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Oxidation of D-Lactate and L-Lactate by *Neisseria meningitidis*: Purification and Cloning of Meningococcal D-Lactate Dehydrogenase

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***Neisseria meningitidis* was found to contain at least two lactate-oxidizing enzymes. One of these was purified 460-fold from spheroplast membranes and found to be specific primarily for D-lactate, with low-affinity activity for L-lactate. The gene for this enzyme (*dld*) was cloned, and a *dld* mutant was constructed by insertional inactivation of the gene. The mutant was unable to grow on D-lactate but retained the ability to grow on L-lactate, providing evidence for a second lactate-oxidizing enzyme with specificity for L-lactate. High-affinity L-lactate-oxidizing activity was detected in intact bacteria of both the *dld*$^+$ and *dld* mutant strains. This L-lactate-oxidizing activity was also seen in sonicated bacteria but was reduced substantially on detergent solubilization or on preparation of spheroplast membranes.**

In 1945, Grossowicz reported that *Neisseria meningitidis* could grow in a simple defined medium containing lactate as a principal carbon source (17). This was confirmed by Catlin and Schloer (8). Juni and Heym described a similar medium for growth of prototrophic *N. gonorrhoeae* (28). The isomer of lactate used in these studies was not specified, and a mixture of isomers was probably used. Since that time, DL-lactate has been used in defined media for growth of both *N. meningitidis* and *N. gonorrhoeae* (6–8, 18, 51), but little is known about the mechanisms of lactate utilization by these organisms. We have found that meningococci are able to grow on either isomer of lactate, and we have begun to study the enzymes required for oxidation of D-lactate and L-lactate.

Lactate-oxidizing activity has been described in several species of bacteria (26). In general, these enzymes are specific for one of the two isomers. In *Escherichia coli*, two enzymes with lactate dehydrogenase (LDH) activity have been well characterized (16, 19). One is specific for the L isomer and is completely unable to oxidize D-lactate. This enzyme is present only when the bacteria are grown with lactate as the principal carbon source (15). The second LDH enzyme in *E. coli* is produced during growth in glucose or in complex media, as well as in lactate. It is specific primarily for D-lactate but also has low-affinity activity toward L-lactate (14, 30), DL-α-hydroxybutyrate (14), and methyl DL-lactate (42). Both of these enzymes are membrane-associated flavoproteins and have been purified from detergent extracts. In each case, the product is pyruvate, and the electrons removed from lactate are thought to enter the electron transport chain. Similar D-specific and L-specific enzymes have been described in another gram-negative bacterium, *Acinetobacter calcoaceticus* (2). In contrast to those of mammalian LDHs, the activities of the bacterial enzymes are not dependent on NAD.

Bhatnagar et al. (3) reported that Triton X-100-solubilized membranes of *N. gonorrhoeae* have NAD-independent enzymatic activity able to oxidize both D-lactate and L-lactate. Their data show that gonococci are also able to oxidize L-phenyllactate and L-hydroxyphenyllactate, and they suggested that a single dehydrogenase with a broad substrate range may oxidize these compounds, as well as both isomers of lactate.

We report here that in *N. meningitidis*, D-lactate-oxidizing activity is carried out by a membrane-associated enzyme that appears to be similar to the D-LDH of *E. coli*. We purified this enzyme several hundredfold, cloned its gene, and constructed a mutant strain of *N. meningitidis* lacking the enzyme. This mutant retained the ability to grow on and oxidize L-lactate, indicating that meningococci possess at least two lactate-oxidizing enzymes that differ in specificity. We found that the L-lactate-oxidizing activity in *N. meningitidis* was quite labile, being lost during detergent solubilization or preparation of spheroplast membranes, procedures that do not reduce D-LDH activity. Thus the L-lactate-oxidizing activity of meningococci appears to differ from that described for *E. coli*.

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this report: PMS, phenazine methosulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BCIP, 5-bromo-4-chloro-3-indolylphosphate, *p*-toluidine salt; NBT, nitroblue tetrazolium.

Chemicals. Except as stated otherwise, chemicals were purchased from Sigma Chemical Company, St. Louis, Mo. Restriction enzymes, T4 ligase, and buffers for their use were purchased from New England BioLabs, Inc., Beverly, Mass. Glycerol, acetic acid, and acrylamide were from Fisher Scientific, Fair Lawn, N.J. Emulphogene BC-720 was a generous gift from Tom Shamper, on behalf of Rhône-Poulenc, Cranbury, N.J. Zwittergent 3-14 was purchased from Calbiochem, La Jolla, Calif.

Bacterial strains and growth. The bacterial strains and plasmids used are listed in Table 1. *N. meningitidis* was maintained on GC agar containing 1% IsoVitaleX (46). The liquid medium used routinely was tryptic soy broth (Difco Laboratories, Detroit, Mich.). The defined medium described by Catlin and Schloer (8) for growth of meningococci was prepared with either D-lactate, L-lactate, or glucose (38 mg/ml) as the principal carbon source. Other media used for meningococci were GC-hepes broth (49) and chocolate agar (pre-

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterium or plasmid	Description	Reference or source
Bacteria		
N. meningitidis BNCV	Nonencapsulated variant of M986 (group B)	C. Frasch
N. meningitidis M1080	Group B, type 1	C. Frasch
N. meningitidis M1080-A	*dld*::Tn*903*(Km)	This work
N. meningitidis M1080-B (*dld*$^+$)	Nonencapsulated, Erm^r	This work
N. meningitidis M1080-C (*dld* mutant)	Nonencapsulated, Erm^r derivative of M1080-A	This work
E. coli Y1090	Δ*lacU169 proA*$^+$ Δ*lon araD139 strA supF* (*trpC22*::Tn*10*)	52
E. coli MC1061	*hsdR mcrB araD139* Δ(*araABC-leu*)*7679* Δ*lacX74 galU galK rpsL thi*	44
E. coli XL-1 Blue	F′::Tn*10 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)M15/*recA1 endA1 gyrA96* (Nal^r) *thi*	44
Plasmids		
pUC19		44
pACYC184		9
pUC4K	Contains kanamycin resistance marker in multiple cloning site	48
pMF121	Contains erythromycin resistance marker flanked by regions from capsular synthesis locus of *N. meningitidis*	12
p3-3	pUC19 containing *dld*	This work

pared from BBL hemoglobin and BBL GC agar base [Becton Dickinson Microbiology Systems, Cockeysville, Md.] in accordance with the manufacturer's instructions). LB medium (44) and NZYCM medium (44) were used for *E. coli*.

Preparation of bacteria or bacterial fractions for LDH assay. (i) Intact bacteria and bacterial lysates. For analysis of LDH activity in unfractionated bacteria, *N. meningitidis* BNCV or M1080 was grown in tryptic soy broth to the late log phase, washed in defined medium (8) lacking a carbon source, and resuspended in defined medium either with or without 5% (vol/vol) Emulphogene BC-720. Suspensions containing detergent were then incubated for 20 min at 37°C. Untreated bacteria were kept on ice until assayed (within 2 h).

(ii) Spheroplast membranes. Membranes were prepared as described previously (47). Briefly, bacteria were treated with lysozyme and EDTA, collected by centrifugation, and disrupted further by repeated freezing and thawing. Membranes were recovered by ultracentrifugation and suspended either in 20 mM glycylglycine (pH 7.0) containing 5 mM $MgCl_2$ or in the same buffer containing 5% Emulphogene BC-720.

(iii) Sonicates. Log-phase bacteria suspended in 50 mM Tris (pH 8.0) containing 2 mM $MgCl_2$ were sonicated for 5 min on ice with a W-380 Ultrasonic Processor (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) fitted with a Microtip. Fifteen seconds of sonication was alternated with 15 s of cooling. Unbroken cells and debris were removed by centrifugation (5 min at 12,000 × *g*).

Assay of LDH activity. LDH was assayed at room temperature by dye reduction as previously described (14, 30). Oxidation of lactate (lithium salts of D-lactate or L-lactate at 5 mM or as indicated) was detected by the coupled reduction of the redox dyes PMS (120 μg/ml) and MTT (60 μg/ml). Solubilized samples were assayed in 50 mM Tris (pH 8.5) containing 0.1% Emulphogene BC-720. For assay of samples containing EDTA, 5 mM $MgCl_2$ was also included. The activity of whole bacteria or of unsolubilized membranes or other bacterial fractions was assayed in 50 mM Tris (pH 8.0) in the presence of 1 mM KCN (14). The change in A_{570} was measured with a Spectronic 3000 Array spectrophotometer (Milton Roy Company, Rochester, N.Y.). LDH activity was expressed as micromoles of MTT reduced per minute on the basis of an extinction coefficient for MTT of 17 mM^{-1} cm^{-1} (30).

For screening of column fractions, the reaction was carried out in microtiter plates, substituting phenazine ethosulfate for PMS because of its lower background (10). Reactions containing enzymatic activity turned purple, and the active fractions were then assayed spectrophotometrically.

Enzyme purification. All purification steps were carried out at 4°C unless indicated otherwise.

(i) Membrane preparation. *N. meningitidis* BNCV was grown overnight in tryptic soy broth (4 liters) at 37°C with shaking (100 rpm). Membranes were prepared as described above and suspended in 20 mM glycylglycine, pH 7.0, containing 5% Emulphogene BC-720, 5 mM $MgCl_2$, and 1 mM DTT.

(ii) Removal of detergent-insoluble material. The solubilized preparation was centrifuged at 100,000 × *g* for 1 h, and the supernatant was subjected to column chromatography.

(iii) Anion-exchange chromatography. The detergent-soluble membrane preparation was dialyzed against 20 mM Tris, pH 8.0, containing 1 mM EDTA, 0.5% Emulphogene BC-720, 15% (vol/vol) glycerol, and 1 mM DTT and then passed over a 25-ml DEAE-Sepharose column (Pharmacia LKB, Uppsala, Sweden). Activity was eluted with sodium chloride.

(iv) Ethanol precipitation and resolubilization in Zwittergent. Active fractions from the DEAE-Sepharose column were pooled and precipitated with ethanol and then resuspended at room temperature in 50 mM sodium acetate, pH 5.5, containing 1 mM EDTA, 5% (wt/vol) Zwittergent 3-14, 15% glycerol, and 1 mM DTT. Insoluble material was removed by centrifugation.

(v) Cation-exchange chromatography. The sample was diluted to 50 ml in 50 mM sodium acetate, pH 5.5, containing 1 mM EDTA, 0.05% Zwittergent 3-14, 15% glycerol, and 1 mM DTT and passed over a 12-ml S-Sepharose column (Pharmacia). Activity was eluted with sodium chloride. Active fractions were pooled and mixed with an equal volume of glycerol. DTT (1 mM) was added, and the preparation was stored at −20°C.

(vi) Phosphocellulose chromatography. The sample was dialyzed against 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.0, containing 1 mM EDTA, 0.05% Zwittergent 3-14, 15% glycerol, and 1 mM DTT and passed over a 2-ml phosphocellulose column (cellulose phosphate P11; Whatman Laboratory Division, Maidstone, England). Activity was eluted with sodium chloride. Active fractions were pooled, and after protein determination, glycerol (50%, vol/vol) and DTT (1 mM) were added. Activity was stable at −20°C for at least 6 months.

Gel electrophoresis. SDS-PAGE was done by the method of Laemmli (31). For two-dimensional gels, the first dimension was run in the absence of SDS. Individual lanes were then

stained for LDH activity (in 50 mM Tris, pH 8.0, containing 120 μg of phenazine ethosulfate per ml and 60 μg of NBT per ml and either 5 mM D-lactate or 25 mM L-lactate) or subjected to SDS-PAGE in the second dimension. Following equilibration in SDS-PAGE loading buffer, the gel section was laid horizontally in a 5.5-cm-wide well in the stacking gel of the second gel, melted agarose (1%) was added to hold the section in place, and SDS-PAGE was carried out as usual.

N-terminal amino acid sequence analysis. Purified D-LDH was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Regions of the membrane containing the 70,000-molecular-weight protein (visualized with Ponceau S) were excised and subjected to N-terminal sequence analysis with an Applied Biosystems 475A protein sequencer (11).

Cloning of the D-LDH gene. (i) Screening of a genomic library and identification of phages with LDH activity. Construction of a λgt11 library with genomic DNA from *N. meningitidis* BNCV has been described previously (41). *E. coli* Y1090 was infected (44) with the library, and the resulting plaques (approximately 5×10^4 PFU per 100-mm-diameter plate) were lifted onto nitrocellulose filters (82-mm diameter, 0.45-μm pore size; Schleicher & Schuell, Keene, N.H.). As discussed in Results, the library was screened by incubation of the filters with BCIP (50 μg/ml) and NBT (50 μg/ml) in 50 mM Tris base containing 20 mM $MgCl_2$. Approximately 5×10^5 plaques were screened, and three positive plaques were identified by development of blue spots (indicating reduction of NBT) at the corresponding sites on the filters. The subsequent determination that these phage encoded D-LDH activity, and not phosphatase activity, is described in Results.

(ii) Construction of a plasmid containing the cloned gene. DNA was isolated from one of the three phages described above, with LambdaSorb (Promega Corporation, Madison, Wis.) and digested with *Eco*RI. The *Eco*RI fragments derived from meningococcal DNA were ligated into pUC19. The resulting plasmid, designated p3-3, was transformed into *E. coli* XL-1 Blue.

Construction of a mutant *N. meningitidis* with an interruption in the cloned gene. A fragment identical to the one used to make p3-3 was cloned into pACYC184, and the gene was interrupted at an internal *Pst*I site with the kanamycin cartridge from pUC4K. *E. coli* MC1061 was used as the host strain for this construction. The resulting plasmid was used to transform *N. meningitidis* M1080 as follows. Piliated M1080 bacteria were suspended in 0.5 ml of GC-hepes broth at 6×10^7 CFU/ml, 5 μg of plasmid DNA was added, and the culture was incubated statically at 37°C for 30 min. A 4.5-ml volume of broth containing DNase I (40 μg/ml) was added, and the culture was incubated at 37°C for 2.5 h with shaking. Serial dilutions were plated on chocolate plates containing kanamycin (25 μg/ml) and incubated overnight at 37°C. Kanamycin-resistant isolates were screened for loss of D-LDH activity. Of 16 kanamycin-resistant isolates screened, 15 had substantially reduced D-LDH activity. The 16th had normal levels of both D-LDH and L-LDH activities. The kanamycin resistance of this isolate was probably due to a spontaneous mutation, since its genomic DNA failed to hybridize to the kanamycin cartridge on Southern hybridization (data not shown). One of the transformants with reduced D-LDH activity, designated M1080-A, was selected for further study.

Production of capsule-deficient mutants. To reduce the risk of laboratory-acquired infection with virulent meningococci, capsule-deficient variants of M1080 and M1080-A were produced by transformation with pMF121, which results in a large deletion at the locus required for synthesis of the capsular polysaccharide (12). The procedure was the same as that described in the previous paragraph, except that 150 ng of plasmid DNA was added to each bacterial suspension and transformants were selected by plating onto GC plates containing erythromycin (7 μg/ml). Erythromycin-resistant transformants were streaked onto group B antiserum plates (tryptic soy medium containing 1% agarose and 1 ml of horse immune serum [1]). Following overnight incubation, the plates were refrigerated for several hours. Halos of precipitin were visible around colonies of M1080 but not around those of capsule-deficient transformants. One capsule-deficient transformant of M1080 and one of M1080-A were selected for further study and designated M1080-B and M1080-C, respectively.

DNA hybridization. Genomic DNA digested with *Hin*dII was electrophoresed through a 0.7% agarose gel and blotted by capillary transfer (44) to two nylon membranes (Hybond N+; Amersham International PLC, Amersham, United Kingdom). Alkali blotting and high-stringency hybridization and washing were done as described in the Amersham protocol booklet supplied with the membranes. Probes were prepared by digesting plasmid DNA and electrophoresing it through low-melting-point agarose. The appropriate bands were excised, and the DNA was labeled with ^{32}P by using the Prime-It II random primer labeling kit from Stratagene (La Jolla, Calif.).

Oxygen uptake. Bacteria grown to the late log phase in tryptic soy broth were suspended in 50 mM sodium phosphate (pH 7.4). Aliquots of the bacterial suspension equivalent to 0.2 to 0.7 mg of protein were added to 4 ml of buffer in the reaction chamber of a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, England). The baseline oxygen consumption was recorded before addition of D- or L-lactate (2.5 mM). Oxygen consumption was reported in nanomoles of O_2 per minute per milligram of bacterial protein.

Protein assay. Protein was assayed by a bicinchoninic acid method, with the BCA Protein Assay Reagent kit from Pierce, Rockford, Ill., or by the Markwell variation of the Lowry assay (35). For determination of the protein contents of bacterial suspensions, bacteria were lysed by addition of Emulphogene BC-720 (5%, vol/vol) and stored at −20°C until assayed.

RESULTS

Characterization of LDH activity in intact meningococci and in spheroplast membranes. When we assayed LDH activity in intact bacteria by using the dye reduction assay that had been developed for assay of LDH in *E. coli*, we found that freshly harvested late-log-phase meningococci oxidized both isomers of lactate at similar rates (Table 2). Disruption of bacteria, either by detergent treatment or by spheroplast formation, altered the kinetics of lactate oxidation substantially, increasing both the maximum rate of metabolism (V_{max}) and the apparent K_m. These effects were different for the two isomers. For disrupted preparations, D-lactate was a much better substrate than L-lactate, especially at low substrate concentrations (Table 2). No lactate-oxidizing activity was recovered in periplasmic or cytoplasmic fractions. LDH activity assayed in particulate systems (i.e., in intact bacteria or in membranes that had not been exposed to detergent) was dependent on incubation (2 min or longer) in reaction buffer containing KCN. When neither KCN nor detergent was present, the onset of dye reduction was delayed several minutes, as described by Massa et al. (36).

Purification of LDH activity from meningococcal membranes. The nonionic detergent Emulphogene BC-720 solubilized both the D-lactate-oxidizing and the L-lactate-oxidizing activities of spheroplast membranes: following ultracentrifuga-

TABLE 2. Oxidation of D-lactate and L-lactate by bacteria and membranes[a]

Enzyme source	D-Lactate		L-Lactate	
	K_m (mM)	V_{max}[b]	K_m (mM)	V_{max}[b]
Intact bacteria	0.1	0.10	0.1	0.06
Detergent-lysed bacteria	1.0	0.70	40	0.35
Spheroplast membranes without detergent	5.1	0.59	14	0.06
Detergent-solubilized spheroplast membranes	0.7	0.39	47	0.25

[a] The data shown are derived from a single experiment and are representative of two or more experiments for each type of preparation. For each preparation, activity was determined by using four or more concentrations of each substrate, each assayed in duplicate. The apparent K_m and the V_{max} were determined by Lineweaver-Burke analysis.

[b] Expressed in micromoles of MTT reduced per minute per milligram of protein.

tion, no activity was recovered in the pellet. The detergent-soluble membrane preparation was subjected to column chromatography (summarized in Table 3). LDH activity in column fractions was determined by dye reduction in the presence of D-lactate (5 mM) or L-lactate (25 mM). L-Lactate was used at a higher concentration because of the low apparent affinity for L-lactate seen in solubilized preparations (Table 2). D-Lactate-oxidizing activity and L-lactate-oxidizing activity comigrated on all columns (Fig. 1).

Several features of the purification require comment. Glycerol and DTT were found to be necessary for maintenance of enzymatic activity. EDTA (1 mM) was included in column buffers to reduce the possibility of poisoning the enzyme with trace metals. It was necessary to change detergents following DEAE chromatography (Table 3, ethanol precipitation and resolubilization in Zwittergent). While Emulphogene treatment of membranes released the enzyme in a form that was not sedimented in the ultracentrifuge, it probably did not solubilize it completely. When we carried out S-Sepharose or phosphocellulose chromatography with buffers containing Emulphogene BC-720, enzymatic activity did not bind to the columns and no purification occurred (data not shown). This suggested that the Emulphogene-treated enzyme might be complexed with other proteins that prevented binding to the columns. Ethanol precipitation and resuspension in Zwittergent apparently solubilized the complexes, since we were able to purify the enzyme nearly to homogeneity by column chromatography in the presence of Zwittergent. This precipitation and resuspension step of the purification scheme also afforded substantial purification, since approximately 87% of the protein in the DEAE peak fractions was not soluble in the pH 5.5 buffer used for S-Sepharose chromatography. When resolubilization in Zwittergent was carried out without changing the pH, all of the protein was soluble in Zwittergent but most of it precipitated when dialyzed against the lower-pH buffer.

SDS-PAGE showed that purification of LDH activity enriched for one protein with an apparent molecular weight of 70,000 (Fig. 2). In the final preparation, several other proteins were also present in small amounts. To evaluate enzyme activity in these proteins, two-dimensional electrophoresis was carried out (data not shown). The first dimension was run under nondenaturing conditions, and individual lanes of the gel were stained for LDH activity. Both D-lactate-oxidizing and L-lactate-oxidizing activities were located in the same region of the gel; no staining was seen when the substrate was omitted. Following SDS-PAGE in the second dimension, the gel was stained with silver. Proteins with apparent molecular weights of approximately 55,000, 46,000, and 32,000 had migrated ahead of the enzyme activity in the first gel, while the remaining proteins, including the principal band at 70,000, migrated with the enzyme activity.

The final enzyme preparation had a specific D-LDH activity of 130 μmol of MTT reduced min^{-1} mg^{-1}, 460-fold greater than that of the solubilized crude membrane; L-lactate-oxidizing activity was increased 520-fold. During purification, we saw no evidence of a second lactate-oxidizing enzyme with specificity for L-lactate.

Amino acid sequence analysis. The N-terminal sequence of the 70,000-molecular weight protein was strongly homologous to a region close to the N terminus of *E. coli* D-LDH, with 9 identical amino acids and 12 conservative replacements among the first 24 (Fig. 3). Comparison was made with the LFASTA program (32).

Characterization of enzymatic activity of purified LDH. The purified enzyme had an apparent K_m for D-lactate of 0.59 mM (V_{max}, 149 μmol min^{-1} mg^{-1}). The apparent K_m for L-lactate was 32.2 mM (V_{max}, 83 μmol min^{-1} mg^{-1}). Addition of NAD (100 μM), flavin adenine dinucleotide (60 μM), or flavin mononucleotide (60 μM) did not affect enzymatic activity.

The substrate specificity of meningococcal D-LDH was compared with the specificities reported for lactate-oxidizing enzymes of *E. coli* and *N. gonorrhoeae* (see introduction) and for some other dehydrogenases. Activity toward 25 mM α-DL-hydroxybutyrate was 10% of that toward 5 mM D-lactate. Activity toward 25 mM D-threonine or L-threonine was between 0.5 and 1% of the D-LDH activity. No activity toward ethanol, glycerol, succinate, D-malate, L-malate, D-tartrate, L-tartrate, D-3-phosphoglycerate, L-phenyllactate, DL-phenyllactate, or DL-hydroxyphenyllactate (all tested at 5 and 25 mM) was seen.

TABLE 3. Purification of D-LDH from meningococcal spheroplast membranes

Procedure	D-LDH activity[a]		Protein content (mg)		Sp act[b]	Fold purification
	Total in fraction	% of initial amt	Total in fraction	% of initial amt		
Membrane preparation	790	100	2,800	100	0.28	1
Detergent extraction	810	103	1,800	65	0.45	1.6
DEAE-Sepharose column chromatography	420	54	250	9.1	1.7	5.4
Ethanol precipitation, resolubilization in Zwittergent	330	42	32	1.2	10	36
S-Sepharose column chromatography	250	31	3.5	0.13	71	250
Phosphocellulose column chromatography	120	15	0.91	0.03	130	460

[a] Expressed in micromoles of MTT reduced per minute.

[b] Expressed in micromoles of MTT reduced per minute per milligram of protein.

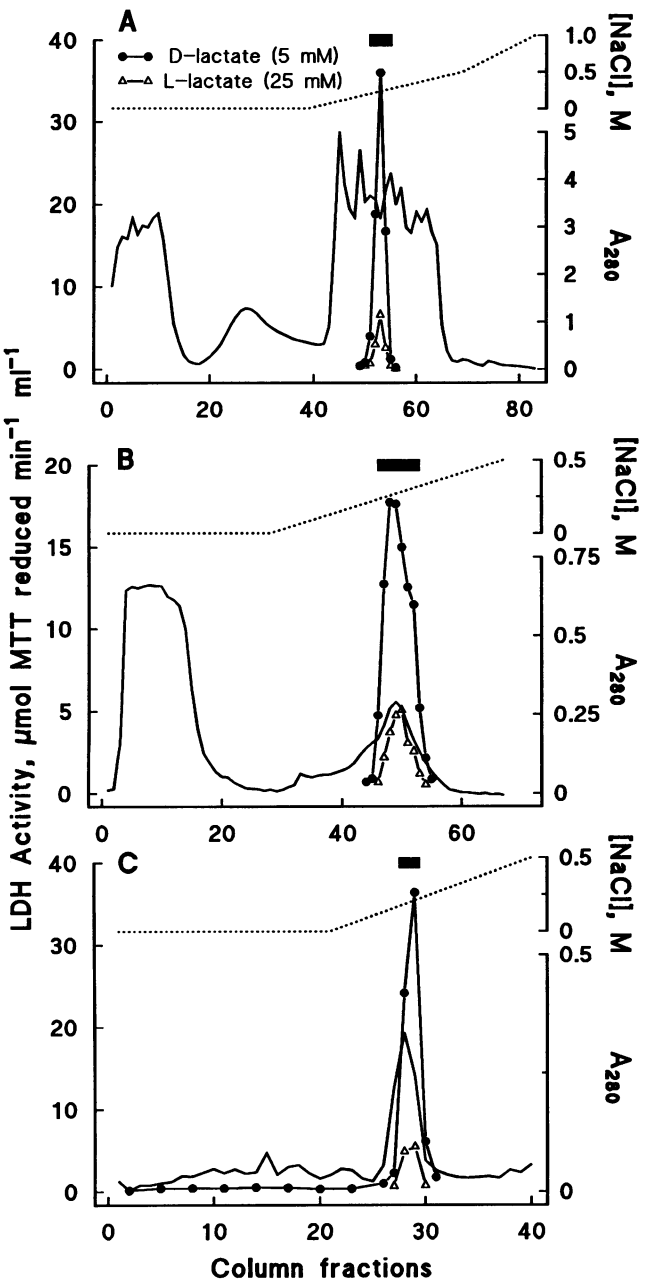

FIG. 1. Purification of D-LDH from meningococcal membranes by column chromatography. Symbols: ●, LDH activity per milliliter of column eluate, measured with 5 mM D-lactate; △, LDH activity measured with 25 mM L-lactate. ———, A_{280}; ·····, NaCl concentration. The horizontal bar above each panel indicates the fractions pooled for the next step. Panels: A, DEAE-Sepharose column; B, S-Sepharose column; C, phosphocellulose column.

Several of the α-hydroxy acids that did not serve as substrates were found to inhibit D-lactate-dependent dye-reducing activity. When these compounds were tested at 20 mM in the presence of 5 mM D-lactate, we found that D-malate, L-malate, and D-tartrate inhibited the reaction completely. L-Tartrate, D-3-phosphoglycerate, L-phenyllactate, DL-phenyllactate, and DL-hydroxyphenyllactate inhibited the reaction to various extents. In addition, pyruvic acid and oxamic acid, tested at 20 mM, also inhibited the reaction, by 74 and 83%, respectively.

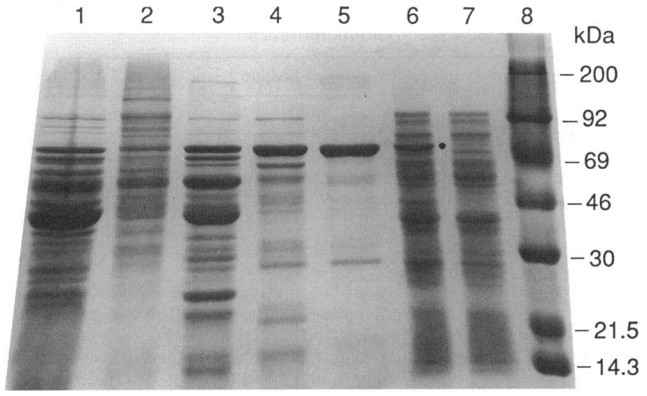

FIG. 2. SDS-PAGE of fractions obtained during purification of D-LDH and comparison with product of cloned DNA. Lanes: 1, total membranes; 2, Emulphogene-soluble membranes; 3, peak fractions from DEAE-Sepharose column; 4, peak fractions from S-Sepharose column; 5, peak fractions from phosphocellulose column; 6, lysate of *E. coli* XL-1 Blue carrying p3-3; 7, lysate of *E. coli* XL-1 Blue carrying pUC19; 8, molecular size markers (Rainbow Markers; Amersham International, Amersham, United Kingdom). A dot to the right of lane 6 identifies the protein expressed by the cloned DNA. The gel was stained with Coomassie blue.

The dependence of enzyme activity on pH (Fig. 4) was affected by the presence of other membrane proteins or a detergent: while the optimum pH for D-LDH activity in Emulphogene-solubilized spheroplast membranes was 8.5, the optimum for the purified enzyme was 8.0. Activity toward L-lactate was less affected by pH. In contrast, for spheroplast membranes assayed in the absence of detergent, the optimal pH was 7.1 or lower. (Since this activity was dependent on cyanide, it was not assayed in acid buffers.)

The role of metal ions was tested by adding metal salts (final concentration, 5 mM) to the assay buffer. When $MgCl_2$ was added, activity was 133% of that seen when no metal was added. $CaCl_2$ and $MgSO_4$ also stimulated activity, to 120% of that seen without added metal ions. Other metals tested inhibited activity to various extents ($MnCl_2$ and $NiCl_2$, 18% of maximal activity; $CoCl_2$, 5%; $ZnCl_2$ and $CuSO_4$, <1%).

Cloning of the gene that encodes meningococcal D-LDH. A clone that encodes meningococcal D-LDH was identified fortuitously during screening of a λgt11 genomic library for phosphatase activity, with a system previously described for histochemical staining of alkaline phosphatase (37) and adapted for use on nitrocellulose (4). In this system, cleavage of the phosphatase substrate BCIP results in reduction of the tetrazolium dye NBT, forming a blue precipitate which identifies the location of the phosphatase. We had identified and plaque purified three phages with presumptive alkaline phos-

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                    5   10   15   20   25   30
E. coli         MSSMTTTDNKAFLNELARLVGSSHLLTDPAK
                ....:. :.. ::.....:::::
N. meningitidis XASQLLSRLTQTVGEKYIITDPAK
                  5   10   15   20
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FIG. 3. N-terminal amino acid sequence analysis. The sequence obtained for the 70,000-molecular-weight protein purified from *N. meningitidis* BNCV is compared with that reported for *E. coli* D-LDH (43). Standard single-letter codes for amino acids are used. The letter X represents a residue whose identity could not be determined. Colons designate identical amino acids; periods indicate conservative replacements.

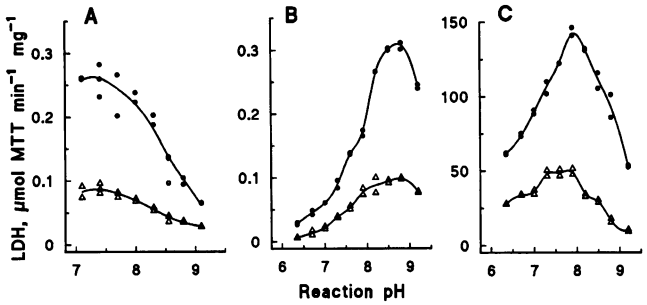

FIG. 4. Effect of pH on LDH activity per milligram of protein. Panels: A, membranes without detergent; B, membranes in buffer containing 5% Emulphogene; C, purified D-LDH. Assays were carried out in 50 mM Tris at various pHs; the substrates were D-lactate (5 mM; ●) and L-lactate (50 mM; △).

phatase activity, when it was discovered that reduction of NBT by plaques was not dependent on the presence of BCIP. This indicated that the activity expressed by these phages was not phosphatase but some other activity that resulted in reduction of NBT, perhaps an enzyme that catalyzes an oxidation-reduction reaction.

Several compounds were tested as potential electron donors. When nitrocellulose filters overlaid on pure phage populations (~50,000 plaques per filter) were incubated with NBT in the presence of D-lactate (5 mM), dark blue spots (indicating reactive plaques) developed rapidly, while in the presence of L-lactate, succinate, or BCIP or in the absence of an electron donor, the blue spots were fainter and developed much more slowly. Plaques of λgt11 did not produce blue spots with any of these substrates. The activity expressed by the phages was thus identified as D-lactate-dependent reduction of NBT. The same reactivity was seen with each of the three phages originally identified by NBT-reducing activity in the presence of BCIP.

The meningococcal insert in one of the three phages was cloned into pUC19. A restriction map of this insert (2.6 kb) is shown in Fig. 5. Analysis of DNAs from the three phages by Southern hybridization, with the cloned insert as a probe, indicated that each insert was slightly different but all had a common region of approximately 2.1 kb (data not shown).

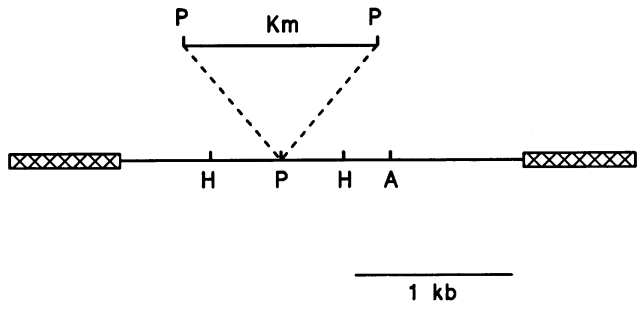

FIG. 5. Restriction map of the cloned DNA and construction of the mutant. The cloned DNA, approximately 2.6 kb long, is indicated by the central horizontal line, and the cross-hatched regions represent flanking vector sequences. The approximate positions of *Hin*dII (H), *Pst*I (P), and *Ava*I (A) restriction sites are indicated. The following enzymes failed to cut within the insert: *Acc*I, *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Pvu*II, *Sac*I, *Sma*I, *Sph*I, and *Xba*I. The upper horizontal line (Km) represents the 1,440-bp *Pst*I fragment of plasmid pUC4K, containing a kanamycin resistance cartridge, which was inserted into the cloned DNA at its *Pst*I site.

D-LDH activity in *E. coli* XL-1 Blue carrying the cloned gene was 70 times greater than that in XL-1 Blue carrying the vector alone. A similar increase in L-lactate-oxidizing activity was also seen, provided a high concentration of L-lactate was used. This is consistent with our analysis of purified meningococcal LDH, which has low-affinity activity toward L-lactate but much-higher-affinity activity toward D-lactate. About half (52%) of the cloned LDH activity was recovered in the membranes of *E. coli*, but part was recovered in the cytoplasm (20%) and periplasm (28%). Analysis by SDS-PAGE of fractions of *E. coli* carrying the cloned gene or carrying the vector alone showed that the cloned gene resulted in production of a protein with an apparent molecular weight of approximately 70,000 (Fig. 2). These findings suggest that expression of the cloned DNA produces the same meningococcal LDH that was purified. The gene was designated *dld*, the name given to the gene for NAD-independent D-LDH of *E. coli* (21, 45).

Phenotype of a *dld* mutant of *N. meningitidis*: evidence for a second LDH specific for L-lactate. A kanamycin resistance marker was inserted into the cloned DNA (Fig. 5), and the plasmid containing the interrupted gene was transformed into *N. meningitidis* M1080. (Strain BNCV, from which the gene is derived, could not be used since it is nonpiliated and consequently not competent for DNA uptake.) Since the plasmid would not be expected to survive in *N. meningitidis*, growth of kanamycin-resistant transformants is presumptive evidence that the transformed DNA has recombined with meningococcal DNA, most likely with the endogenous gene for D-LDH. Study of the phenotype of the presumptive *dld* mutant was carried out with nonencapsulated variants of the mutant and M1080 (strains M1080-C and M1080-B, respectively). For simplicity, these will be referred to below as the *dld* mutant and *dld*$^+$ strains.

Analysis of genomic DNA from these strains by Southern hybridization, with the cloned DNA and the kanamycin resistance cartridge as probes, was consistent with insertion of the kanamycin gene into the *dld* gene (Fig. 6).

The *dld*$^+$ strain grew in either isomer of lactate and also in glucose (Fig. 7). The *dld* mutant strain was unable to grow in defined medium containing D-lactate as the principal carbon source. It was, however, able to grow in L-lactate, and growth in glucose was unchanged.

Further evidence of the ability of the *dld* mutant strain to oxidize L-lactate was obtained by studies of lactate-dependent oxygen utilization. Oxygen uptake by the *dld*$^+$ strain in the presence of D-lactate was 369 ± 80 nmol min^{-1} mg of $protein^{-1}$; in the presence of L-lactate, it was 317 ± 80 nmol min^{-1} mg^{-1}. Addition of D-lactate to suspensions of the *dld* mutant strain resulted in no increase in oxygen consumption (-12 ± 24 nmol min^{-1} mg^{-1}), but addition of L-lactate to the *dld* mutant strain produced oxygen uptake that was two-thirds that of the *dld*$^+$ strain, 213 ± 52 nmol min^{-1} mg^{-1}. (These data are means ± standard deviations of six or seven observations from two experiments.)

Oxidation of lactate by intact bacteria of the *dld*$^+$ strain and the *dld* mutant strain was also assayed by dye reduction (Fig. 8A). As seen previously for strain BNCV, the *dld*$^+$ strain was able to oxidize both isomers of lactate, with the maximal rate for D-lactate about twice that seen for L-lactate. The *dld* mutant strain was able to oxidize L-lactate at rates close to those seen for the *dld*$^+$ strain but did not oxidize D-lactate. However, detergent lysis of the same bacteria gave somewhat different results (Fig. 8B). Lysates of the *dld* mutant strain oxidized neither isomer of lactate. Lysates of the *dld*$^+$ strain oxidized both isomers, with much greater activity for D-lactate than for L-lactate. Thus, L-LDH activity of the *dld* mutant

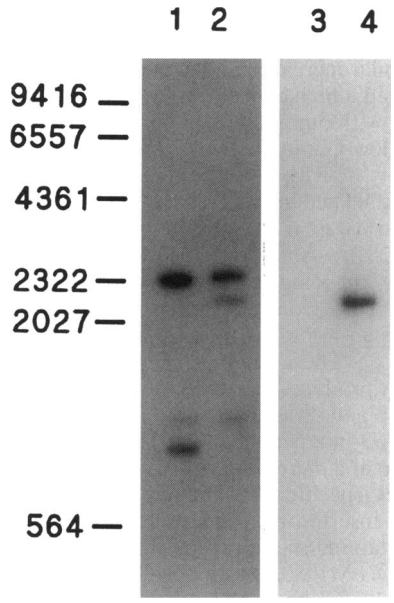

FIG. 6. Analysis of the insertional mutation by Southern hybridization. Autoradiographs of duplicate blots from a single gel containing *Hin*dII-digested genomic DNAs from M1080-B and M1080-C are shown. The blot shown on the left was probed with the cloned DNA, containing the *dld* gene, which hybridized to three bands in each lane. A band of approximately 850 bp in M1080-B (lane 1) was replaced in M1080-C (lane 2) by a larger band, consistent with insertion of the kanamycin cartridge into an internal *Hin*dII fragment (see the map in Fig. 5). The duplicate blot, shown on the right, was probed with a *Pst*I fragment from pUC4K, containing the kanamycin cartridge. This probe did not hybridize to M1080-B (lane 3) and hybridized to a single band in M1080-C (lane 4), corresponding to the new band seen in lane 2. The numbers on the left are molecular sizes in base pairs.

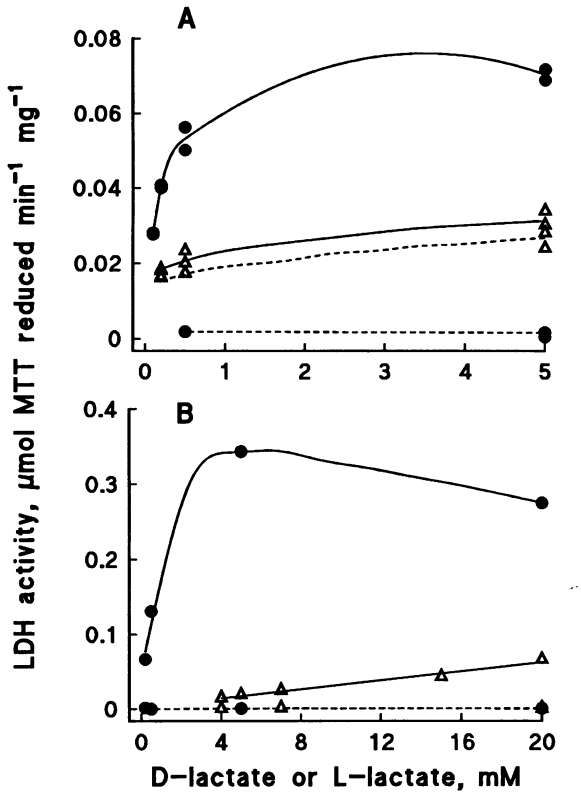

FIG. 8. Effect of the *dld* mutation on D-LDH and L-LDH activities per milligram of protein. The LDH activities of strains M1080-B (*dld*$^+$; solid lines) and M1080-C (*dld* mutant; dashed lines) were assayed by using various concentrations of D-lactate (●) or L-lactate (△). Panels: A, intact bacteria; B, detergent-lysed bacteria.

strain was demonstrable in intact bacteria but was lost on detergent lysis. The L-lactate-oxidizing activity in lysates of the *dld*$^+$ strain can be attributed to the low-affinity activity of the D-LDH enzyme for L-lactate, demonstrated for the purified enzyme.

The L-LDH activity of the *dld* mutant was also reduced substantially during preparation of spheroplast membranes. Formation of spheroplasts by lysozyme treatment resulted in a substantial increase in D-LDH activity of the *dld*$^+$ strain, compared with the activity seen in intact bacteria (Table 4, spheroplast preparation). This is consistent with the previous finding that spheroplast membranes from strain BNCV have a greater V_{max} for D-lactate than do the corresponding intact bacteria (Table 2). For the *dld* mutant strain, spheroplast L-LDH activity was the same as that seen in intact bacteria. When spheroplasts were disrupted by freezing and thawing, D-LDH activity of the *dld*$^+$ strain was reduced only slightly but the L-LDH activity of the *dld* mutant strain was reduced to nearly undetectable levels (Table 4, spheroplast preparation).

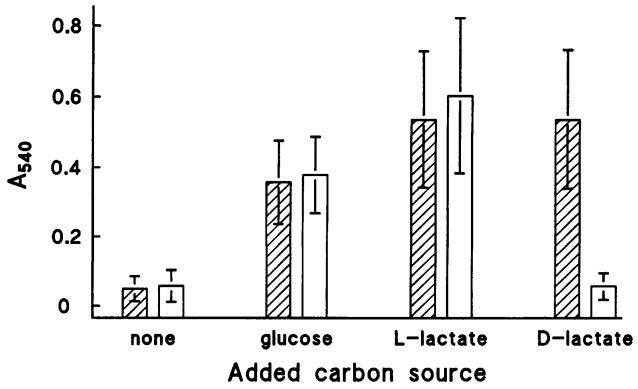

FIG. 7. Effect of the *dld* mutation on growth in lactate. Strains M1080-B (dld$^+$, shaded bars) and M1080-C (*dld* mutant, open bars) were grown in defined media containing glucose, L-lactate, D-lactate, or no added carbon source. The A_{540} of each culture after 7 h of incubation is shown. Each bar represents the mean ± the standard deviation data from three experiments.

Sonicated bacteria retain L-LDH activity. When meningococci were disrupted by sonication, the D-LDH activity of the *dld*$^+$ strain was increased substantially, as had been seen for disruption by spheroplast formation. The L-LDH activity of the *dld* mutant strain was increased slightly following sonication (Table 4, sonication). Most of the D-LDH and L-LDH activities remained in the supernatants following low-speed centrifugation to remove unbroken bacteria.

Further characterization of the LDH activities of sonicated bacteria was done with cell-free supernatants. As seen for the purified enzyme, the D-LDH activity of a sonicate of the *dld*$^+$ strain had a pH optimum of 8. In contrast, the L-LDH activities of both the *dld*$^+$ and *dld* mutant strains had pH optima at 8.8 or higher (Fig. 9). In a separate experiment, a sonicate of the *dld* mutant strain was found to have an apparent K_m for

TABLE 4. LDH activity in meningococci disrupted by lysozyme treatment or sonication

Enzyme source	LDH activity[a] of following strain[b] on substrate indicated:			
	dld$^+$		*dld* mutant	
	D-Lactate	L-Lactate	D-Lactate	L-Lactate
Intact bacteria	0.046	0.021	<0.004	0.020
Spheroplast preparation[d]				
Step 1	0.364	0.041	ND[c]	0.022
Step 2	0.348	0.028	ND	0.008
Sonication[e]				
Step 1	0.282	0.031	ND	0.030
Step 2	0.252	0.028	0.006	0.024

[a] LDH activity is expressed in micromoles of MTT reduced per minute per milligram of protein. Since the D-LDH activity of strain M1080-B varied from one experiment to another, the data presented are means of duplicate assays from a single experiment, which is representative of at least four preparations of spheroplasts or sonicates from these strains.

[b] Strains: *dld*$^+$, M1080-B; *dld* mutant, M1080-C. Both substrates were used at 5 mM.

[c] ND, not determined.

[d] Spheroplast preparation steps (see Materials and Methods for details): 1, following lysozyme treatment, spheroplasts were pelleted and then resuspended in $MgCl_2$-DNase; 2, spheroplasts from step 1 were frozen and thawed four times.

[e] Sonication steps: 1, bacteria were sonicated; 2, intact bacteria were removed by centrifugation at 12,000 × *g*.

L-lactate of 0.09 mM (V_{max}, 0.045 μmol of MTT min^{-1} mg^{-1}), similar to that of intact bacteria (Table 2). Both L-LDH and D-LDH activities of sonicates were dependent on the presence of KCN and did not require addition of NAD. The L-LDH activity of sonicates was unstable, with 50 to 80% of the activity lost during overnight storage at 4 or −20°C.

DISCUSSION

While previous studies have shown that meningococci can grow on DL-lactate, ours is the first to examine utilization of the individual isomers. We found that meningococci were able to grow on either D-lactate or L-lactate at least as well as on glucose. This finding may have implications for meningococcal physiology, since the two isomers of lactate may be available at different sites within a human host. Lactate produced by mammalian cells is the L isomer (33). While D-lactate can be detected in human blood, perhaps absorbed from the gut, its concentration in serum is only 1 to 5% of that of L-lactate (38). In contrast, D-lactate can be produced as a byproduct of glucose metabolism by some lactic acid bacteria, as well as by *E. coli*, so it may be available on the mucosal surfaces that pathogenic neisseriae colonize.

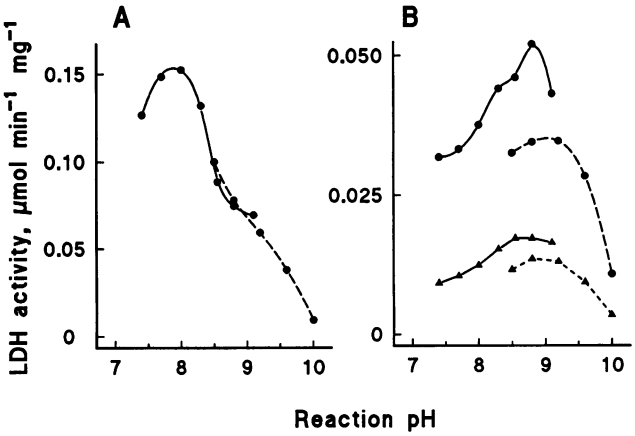

FIG. 9. Effect of pH on LDH activity per milligram of protein of sonicates (supernatant following centrifugation at 12,000 × *g*). Activity was measured by using 5 mM D-lactate (A) or 5 mM L-lactate (B). Sonicates were derived from strains M1080-B (*dld*$^+$) (●) and M1080-C (*dld* mutant) (▲). ———, Tris buffers (50 mM); – – –, glycine-NaOH buffers (50 mM glycine).

Our data indicate that meningococci contain at least two lactate-oxidizing enzymes. We purified one of these from detergent-solubilized spheroplast membranes and found it to be specific primarily for the D isomer, with a low-affinity activity toward L-lactate. Three lines of evidence suggest that this L-lactate-oxidizing activity is a property of the D-LDH enzyme and not of a second LDH. (i) The relative proportions of D-lactate-oxidizing and L-lactate-oxidizing activities in detergent-solubilized membranes are the same as those in the purified enzyme. Following the initial loss of L-LDH activity during membrane preparation and solubilization (as seen in Table 2), the ratio of L-lactate-oxidizing activity to D-lactate-oxidizing activity remained constant while the D-LDH activity was purified approximately 500-fold. The two activities also comigrated during further separation on a nondenaturing gel. (ii) Expression of the cloned DNA in *E. coli* resulted in similar increases toward D-lactate and L-lactate. (iii) A detergent lysate of the *dld* mutant did not oxidize either L-lactate or D-lactate (Fig. 8). In each of these cases, the L-lactate-oxidizing activity was evident only when high concentrations of the substrate were used. It seems likely, therefore, that meningococcal D-LDH is similar to *E. coli* D-LDH (14, 30) in having a low-affinity activity toward the opposite isomer.

We were unable to demonstrate a higher-affinity L-LDH in spheroplast membranes, leading to the hypothesis that in meningococci a single enzyme is responsible for oxidizing both isomers of lactate. This hypothesis was tested by constructing a mutant in which the gene for D-LDH was interrupted. The mutant was no longer able to utilize D-lactate or oxidize D-lactate, as predicted. However, it retained the ability to grow in L-lactate. Oxidation of lactate, measured by using either oxygen or redox dyes as the electron acceptor, was reduced only slightly. Thus, the hypothesis was disproven. Our inability to demonstrate a distinct L-LDH during enzyme purification was explained by the finding that L-LDH activity in the mutant was largely destroyed by detergent lysis (Fig. 8) or by preparation of spheroplast membranes (Table 4). We were, however, able to demonstrate cell-free L-LDH activity in sonicates of meningococci. This activity had a high affinity for L-lactate and a higher pH optimum than D-LDH—clearly distinguishing it from the low-affinity L-lactate-oxidizing activity possessed by spheroplast membranes and by purified D-LDH.

The D-lactate-oxidizing enzyme we purified and cloned from meningococci seems to be similar to the D-LDH of *E. coli*, both biochemically and by molecular analysis. The predominant protein in the purified preparation has an apparent molecular weight of 70,000; *E. coli* D-LDH has a molecular weight of 65,000 (19). Partial amino acid sequence analysis of the 70,000-molecular-weight meningococcal protein indicated structural similarity to *E. coli* D-LDH.

The meningococcal enzyme is membrane associated, and since it is solubilized by treatment with nonionic detergent in the presence of divalent cations, it is likely to be associated with the cytoplasmic rather than the outer membrane. This notion is supported by the previous finding of D-LDH activity

in the cytoplasmic membrane (separated by sucrose density gradient centrifugation) of *N. gonorrhoeae* (27). Like *E. coli* D-LDH (14, 30), the meningococcal enzyme can utilize 2,6-dichlorophenol-indophenol as an electron acceptor in place of PMS-MTT (data not shown). We found also that while electrons could not be transferred directly from the enzyme to MTT, coenzyme Q_0, a ubiquinone, could replace PMS as an initial electron acceptor (data not shown). This is consistent with a role in electron transport, as for *E. coli*. The requirement for KCN in preparations lacking detergent also supports this idea. The *E. coli* enzyme is activated by nonionic detergents (19). The meningococcal D-LDH activity was increased following solubilization of bacteria or membranes in Emulphogene BC-720 (Table 2), and activity of the Zwittergent-solubilized enzyme was greater when measured in the presence of Emulphogene BC-720 (data not shown).

For both the *E. coli* and *N. meningitidis* activities, D-LDH in spheroplast membranes (without detergent) has optimal activity below pH 7; following purification, both enzymes have optimum activity at pH 8 or higher (Fig. 3) (14, 19). While the purified enzymes presumably transfer electrons directly to PMS, oxidation of lactate by spheroplast membranes may result in flux of electrons from the enzyme through additional electron transport components before transfer to the dye. Such additional steps might alter the pH dependence of the observed reaction.

In contrast, meningococcal L-LDH appears to be quite different from that of *E. coli*. (i) It is produced constitutively, being present in bacteria grown in complex media, whereas *E. coli* L-LDH is not present unless induced by growth in lactate (15). (ii) The meningococcal activity is labile, being lost during procedures (physical disruption or detergent solubilization) that do not affect *E. coli* L-LDH activity.

Our findings on meningococcal L-LDH activity are consistent with those of Hoshino et al. (25). They studied lactate oxidation by a *Neisseria* species isolated from dental plaque and found that while whole cells converted similar amounts of radiolabeled D-lactate and L-lactate to pyruvate, cell extracts had nine times as much D-LDH activity as L-LDH activity. They saw similar results with 20 of 23 other neisserial strains (23), with *Veillonella dispar* (22), and with *V. alcalescens* and *V. parvula* (24). These reports support our finding of relative loss of L-LDH activity on disruption of meningococci and suggest that this phenomenon is not confined to *Neisseria* spp.

Previous work on lactate metabolism of meningococci was carried out by Holten and Jyssum (20, 29). They assayed oxidation of lactate by monitoring the reduction of NAD. In contrast, we were not able to detect D-lactate- or L-lactate-dependent reduction of NAD, either in sonicates or in spheroplast membranes. It is possible that the NAD-linked LDH activity they detected is regulated and is not produced under our culture conditions. Holten and Jyssum reported that meningococcal sonicates were also able to carry out the reverse reaction, reduction of pyruvate accompanied by oxidation of NADH (20, 29). Our experiments confirmed this (data not shown).

Lactate-oxidizing activities of *N. gonorrhoeae* have been reported but have not been well characterized. Intact gonococci have been reported to oxidize both D-lactate (5) and L-lactate (5, 13, 34). Membranes prepared from gonococci have also been reported to oxidize D-lactate (27) and L-lactate (40, 50). We have found no reports in which both D-LDH and L-LDH activities were assayed in a single gonococcal membrane preparation. Preliminary data obtained in our laboratory suggest that gonococci are similar to meningococci in that the primary L-lactate-oxidizing enzyme is very labile and that D-LDH also has low-affinity activity toward L-lactate. It is possible that the L-lactate-oxidizing activity of gonococcal membranes described previously was the result of the latter enzyme or that the methods used by others for membrane preparation preserved the labile L-LDH better than our methods have.

As noted earlier, Bhatnagar et al. (3) reported that gonococci have L-phenyllactate dehydrogenase and L-hydroxyphenyllactate dehydrogenase activities and suggested that these activities, as well as oxidation of D-lactate, may be catalyzed by a single LDH with a broad substrate range. This is clearly not the case for meningococci, since we were able to inactivate the D-LDH gene without affecting L-LDH activity. Development of techniques for solubilization of meningococcal L-LDH is necessary before its substrate range can be determined.

Pathogenic *Neisseria* spp. are able to metabolize only a few carbohydrate sources (39). The ability of these fastidious organisms to oxidize D-lactate and L-lactate suggests that these compounds can serve as carbon and energy sources in vivo. Further characterization of D-LDH and L-LDH activities of meningococci and gonococci, and generation of additional mutants, may allow evaluation of the role of lactate metabolism in neisserial physiology and pathogenesis.

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