$^-$ mutant membranes and their presence for the wild-type are shown in Fig. 1A. That the cytochrome *bd* is still present in this mutant is shown by the absorption maxima at 627, 560, and 530 nm in reduced minus oxidized difference spectra (Fig. 1B); CO difference spectra of cytochrome *bd* show little absorption in the Soret region, but a characteristic peak at 642 nm (not shown) was observed. These results are consistent with the presence of only one terminal oxidase in the *cyo*$^-$ mutant, the cytochrome *bd* terminal oxidase, in contrast to the *Vitreoscilla* wild-type and *E. coli* JM103, which contain both cytochromes *bo* and *bd*. In *E. coli* the latter cytochrome increases in concentration under oxygen

limiting conditions, but in *Vitreoscilla* the ratio of the two terminal oxidases does not appear to vary much with the oxygen concentration during growth (preliminary unpublished observations).

CO difference spectra of intact cells of the wild type *Vitreoscilla* and *cyo*⁻ mutant confirmed that the latter is a mutant strain of *Vitreoscilla* (Fig. 2). The broader spectrum of the wild type (Fig. 2A) has Soret absorption maxima at 416 nm (cytochrome *bo*) and 418 nm (*Vitreoscilla* hemoglobin), whereas the mutant shows only one maximum at 418 nm (Fig. 2B); this hemoglobin spectrum is characteristic of *Vitreoscilla*.

A plasmid was detected in the *cyo*⁻ mutant unexpectedly, since pUT is a suicide plasmid. This plasmid (pUT-KJS) was examined by restriction mapping analysis, which showed that it had the pUT portion of pUT-mini-Tn5*Cm* and an unknown 4.8 kb insert which was larger than the 4.0 kb mini-Tn5*Cm* portion (Fig. 3A). Transformation of pUT-KJS into *E. coli* strain cc118 was performed and antibiotic resistance was determined in this transformed strain along with various controls (Table I). *E. coli* strain cc118 bearing pUT-mini-Tn5*Cm* and the *Vitreoscilla cyo*⁻ mutant were resistant to both ampicillin and chloramphenicol, but *E. coli* strain cc118 bearing pUT-KJS was resistant to only ampicillin. This indicates that the chloramphenicol resistance of the *cyo*⁻ mutant was due to the integration of mini-Tn5*Cm* into the *Vitreoscilla* chromosome. The existence of the mini-Tn5*Cm* fragment in the *cyo*⁻ mutant chromosome was further confirmed by Southern hybridization using pUT-mini-Tn5*Cm* as a probe (Fig. 3B). This produced signals from the positive control (pUT-mini-Tn5*Cm*, lanes 2–4), purified pUT-KJS (lanes 13–15), and *cyo*⁻ total DNA (lanes 9–12), but not from *Vitreoscilla* wild type total DNA (lanes 5–8). Restriction fragments corresponding to those from pUT-KJS (see Fig. 3A) are also visible on Southern hybridization of the restriction digests of the *cyo*⁻ total DNA (lanes 10–12, Fig. 3B), but other signals are observed for larger fragments of this DNA, both in the uncut (lane 9) and restriction digested samples. This is evidence of the integration of the mini-Tn5*Cm* fragment into the *cyo*⁻ mutant chromosome. The hybridization of all fragments of pUT-KJS, including the 4.8 kb insert, tells us that pUT-

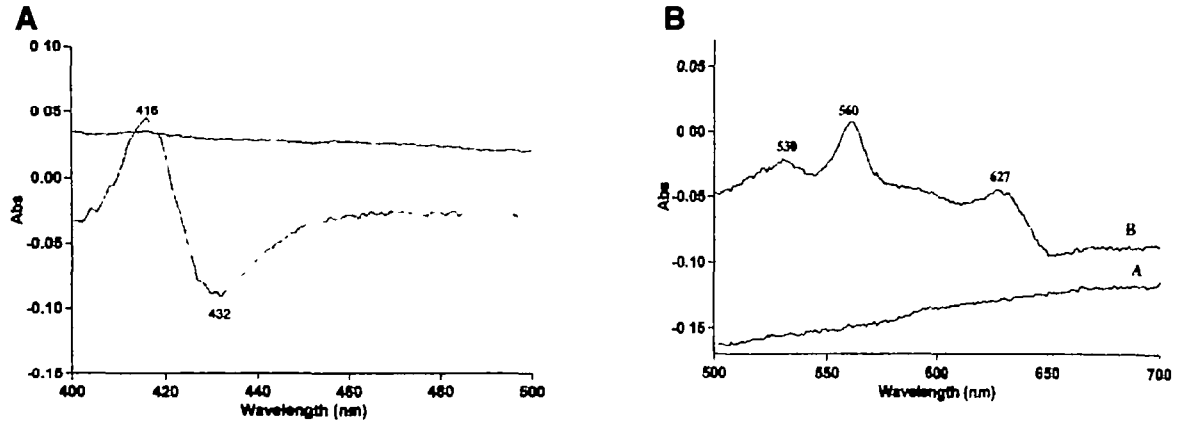

Fig 1. **Difference spectra of membranes of the *Vitreoscilla* wild-type and *cyo*⁻ mutant.** A: CO difference spectra. The wild-type membranes (lower curve) showed a peak at 416 nm and a trough at 432 nm characteristic of cytochrome *bo*, both of which were absent for the *cyo*⁻ mutant membranes (upper curve). B: The bottom curve (A) is the base line, and the top curve (B) is the reduced minus oxidized difference spectrum The *cyo*⁻ mutant shows peaks at 627, 560, and 530 nm, which are characteristic of cytochrome *bd*. The membrane fragments (1 5 mg protein/ml in A and 15 mg protein/ml in B) were suspended in 0 1 M potassium phosphate, pH 7.2, containing 2 M sucrose

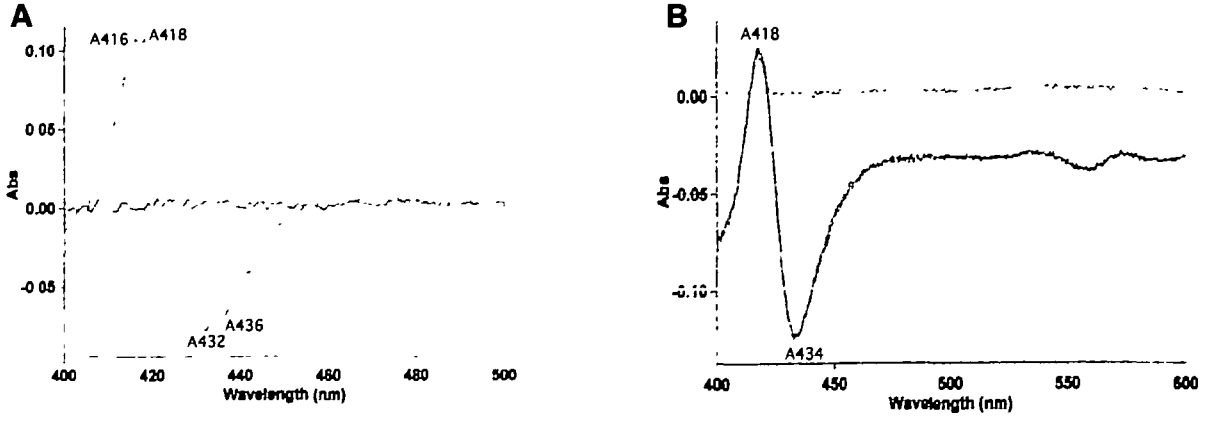

Fig 2. **CO difference spectra of whole cells of the *Vitreoscilla* wild-type (A) and *cyo*⁻ mutant (B).** The cell concentration was 50 mg wet weight per ml of 0.1 M sodium phosphate, pH 7.5 CO was bubbled slowly into the sample cell suspension for two min

KJS may contain part of the mini-Tn5*Cm* sequence but not all of it because, as described above, *E. coli* strain cc118 bearing pUT-KJS was not resistant to chloramphenicol. A less likely possibility is that the 4.8 kb fragment is some kind of repeat of the pUT sequence only.

The growth of the *Vitreoscilla cyo*⁻ mutant was slower than that of the wild-type; the generation times were 3.5 and 2 h, respectively (Fig. 4). Additionally, the *cyo*⁻ mutant had a longer lag phase than the wild-type.

The NADH oxidase specific activity (without added NaCl) of the *Vitreoscilla cyo*⁻ mutant membranes was 1.28 nmol NADH oxidized/min/mg protein, which was less than those of the wild-type (2.04 nmol/min/mg) and *E. coli* JM103 (1.77 nmol/min/mg) membranes (Fig. 5A). More importantly, in the presence of 75 mM NaCl the activity of *Vitreoscilla* wild-type membranes was stimulated 53%, while the *Vitreoscilla cyo*⁻ mutant and *E. coli* JM103 membranes were stimulated less than 10% (Fig. 5A). The KCl control in the same figure shows the specificity for NaCl; the stimulation was only 6 to 9% for all three strains, including the *Vitreoscilla* wild type. The plot of percent stimulation of NADH oxidase activity *versus* Na^+ concentration showed that the response of *Vitreoscilla* wild-type membranes to increasing Na^+ concentrations was roughly linear to 75 mM, while those of the *Vitreoscilla cyo*⁻ mutant and *E. coli* JM103 membranes leveled off at around 25 mM Na^+ (Fig. 5B).

The ubiquinol-1 oxidase activity assay gave similar results. The specific activity of the *Vitreoscilla cyo*⁻ mutant membranes was less (109 nmol O_2 consumed/min/mg protein) than those of the wild-type (133 nmol/min/mg protein) and *E. coli* JM103 (128 nmol/min/mg protein) membranes without added NaCl (Fig. 6A). When 75 mM NaCl was added, the *Vitreoscilla* wild-type membranes were stimulated 63%, but the *cyo*⁻ mutant and *E. coli* JM103 membranes were only stimulated 10% (Fig. 6A). The dose-responses of the ubiquinol-1 oxidase activities of all three strains as to Na^+ concentration (Fig. 6B) were similar to those of the NADH oxidase activities (Fig. 5B). In the presence of 10 mM KCN, the ubiquinol-1 oxidase activity of the *Vitreoscilla cyo*⁻ mutant membranes was inhibited only 50%, while the inhibition was 80 and 85%, respectively, for the *Vitreoscilla* wild-type and *E. coli* JM103 membranes (Fig. 6A). Since in *E. coli* the cytochrome *bd* oxidase is less sensitive to KCN than the cytochrome *bo* oxidase (*10*), this result is consistent with the cytochrome *bo* deficiency in the *cyo*⁻ mutant, the ubiquinol-1 oxidase activity being due solely to the cytochrome *bd* terminal oxidase. This assumes, of course, that the *Vitreoscilla* cytochrome *bd* is also less sensitive to KCN than the *Vitreoscilla* cytochrome *bo*.

The endogenous respiration of all three strains was approximately equal, but the *Vitreoscilla cyo*⁻ mutant cells responded less strongly to exogenously added succinate

TABLE I **Antibiotic resistance test.** The strains listed in this table were examined for ampicillin (50 μg/ml) and chloramphenicol (50 μg/ml) resistance. + = growth, − = no growth.

Strain	Ampicillin	Chloramphenicol
E. coli cc118 wild-type	−	−
E. coli cc118 bearing pUT-mini-Tn5*Cm*	+	+
E. coli cc118 bearing pUT-KJS	+	−
Vitreoscilla cyo⁻ mutant	+	+
Vitreoscilla wild-type	−	−

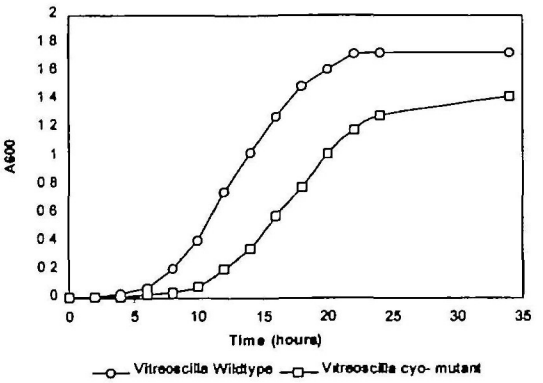

Fig 4. Growth of the *Vitreoscilla* wild-type and *cyo*⁻ mutant in PY medium.

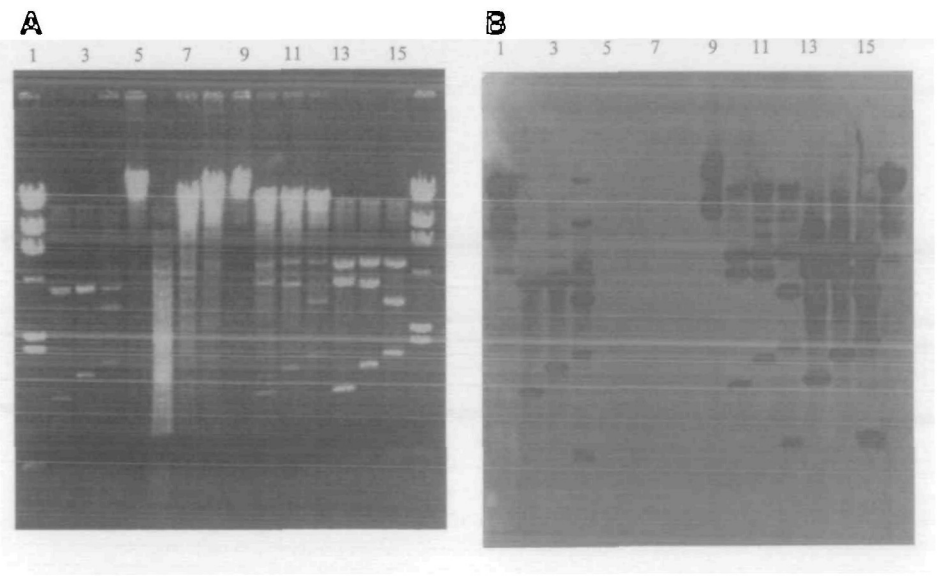

Fig. 3 **A: Agarose gel electrophoretic analysis of pUT-mini-Tn5*Cm*, *Vitreoscilla* wild-type total DNA, *Vitreoscilla cyo*⁻ mutant total DNA, and pUT-KJS.** Lanes 1 and 16, λ *Hin*dIII markers; lanes 2–4, pUT-mini-Tn5*Cm* digested with *Hin*dIII, *Sal*I, and *Bam*HI, respectively; lanes 5–8, *Vitreoscilla* wild-type total DNA uncut, and digested with *Hin*dIII, *Sal*I, and *Bam*HI, respectively; lanes 9–12, *Vitreoscilla cyo*⁻ mutant total DNA uncut, and digested with *Hin*dIII, *Sal*I, and *Bam*HI, respectively; lanes 13–15, pUT-KJS digested with *Hin*dIII, *Sal*I, and *Bam*HI, respectively **B: Southern hybridization of the gel in A using a mixture of pUT-mini-Tn5*Cm* and λ DNA as probes.** A signal was observed for the *Vitreoscilla cyo*⁻ mutant chromosomal DNA as well as all the fragments of pUT-mini-Tn5*Cm* and pUT-KJS.

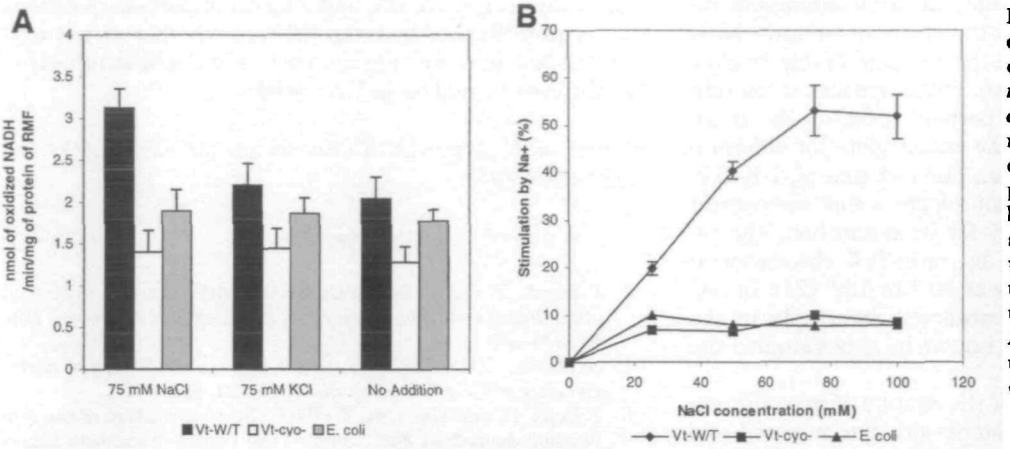

Fig. 5. **A: Effect of 75 mM Na^+ on the NADH oxidase activity of *Vitreoscilla* wild-type, *Vitreoscilla cyo*⁻ mutant, and *E. coli* JM103 membranes.** All membranes were prepared from cells grown to the early stationary phase. RMF, respiratory membrane fragments. **B: Dose-response of NADH oxidase activity *versus* Na^+ concentration for membranes of all three strains.** The values in both A and B are averages of 3 individual measurements; error bars indicate standard deviations.

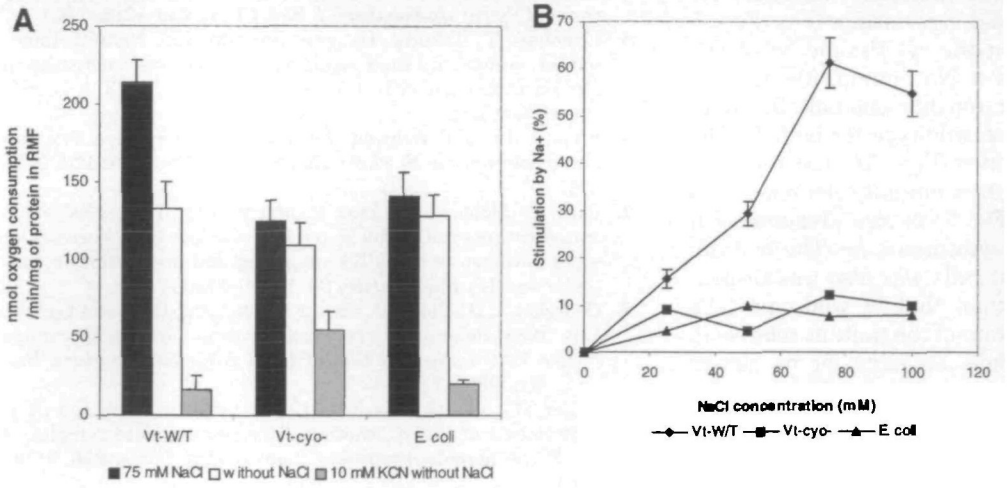

Fig. 6. **A: Ubiquinol-1 oxidase activity of membranes of the *Vitreoscilla* wild-type, the *Vitreoscilla cyo*⁻ mutant, and *E. coli* JM103 without NaCl, with 75 mM NaCl, and with 10 mM KCN (assayed without NaCl).** RMF, respiratory membrane fragments. **B: Dose-response of the ubiquinol-1 oxidase activity of membranes of all three strains to the Na^+ concentration.** Values for both A and B are averages of 3 individual measurements; error bars indicate standard deviations

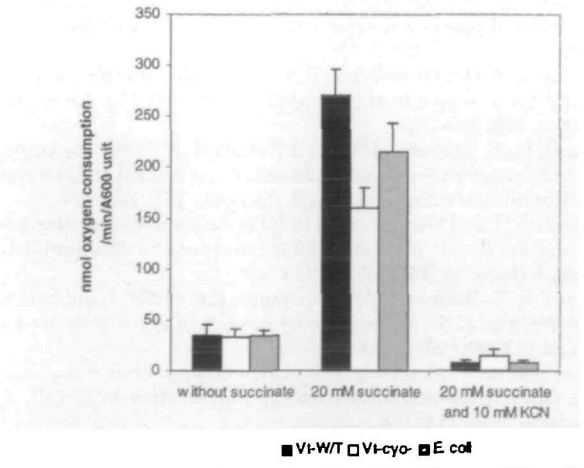

Fig. 7. **Respiration of intact cells of the *Vitreoscilla* wild-type, *Vitreoscilla cyo*⁻ mutant, and *E. coli* JM103 with and without 20 mM succinate.** Inhibition by 10 mM KCN was measured in the presence of succinate. Values are averages of 3 individual measurements, error bars indicate standard deviations.

(160 nmol O_2 consumed/min/A_{600} unit) than *Vitreoscilla* wild-type cells (271 nmol/min/A_{600} unit) and *E. coli* JM103 cells (215 nmol/min/A_{600} unit) (Fig. 7). This succinate-stimulated respiration is presumably a good indication of the maximum attainable respiration of the cells. The succinate-stimulated respiration of the mutant was somewhat less inhibited by 10 mM KCN than that of the wild-type, but the difference (90 *versus* 97%) was not as dramatic as that observed for the NADH oxidase activity of membranes (Fig. 6A). This stronger inhibition by cyanide of the succinate oxidase activity of intact cells may be due to an additional effect on another enzyme, *e.g.*, succinic dehydrogenase.

DISCUSSION

The inactivation of *cyo* in *Vitreoscilla* by the transposon mini-Tn*5Cm* is supported by the lack of any spectral evidence of cytochrome *bo* in membrane preparations from the mutant in contrast to the wild-type (Fig. 1). The presence of the vector and/or transposon in the mutant but not in the wild type chromosome was indicated by Southern blotting (Fig. 3). The resistance of the mutant *Vitreoscilla* strain to both ampicillin and chloramphenicol (Table I) is further evidence of the presence of pUT-mini-Tn*5Cm* DNA in this strain. The only uncertainty regarding the latter was the unexpected presence of a plasmid (pUT-KJS) in the mutant strain: isolation of pUT-KJS and its transformation into *E.*

coli showed that this plasmid did not carry chloramphenicol resistance, indicating that this trait must have been integrated into the *Vitreoscilla* chromosome (Table I). How this plasmid arose is not known, but it appears to contain the pUT suicide vector and perhaps part of the mini-Tn5*Cm* transposon (but not the intact gene for chloramphenicol resistance of the latter). The fact that pUT-KJS is stable in the *Vitreoscilla* mutant suggests that *Vitreoscilla* contains the π factor necessary for its replication. The reversion of mutants produced by mini-Tn5 chromosomal insertion occurs at frequencies of 10^{-4} to 10^{-6} (*21*). In our studies with mini-Tn5*Cm* we observed revertants in the absence of selective antibiotics, but we have not studied the frequencies of these reversions.

The respiratory properties of the mutant *Vitreoscilla* examined in this work are consistent with the proposed role of cytochrome *bo* as a Na^+ pump in this organism. Both the NADH oxidase and ubiquinol-1 oxidase activities of membranes from the mutant showed markedly decreased responses to Na^+ compared to wild type membranes (Figs. 5 and 6). This also suggests that the cytochrome *bd* of *Vitreoscilla* does not function as a Na^+ pump. Membranes from the *cyo*$^-$ mutant exhibit roughly one-half the total activity of membranes from the wild-type for both NADH and ubiquinol-1 oxidase activities (Figs. 5A and 6A), and the lower respiratory rate of intact mutant cells relative to that of the wild-type strain (Fig. 7) is also presumably a manifestation of the missing cytochrome *bo* The endogenous respiration of the mutant cells was also less responsive to exogenous succinate than that of wild-type cells (Fig. 7). The lower respiratory rate of the mutant relative to that of the wild-type most likely accounts for its slower growth (Fig. 4); nevertheless the mutant could grow reasonably well without one of its two oxidases. This was also observed for *E. coli* (*12*, *13*).

It has been found that cytochrome *bd* in *E. coli* is synthesized and functions primarily at lower oxygen tension (*22*, *23*). It can be estimated from published data (*23*), for example, that the content of cytochrome *bd* in cells grown aerobicallly increases from 0.027 nmol/mg membrane protein in the log phase to 0.138 nmol/mg membrane protein in the stationary phase, *i.e.* a five-fold increase. The cytochrome *bo* content remained relatively constant under these conditions, 1.56 *versus* 2.06 nmol/mg membrane protein, respectively. Similarly, the cytochrome *bo* and *bd* contents of *Vitreoscilla* estimated in previous work (*11*) were 0.28 and 0.11 nmol/mg membrane protein, respectively. Since both the wild-type *Vitreoscilla* and *E. coli* have a ratio of cytochrome *bo* to cytochrome *bd* greater than 1 (roughly 3 and 15 in the stationary phase, respectively), the inhibition by 10 mM KCN is more than, would occur if the ratio were 1, as estimated using the K_is determined using the purified enzymes from *E. coli* (*10*). However, the sensitivity of the membrane-bound oxidases to KCN may differ from that of the purified proteins, and there may be other cyanide-sensitive respiratory enzymes in the membranes. In Fig. 1 in this paper, the wild-type *Vitreoscilla* content of cytochome *bo* was slightly higher, *i.e.* 0.55 nmol/mg membrane protein, and the *cyo*$^-$ mutant content of cytochrome *bd* was 0.39 nmol/mg membrane protein, *i.e.* a 3.5-fold increase relative to the wild-type. This increased level of cytochrome *bd* in the mutant presumably accounts for the relatively respectable respiratory rates compared to the wild-type in the various assays (Figs. 5A, 6A, and 7). Two important questions that require further investigation are whether or not oxygen and Na^+ have any regulatory role in the biosynthesis of cytochromes *bo* and *bd* in *Vitreoscilla*.

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