Nitrate Reductase Activities in Lysogenic and Nonlysogenic Strains of *Corynebacterium diphtheriae* and Related Species

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Corynebacterium diphtheriae and closely related corynebacteria are gram-positive, pleomorphic, facultatively anaerobic bacteria which have been classified (in part) according to their capacity to reduce or not reduce nitrates to nitrites. An investigation of the presence or absence of nitrate reductase activity in *C. diphtheriae*, in particular, and in *C. belfanti*, *C. ulcerans*, *C. ovis*, *C. hofmanni*, and *C. xerosis*, in general, indicates that (i) the control of the synthesis of the enzyme is a readily mutable property, (ii) enzyme synthesis occurs under anaerobic conditions, and (iii) maximal activity is associated with the "pellet" or "membrane" fraction. Additional evidence indicates that the gene *N-red* is not on the phage chromosome and, therefore, is not linked to the gene governing the synthesis of diphtherial toxin (*tox*) as previously claimed.

The bacterial nitrate reductases, which reduce nitrates to nitrites, serve one of two physiological functions: (i) that of nitrate assimilation in which nitrate is ultimately reduced to ammonium ion and is used as the cell's sole source of nitrogen under aerobic and anaerobic conditions, and (ii) that of nitrate respiration (dissimilation), an energy process in which nitrate is utilized in place of oxygen as the terminal electron acceptor, under anaerobic conditions. The latter process has been referred to as "nitrate or anaerobic respiration" (8, 22).

The capacity to reduce nitrates to nitrites has been used as a key property of *Corynebacterium diphtheriae* (7), separating it from a corynebacterium of ozaena, *C. belfanti* (5). It has been reported that corynebacteriophage β^{tox+} , when used to lysogenize *C. belfanti*, converts it to nitrate positivity (*N-red*⁺) and toxinogenicity (*tox*⁺) (11, 12). This putative lysogenic co-conversion to two positive properties aroused our interest. Here we describe certain properties of corynebacterial nitrate reductase(s) and show that the genetic control of the synthesis of this enzyme is not linked to the gene controlling the synthesis of diphtherial toxin.

MATERIALS AND METHODS

Bacterial strains. The strains of corynebacteria used were from this laboratory, except where otherwise indicated: *Corynebacterium diphtheriae*, *mitis*, C7_s(-)^{yo_x-} (hereafter referred to as C7), C7_s(β)^{yo_x+} (19), C4_s(-)^{tox-} (9) and PW8_s(P)^{tox+} (16); *gravis*, C8_s(-)^{yo_x-} (Halifax flat), C8_s(ϕ)^{yo_x+} (Halifax) (3); *intermedius*, E.B. 79 (New York University School of Medicine, The Class Collection of the Department of Microbiology) and N.S. 1057 (U.S. Armed Forces

Institute of Pathology, Washington, D.C.); *C. belfanti* strains B1, B6, B8, 1170/Ore (received from Wenche Blix Gundersen; 12); 106, 107, 109 (received from N. B. Groman, University of Washington, Seattle) and 1030 (received from A. Saragea, Bucharest, Rumania; 20); *C. ulcerans* strains 603/50 D.L.C. (13) and 842/50 D.L.C. (27); *C. ovis* strains CSIR 6 (received from H. R. Carne; 7) and 21 (received from A. Saragea; 21); *C. hofmanni* strains ATCC 10700 and 10701 and E.B. 80 (New York University Department of Microbiology); and *C. xerosis* strains ATCC 373, 7094, 7711 and 9016.

Bacteriophages. Phages β^{tox+} , $\beta^{vir\ tox+}$ ($\beta^{HV64\ tox+}$) and 1^{tox+} have been described elsewhere (14). Phage 76c/*ovis* 21, originating from a *gravis* strain, was received from A. Saragea (21). It is hereafter referred to as Z^{tox+} .

Procedures for the preparation of lysogenic corynebacteria and phage stocks, phage assays, spot testing for the detection of bacteriophage, and measurement of phage adsorption were carried out as described by Holmes and Barksdale (14) and Lampidis and Barksdale (16).

Media and general methods. The compositions of the PGT medium, maltose and glucose supplements, and tryptose agars, methods of cultivation of bacteria, and the intracutaneous test for toxinogenicity have been described elsewhere (14, 16). Tryptose-yeast extract broth contained per liter: tryptose, 10 g; yeast extract, 3 g; and phenol red, 0.005 g.

Assays for nitrate reductase activity. (i) **Qualitative determination.** Organisms to be screened for nitrate reductase activity were inoculated (in duplicate sets) into PGT liquid media or swabbed onto 1.5% (wt/vol) tryptose agar each containing 0.5% (wt/vol) maltose and 0.2% (wt/vol) potassium nitrate. Tubes and plates were incubated at 37 °C for 3 to 5 days aerobically (standard incubator atmosphere) and anaerobically. Anaerobic conditions were obtained by placing the tubes and plates in a Torbal jar (model AJ-2, Torsion Balance Company, Clifton, N.J.) and flushing for 5 min with 10% carbon dioxide in nitrogen. Three pounds of gas pres-

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sure per square inch were maintained during incubation of the jar.

The product of the nitrate reductase reaction, nitrite, was assayed by the addition to the tubes or plates of a solution consisting of equal parts of sulfanilic acid (8 g/liter of 5 N acetic acid) and of α -naphthylamine (5 g/liter of 5 N acetic acid) (29).

(ii) **Quantitative determinations.** When the nitrate reductase activity of a growing culture was being examined, organisms were inoculated into complete PGT medium containing 2% (wt/vol) maltose supplement and incubated at 37 C under three different conditions: (i) aerobically in a shaking culture (300 rpm), (ii) anaerobically by continuous sparging with nitrogen, and (iii) under quasi-anaerobic conditions employing a magnetic stirrer in a stoppered bottle. Potassium nitrate at a final concentration of 0.8% (wt/vol) was added to each culture at zero time. At specific intervals 2.5-ml samples were removed to small glass tubes and heated in a boiling water bath for 2.5 min to stop enzymatic activity. The samples were clarified by centrifugation at $1,200 \times g$ for 5 min.

When cell-free sonic extracts or their fractions were being assayed for activity, a modification of the assay procedures of Showe and DeMoss (28) and of Ruiz-Herrera et al. (26) was used. Extracts plus 0.125 M potassium nitrate and 1.25×10^{-4} M methyl viologen (Schwarz/Mann) were made up to a final volume of 2.4 ml with 0.05 M K_2HPO_4 - KH_2PO_4 buffer (pH 7.3) in test tubes (1.5 by 15 cm). The tubes were flushed for 30 s with argon, stoppered with plastic caps, and incubated for 5 min at 35 C. The reaction was initiated by the quick addition of 0.1 ml of a freshly made solution of 50 mg of $Na_2S_2O_4$ in 10 ml of 0.01 N NaOH. After 10 min at 35 C the reaction was terminated by vigorously shaking the mixture until the $Na_2S_2O_4$ was completely oxidized as indicated by the disappearance of the blue color of the reduced methyl viologen. Clarification was carried out as already indicated.

Nitrite content was measured by the method of Showe and DeMoss (28). To 2.5 ml of the nitrite-containing samples, 0.75 ml of a solution consisting of two parts of 4% (wt/vol) sulfanilamide in 25% (vol/vol) HCl and one part of an aqueous solution of 0.08% (wt/vol) *N*-1-naphthylethylenediamine dihydrochloride was added. After 10 min at room temperature the absorbancy at 540 nm was read on a Beckman model DU spectrophotometer and determinations made using a standard curve prepared using potassium nitrite.

Nitrate reductase activity was followed in growing cultures by periodic sampling. Nitrite determinations were made on bacteria-free samples as indicated above. To ascertain the nanomoles of nitrite released per microgram of bacterial nitrogen, optical density (OD) measurements (590 nm, Bausch and Lomb Spectronic 20) were determined at each interval (optical density of $0.1 \approx 1.32 \times 10^8$ bacteria per ml $\approx 5.6 \mu g$ of bacterial nitrogen per ml (4)).

In the case of sonic extracts or their fractions (see below), one unit of enzyme activity is defined as the amount of enzyme which will convert 1 μmol of nitrate to nitrite per min. The specific activity is

expressed as units of enzyme per milligram of protein (28).

Qualitative effect of pH and glucose on nitrate reductase activity. Duplicate cultures of C7 grown in complete PGT medium were centrifuged, suspended in 25 ml of PGT plus 1% (wt/vol) glucose supplement and 0.0005% (wt/vol) phenol red (final pH 6.8) to an OD of 0.5 to 1.0 in 125-ml Erlenmeyer flasks, and incubated in a water bath shaker at 35 C, 120 rpm (quasi-anaerobiosis). As the glucose was fermented and the pH was lowered, potassium hydroxide was added to one flask to neutralize the acidity. At 1-h intervals, over a 7- to 8-h period, 1.5-ml samples were removed from each flask and frozen. The samples were melted and qualitatively assayed for nitrite. In addition, pH values were determined on each sample with pHDrion papers (Micro Essential Laboratories, Brooklyn, N.Y.).

The effect of pH on nitrate reductase activity was determined by inoculating C7 to an OD of 1.0 into tryptose-yeast extract broth containing 0.5% (wt/vol) potassium nitrate unbuffered, as well as buffered with tris(hydroxymethyl)aminomethane (Tris) maleate to pH 5.5, 6.3, 7.1, and 8.2 in screw-cap tubes to one-half volume capacity and incubated at 37 C (without shaking). After 1, 2, and 3 days, samples were taken and qualitatively assayed for nitrite.

Protein determinations. Protein was precipitated overnight at 4 C from cell-free sonic extracts or their fractions by the addition of an equal volume of 10% (wt/vol) trichloroacetic acid. Precipitates were centrifuged and washed two times in 5-ml portions of cold 2.5% (wt/vol) trichloroacetic acid and redissolved in 0.1 N NaOH. Protein content was measured by the method of Lowry et al. (18) using bovine serum albumin as a standard.

Growth and disruption of bacteria. Cultures of *C. diphtheriae* C7 were inoculated into 1-liter Erlenmeyer flasks in 800-ml volumes containing PGT medium plus 0.5% (wt/vol) maltose and 1% (wt/vol) potassium nitrate. To maintain a low oxygen tension, the flasks were incubated as still cultures at 37 C. After 4 days the cultures were harvested, centrifuged (at 4 C) at $9,000 \times g$ for 20 min and washed two times with 0.05 M K_2HPO_4 - KH_2PO_4 buffer (pH 7.3). The packed, washed cells were resuspended in 9 to 10 ml of the same buffer. The concentration of bacteria was always equivalent to at least 3 mg of bacterial N per ml (4). Powdered glass beads (type 110 5005, 3M Co., St. Paul, Minn.) were added to the resuspended cells which were then subjected to sonic vibration (Sonifier Cell Disruptor, model W185, Heat Systems-Ultrasonics, Inc., Plainview, Long Island, N.Y.) in an iced container for two 2-min intervals, spaced 1 min apart. The material from disrupted cells was clarified by centrifugation at $25,000 \times g$ for 30 min. The supernatant was further fractionated by centrifugation at $100,000 \times g$ for 240 min at 4 C. Sedimented material was suspended in 0.05 M K_2HPO_4 - KH_2PO_4 buffer (pH 7.3) and is referred to as the "pellet" or "membrane" fraction; the supernatant fluid is referred to as the soluble fraction.

Isolation of nitrate reductase-deficient mutants. Under anaerobic conditions the nitrate reduc-

tases (of certain members of the *Enterobacteriaceae*) catalyze the reduction of nitrate to nitrite and of chlorate to chlorite. This latter product when formed in amounts lethal for the bacteria serves to select nitrate reductase-deficient mutants, because only those cells lacking this enzyme can survive under anaerobic conditions in the presence of chlorate (23).

C. diphtheriae strains C7 and C7(β) were plated out onto tryptose agar containing 0.5% (wt/vol) potassium nitrate and 0.2 to 0.5 M potassium chlorate and incubated at 37 C for 5 days under anaerobic conditions. The plates were incubated aerobically for an additional 2 to 3 days to allow the colonies to increase in size. Isolated colonies were picked and tested for nitrate-reducing activity.

In addition to this procedure, *N-red*⁻ mutants of C7 were obtained by treatment with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (K and K Laboratories, Plainview, N.Y.). An overnight culture of C7 was subcultured into complete PGT medium to an OD of 0.1 to 0.2 and grown up to approximately 8×10^8 cells/ml (OD $\approx$ 0.6). A 10-ml volume of culture was centrifuged, washed once in 10 ml of special Tris-maleate buffer (T-M), pH 6.0 (1), resuspended in 10 ml of T-M buffer containing 100 to 300 μ g of NTG per ml, and incubated for 15 min, at 35 C, and at 200 rpm. The treated culture was then centrifuged (4 C), washed once in cold T-M buffer, and suspended in saline to an OD of 0.5. These cells were serially diluted, plated out onto tryptose agar (plus or minus potassium chlorate), and incubated anaerobically as above. Isolated colonies were scored for nitrate reductase activity. Selected mutants were cloned and retained for further study.

RESULTS

Nitrate reductase activity among species of *Corynebacterium*. Twenty-six strains of *Corynebacterium* examined under aerobic and anaerobic conditions with regard to nitrate reductase activities are listed in Table 1. All seven *Corynebacterium diphtheriae* strains tested, *mitis*, *gravis*, and *intermedius*, as well as *C. hofmanni* strains and two strains of *C. xerosis*, showed strong activity anaerobically and weak (to moderate) activity aerobically, whereas two strains each of *C. ulcerans*, *C. ovis*, and *C. xerosis* and eight *C. belfanti* strains were negative under both sets of conditions.

***Corynebacterial* nitrate reductase.** When C7(-)^{tox}-*N-red*⁺ was grown in complete PGT medium containing 0.8% (wt/vol) potassium nitrate and incubated (i) aerobically in a shaking culture (300 rpm), (ii) anaerobically by continuous sparging with nitrogen, and (iii) employing a magnetic stirrer in a stoppered bottle under quasi-anaerobic conditions, the greatest amount of nitrate reductase activity was found under conditions of reduced oxygen tension (conditions ii and iii) (Fig. 1).

It has been reported that when glucose is used as a carbon source for *Citrobacter* sp. nitrate reductase activity is repressed (15). Preliminary tests with glucose and maltose indi-

TABLE 1. Nitrate reductase activity of various corynebacterial "species"^a

Bacterial strains	Nitrate reductase activity	
	Aerobic ^b	Anaerobic ^b
<i>Corynebacterium diphtheriae</i>		
<i>mitis</i>		
C7 _s (-) ^{tox-} , C7 _s (β) ^{tox+} , PW8 _s (P) ^{tox+}	Weak to moderate	Strong
<i>gravis</i>		
C8 _s (-) ^{tox-} , C8 _s (ϕ) ^{tox+}	Weak to moderate	Strong
<i>intermedius</i>		
E.B. 79, NS 1057	Weak to moderate	Strong
<i>C. ulcerans</i>		
603/50, 842/50	Negative	Negative
<i>C. ovis</i>		
21, CSIR 6	Negative	Negative
<i>C. belfanti</i>		
B1, B6, B8, 1170/Ore, 106, 107, 109, 1030	Negative	Negative
<i>C. xerosis</i>		
9016, 373	Weak to moderate	Strong
7094, 7711	Negative	Negative
<i>C. hofmanni</i>		
10700, 10701, E.B. 80	Weak to moderate	Strong

^a Qualitative nitrate reductase activity was determined by the intensity of the reddish color formed as a result of the diazo-coupling reagents added. Negative, no color change; weak, pink; moderate, red; strong, dark reddish-brown.

^b Condition of growth.

FIG. 1. Reduction of nitrate by $C7_s$ ($-$) $^{tox^-N-red^+}$ grown at 37°C under three different conditions of incubation: (i) aerobically in a shaking culture (300 rpm), ○; (ii) anaerobically by continuous sparging with nitrogen, ■; and (iii) under quasi-anaerobic conditions employing a magnetic stirrer in a stoppered bottle, ●.

cated that much less nitrate reductase activity was associated with glucose-grown cells. However, the final pH of the medium of the glucose-grown cells was 5.5 or lower. $C7$ grown in PGT medium containing 0.5% (wt/vol) potassium nitrate and 1% (wt/vol) glucose (under quasi-anaerobic conditions) and continually neutralized with potassium hydroxide showed nitrate reductase activity qualitatively similar to that produced by maltose-grown cells (Tables 2 and 3). In the unneutralized glucose-containing culture, the pH fell to 5.5 and there was negligible activity.

Maximal activity in the tryptose-yeast extract-nitrate broth was observed with the unbuffered culture as well as the cultures buffered at pH 7.1 and 8.2; a slightly decreased activity was observed at pH 6.3; and negligible activity was observed at pH 5.5.

When the specific nitrate reductase activity of a whole-cell sonic extract of strain $C7(-)^{tox^-N-red^+}$ and that of the soluble (supernatant) and "pellet" or "membrane" fractions were compared, about 25% of the original activity remained in the soluble fraction (see Table 4). Thus, the major nitrate reductase activity appears to be located in the "pellet" or "membrane" fraction of *C. diphtheriae* $C7$.

Lysogenization of $N-red^-$ corynebacteria. When the nitrate reductase-negative mutant $C7(-)^{tox^-N-red^-}$ was lysogenized with β^{tox^+} (1^{tox^+} or Z^{tox^+}), it became a toxin producer but re-

tained its $N-red^-$ character. $N-red^-$ mutants of $C7(\beta)^{tox^+}$ were still lysogenic and maintained their tox^+ status (Fig. 2).

TABLE 2. Effect of glucose and maltose on pH and on yields (qualitative) of nitrate reductase^a

Sugar supplement	Initial pH	Periodic neutralization with KOH	Final pH	Nitrate reductase activity (at 8 h)
Glucose	6.85	—	5.5	Negligible
Glucose	6.85	+	6.8–7.0	Strong
Maltose	6.85	—	6.6–6.8	Strong

^a $C7$ cells were inoculated to an OD of 1.0 into PGT medium containing 0.5% (wt/vol) KNO_3 supplemented with 1.0% (wt/vol) sugar and incubated under quasi-anaerobic conditions in a 37°C water bath at 120 rpm. One glucose-containing culture was periodically neutralized with KOH. The pH of each culture was determined at 0 and 8 h. Nitrate reductase activity was qualitatively determined at 8 h and scored as in Table 1.

TABLE 3. Effect of pH on yields (qualitative) of nitrate reductase^a

Tryptose-yeast extract broth	Initial pH	Final pH	Nitrate reductase activity (at 2 days)
(Unbuffered)	6.8	6.4–6.8	Strong
+ Tris-maleate	5.5	5.5	Negligible
+ Tris-maleate	6.3	6.3	Moderate to strong
+ Tris-maleate	7.1	7.1	Strong
+ Tris-maleate	8.2	8.2	Strong

^a $C7$ cells were inoculated to an OD of 1.0 into tryptose-yeast extract broths containing 0.5% (wt/vol) KNO_3 , unbuffered, and buffered with Tris-maleate (0.05 M):NaOH to pH 5.5, 6.3, 7.1, and 8.2 and incubated as still cultures at 37°C. The pH of each culture was determined at 0 and 2 days. Nitrate reductase activity was qualitatively determined at 2 days and scored as in Table 1.

TABLE 4. Localization of nitrate reductase activity in fractions of a cell-free sonic extract of *Corynebacterium diphtheriae* strain $C7$

Determination	Sp act ^a ($\times 10^{-3}$)
Extract from sonically disrupted <i>C. diphtheriae</i> $C7$	0.746
Soluble (supernatant) fraction	0.182
"Pellet" or "membrane" fraction ...	1.348

^a Specific activity is defined as enzyme units per milligram of protein. One enzyme unit is defined as the amount of enzyme which will convert 1 μ mol of nitrate to nitrite per min. Electron microscopic monitorings of the soluble fraction and the "pellet" fraction comprise Fig. 4.

Two other *tox*⁻, *N-red*⁻ corynebacteria, *C. ulcerans* 603 and *C. belfanti* 1030 when lysogenized with β^{tox+} gained the capacity to synthesize diphtherial toxin but remained *N-red*⁻. *C. ovis* (*N-red*⁻), strain 21, was not sensitive to phage β^{tox+} but could be lysogenized with phage Z^{tox+} . Strain 21(*Z*)^{tox+} was still *N-red*⁻. Thus, although lysogenicity was followed in each of these four cases by conversion to toxinogenicity, there was no change in the inability to reduce nitrates to nitrites (Fig. 3).

Adsorption to, but not lysis of, *C. belfanti* strains by phage β^{tox+} . Although seven *C. belfanti* strains were capable of removing β^{tox+} phage particles in a standard adsorption experiment with an efficiency sometimes equalling that of the C7 reference strain, they were incapable of supporting the growth of the three *tox*⁺ corynebacteriophages β , β^{vir} and 1, either in soft agar overlays on plates or during continuous subculture in the presence of excess phage (Table 5).

DISCUSSION

Our initial studies concerning the presence of the anaerobic nitrate reductase were in relation to the proposed linkage of the gene controlling its synthesis to the corynebacteriophage gene, *tox*, which governs the synthesis of diphtherial

FIG. 2. Established bacterial genomes which indicate that *tox*⁺ and *N-red*⁺ are separate and independent.

FIG. 3. Expression of *tox*⁺ in relation to *N-red*⁺ in four corynebacterial "species."

TABLE 5. Behavior of *C. belfanti*, *C. diphtheriae*, and *C. ulcerans* in the presence of various concentrations of corynebacteriophages^a

Strain	Lysis by drops (0.05 ml) of phages β , β^{vir} , 1		Formation of plaques by β , β^{vir} , 1 (replicates in)	Efficiency of adsorption of β^{tox+}
	5×10^6 PFU/ml	1×10^6 PFU/ml		
<i>C. belfanti</i> ^b				
Strain B-1	+	-	-	0.9
B-6	+	-	-	1.0
B-8	+	-	-	0.9
1170/Ore	+	-	-	0.8
106	+	-	-	1.0
107	+	-	-	1.0
109	+	-	-	1.0
<i>C. diphtheriae</i>				
Strain C7s(-) ^{tox-c}	+	+	+	1.0
C4s(-) ^{tox-}	+	+	+	1.1
<i>C. ulcerans</i>				
Strain 603	+	+	+	0.6

^a PFU, Plaque-forming units; +, lysis; -, no lysis.

^b Strains of *C. belfanti* undergo lysis from without (first two columns), but do not support phage replication (column 3). The enzymatic, nonproductive lysis which is observed results from the action of murolitic enzymes in the phage stocks.

^c Standard reference strain, C7s(-)^{tox-}, which adsorbs β^{tox+} phage with an efficiency of 1.0.

FIG. 4. Electron micrographs (prepared by K. S. Kim) of a bacteria-free sonic extract ("pellet" and soluble fractions) of *Corynebacterium diphtheriae* C7₈(-)^{tox}-N-red⁺ negatively stained with 2% (wt/vol) ammonium molybdate. (A) 100,000 × g "pellet" fraction consisting mostly of small membrane fragments associated with major nitrate reductase activity. (B) 100,000 × g soluble (supernatant) fraction consisting mostly of ribosome-like particles (±15 nm in diameter). There are no bacterial cell membrane structures. See text concerning residual activity associated with this soluble fraction.

toxin. If this linkage existed, one would expect that phage β^{tox+} would co-convert a nontoxinogenic (tox^-), nitrate reductase-deficient ($N-red^-$) corynebacterium to the ability to synthesize both diphtherial toxin and nitrate reductase. Two independent investigations employing a number of strains of the $N-red^-$ *Corynebacterium belfanti*, plus phage β^{tox+} , purportedly achieved this co-conversion in one of two ways: (i) by infecting strains with high multiplicities of β or (ii) following continuous subculture of *C. belfanti* in the presence of this same phage (17, 19; personal communication to L. B. from N. B. Groman, 1964). The $N-red^-$ strains *C. ulcerans* 603, *C. belfanti* 1030, and a *C. diphtheriae* C7 mutant are tox^- and sensitive to phage β^{tox+} . The $N-red^-$, tox^- *C. ovis*, strain 21, is sensitive to phage Z^{tox+} . Lysogenic strains prepared from each of the foregoing corynebacteria and corynebacteriophages were all tox^+ and $N-red^-$ (Fig. 3). There seems, then, no correlation between the presence of a tox^+ prophage and the capacity to reduce nitrates. Were there such a correlation, the converted $N-red^+$ strains would be heterogenotes since the nonlysogenic strains independently undergo mutation with regard to nitrate reductase activity.

Through further investigation of the corynebacterial nitrate reductase, in particular that of *C. diphtheriae* strain C7, we have shown that it is membrane associated. It has also been observed that its activity is "repressed" when cells are grown in the presence of glucose. This is actually a pH effect since it can be obtained by growing C7 in a medium having a pH of 5.5 or below. Whereas a drop in pH is always associated with glucose-grown cells, continuous neutralization of them with potassium hydroxide leads to the formation of reasonable amounts of enzyme. It appears that the corynebacterial nitrate reductase is a dissimilatory one since (i) the media being used contain organic nitrogen sources, and one would expect these to be preferentially utilized to the inorganic nitrogen source (potassium nitrate), and (ii) maximal activity is observed under conditions of anaerobiosis or reduced oxygen tension.

The capacity to reduce nitrates was early exploited as a character useful in the taxonomy of corynebacteria (6) and mycobacteria (32). In *Escherichia coli* the nitrate reductase complex appears to be controlled by several genetic loci (10). Nothing is known about the genetic control of nitrate reductase in *Corynebacterium*. The mutation from $N-red^+$ to $N-red^-$ is not uncommon. Yet, somehow in nature it does not occur very frequently or there would be more reports of isolates of *C. diphtheriae* which lack the activity. In bacterial taxonomy, trivial prop-

erties can often be successfully exploited because mutations in them or affecting their expression are selected against in the natural environment. An example in kind is the usefulness of lac^- in the characterization of *Shigella dysenteriae* and *Shigella sonnei*. Both organisms carry the *Z* gene controlling the synthesis of β -galactosidase but lack entirely the *E. coli* permease (17, 25). In the laboratory, mutants which are lac^+ are easily obtained. Yet these mutants are often susceptible to such counter-selecting agents as bacteriophages and seem never to be isolated from the wild. In the case of *C. diphtheriae* in the wild there may be a special advantage to being able to turn on nitrate respiration with the result that selective pressures strongly favor $N-red^+$ strains. This would especially be the case if, as is true for a number of gram-negative bacteria (2, 24, 30, 31), nitrate respiration in corynebacteria were cofunctional with certain other essential reductases.

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REPRINT REQUESTS

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