Cloning of the Gene for the Respiratory D-Lactate Dehydrogenase of *Escherichia coli*†

Ian G. Young,* Anthony Jaworowski, and Maria Poulis

ABSTRACT: During attempts to clone the gene coding for the respiratory NADH dehydrogenase of *Escherichia coli*, two hybrid plasmids were constructed from *E. coli* chromosomal DNA [Young, I. G., Jaworowski, A., & Poulis, M. I. (1978) *Gene 4*, 25–36]. One of these plasmids, pIY1, derived from *Eco*RI-digested chromosomal DNA, was studied in detail and shown to possess the gene coding for the NADH dehydrogenase of the aerobic respiratory chain of *E. coli*. We now report the characterization of the other hybrid plasmid, pIY2, derived from *Hin*dIII-digested chromosomal DNA, and show that it complements *ndh* mutants not by virtue of carrying the *ndh* gene but because it carries the gene coding for the respiratory D-lactate dehydrogenase. Cells carrying this hybrid plasmid overproduce the respiratory D-lactate dehydrogenase in their cell membranes by 15–20-fold with negligible activity appearing in the cytoplasm. This results in an amplification of the levels of the D-lactate oxidase. The amplified D-lactate oxidase activity, coupled with the pyridine nucleotide linked D-lactate dehydrogenase, apparently provides a new pathway for the oxidation of reduced nicotinamide adenine dinucleotide (NADH) in the cell, independent of the respiratory NADH oxidase.

The respiratory D-lactate dehydrogenase of *Escherichia coli* is the first component of the membrane-bound D-lactate oxidase of this organism and plays an important role in generating the energy used for the active transport of various sugars and amino acids in *E. coli* [see Kaback (1974)]. This enzyme has been solubilized and purified by several different laboratories (Futai, 1973; Kohn & Kaback, 1973; Pratt et al., 1979) and shown to consist of a single subunit of $M_r \sim 74000$. It is located together with the other respiratory components in the inner or cytoplasmic membrane of *E. coli*.

We have previously reported the characterization of a hybrid plasmid (pIY1) that carries the gene coding for the respiratory NADH dehydrogenase of *E. coli* (Young et al., 1978, 1981; Poulis et al., 1981). This plasmid was isolated from an *E. coli* chromosomal library by its ability to complement an *ndh* mutant, which is defective in NADH dehydrogenase (Young & Wallace, 1976). A second plasmid (pIY2) was also isolated that had the same property but did not carry the *ndh* gene. In the present work we describe the characterization of the plasmid pIY2 and show that it carries the gene coding for the respiratory D-lactate dehydrogenase. The unexpected property of this plasmid of overcoming the defect in reduced nicotinamide adenine dinucleotide (NADH) oxidation in *ndh* mutants can be attributed to the overproduction of D-lactate dehydrogenase, which appears to generate an alternative route for NADH oxidation.

Experimental Procedures

Bacterial Strains. All strains used were derivatives of *E. coli* K12. The construction of strains IY35 and IY36, both derived from IY12 (*thi*, *his*, *ilv*, *trp*, *rps*L, *ndh*) and carrying the plasmids pIY1 and pIY2, respectively, has been described previously (Young et al., 1978). IY13 (*thi*, *his*, *ilv*, *trp*, *rps*L) is an isogenic *ndh*$^+$ transductant of IY12. IY65, derived from IY13, carries pGM706.

Media, Chemicals, and Enzymes. The mineral salt medium and the concentration of supplements used have been described previously (Stroobant et al., 1972). The complete medium was brain heart infusion (Oxoid). Chemicals and enzymes used were obtained from the following sources: ethidium bromide, agarose, chloramphenicol, D(−)-lactic acid (lithium salt), lysozyme, and L-lactate dehydrogenase (rabbit muscle), Sigma Chemical Co. (St. Louis, MO); CsCl, BDH Chemicals Ltd. (Poole, England); *Eco*RI and *Hin*dIII restriction endonucleases, Miles Research Products (Elkhart, IN); L(+)-lactic acid (lithium salt), Calbiochem (San Diego, CA); D-lactate dehydrogenase (*Lactobacillus leichmanii*), Boehringer Mannheim GmbH (West Germany). Purified D-lactate dehydrogenase from *E. coli* was generously donated by Dr. H. R. Kaback.

Isolation and Restriction Analysis of Plasmid DNA. The procedures for isolation of plasmid DNA and restriction with *Eco*RI and *Hin*dIII have been described previously (Young et al., 1978). The results of endonuclease digestions were monitored by electrophoresis on 0.8% agarose gels (Young et al., 1978); 1 μg of DNA was used unless otherwise indicated.

DNA Transfer and Hybridization. Chromosomal DNA from *E. coli* was prepared according to Marmur (1961). A limit digest of the DNA was prepared with *Hin*dIII restriction endonuclease, and three samples each containing 5 μg of DNA were separated by electrophoresis on a 0.6% agarose slab gel (Young et al., 1978). After electrophoresis the DNA was transferred to a sheet of nitrocellulose by the procedures of Southern (1979) and the membrane dried for 2 h at 80 °C in a vacuum oven. Three strips (2 × 10 cm), corresponding to the three lanes of the original gel, were cut and preincubated for 24 h in 10 × Denhardt's solution (Denhardt, 1966). The strips were hybridized at 65 °C in 10 × Denhardt's solution with 30 ng of heat-denatured probe that had been labeled with [α-^{32}P]dATP by nick translation (Rigby et al., 1977). Three different probes were used: the purified 1.6-megadalton cloned DNA fragment of pIY1 (Poulis et al., 1981), pIY2, and pGM706. After hybridization for 16 h at 65 °C the strips were washed 3 times at 65 °C with 200 mL of 3 × SSC (SSC is 0.15 M NaCl–0.015 M sodium citrate, pH 7.0), followed by one wash in 0.75 × SSC. Excess fluid was removed and the strips were wrapped in Saran wrap and autoradiographed.

Preparation of Membranes and Enzyme Assays. For the preparation of membranes, cells were grown in 10-L fermenters with mannitol (30 mM) as the carbon source and were harvested in late exponential phase. Membranes were prepared and assayed for oxidase activities as described (Wallace &

† From the Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra City, A.C.T. 2601, Australia. *Received May 1, 1981.*

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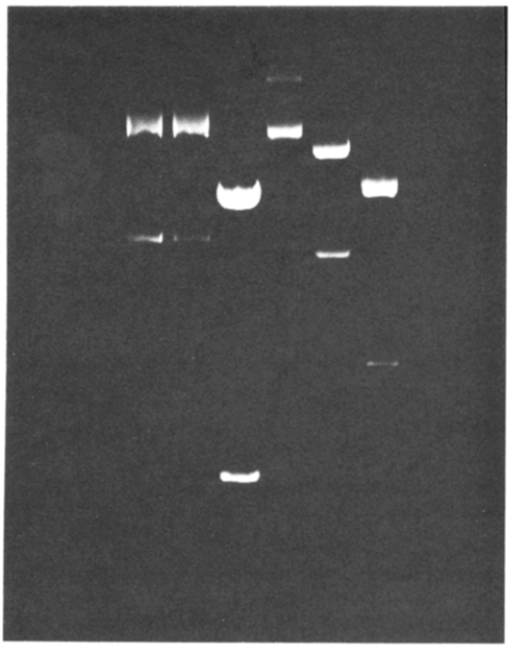

FIGURE 1: Electrophoresis of plasmid DNAs on 0.8% agarose gels. The direction of migration is from top to bottom. Samples: (a) pIY1; (b) pIY1 digested with *Hin*dIII; (c) pIY1 digested with *Eco*RI; (d) pIY2; (e) pIY2 digested with *Hin*dIII; (f) pIY2 digested with *Eco*RI. The two bands in (a), (b), and (d) correspond to the covalently closed and open circular forms of the respective hybrid plasmid DNAs. The open circular forms are in each case closer to the origin. The two bands in (c) and (e) correspond to the cloned DNA (leading bands) and the parental plasmid DNA of pIY1 and pIY2, respectively.

Young, 1977a), except that 0.8 mM NADH and 20 mM D(−)-lactate were used for their respective oxidase measurements. D-Lactate dehydrogenase was assayed by measuring the phenazine methosulfate coupled reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Futai, 1973). Details for the determination of quinones and cytochromes have been presented (Wallace & Young, 1977a).

Growth Tests. Measurements of growth rates were carried out in 125-mL flasks that contained 10 mL of growth medium and were shaken in a water bath at 37 °C. The flasks were fitted with a side arm that allowed the turbidity of the culture to be followed with a Klett-Summerson colorimeter fitted with a blue filter.

Other Techniques. Sodium dodecyl sulfate (NaDodSO$_4$)–polyacrylamide gel electrophoresis was carried out on slab gels consisting of a linear 10–25% concentration gradient of acrylamide as previously described (Jaworowski et al., 1981). Culture supernatants were assayed for volatile and nonvolatile products as described previously (Wallace & Young, 1977b).

Results

Comparison of Cloned DNA Fragments of pIY1 and pIY2. The hybrid plasmid pIY1 possesses a 1.6-megadalton cloned DNA fragment, which carries the *ndh* gene, inserted into the *Eco*RI site of pSF2124. The plasmid pIY2 possesses a 4.6-megadalton DNA fragment inserted into the *Hin*dIII site of pGM706 (Young et al., 1978). Since both plasmids were known to complement *ndh* mutants, a comparison was made with restriction enzymes to determine if the two cloned DNA fragments had a region in common. To establish whether the insert in pIY1 overlapped that of pIY2 at either end, we tested pIY1 to see if it carried a *Hin*dIII site. Since the parent plasmid pSF2124 does not possess a *Hin*dIII site, this was conveniently tested by seeing whether pIY1 was converted to a linear form by incubation with *Hin*dIII. Since no digestion occurred (Figure 1), it was clear that the pIY1 DNA fragment did not overlap that of pIY2. This left the possibility that the pIY1 fragment was a subset of the larger pIY2 fragment. However, a 1.6-megadalton fragment was not generated from pIY2 after complete digestion with *Eco*RI (cf. lanes c and f, Figure 1), indicating that the two DNA fragments are unrelated and that therefore pIY2 does not carry the *ndh* gene.

Table I: Respiratory Activities in Wild-Type and Plasmid-Containing Strains

strain	plasmid	respiratory activities[a] D-lactate dehydrogenase	D-lactate oxidase	NADH oxidase	succinate oxidase
IY13(*ndh*$^+$)		0.09	0.10	0.78	0.09
IY36(*ndh*)	pIY2	2.33	1.03	<0.02	0.06
IY65(*ndh*$^+$)	pGM706	0.08	0.09	NT	NT

[a] The dehydrogenase rate is expressed as micromoles of MTT reduced per minute per milligram. All oxidase rates are expressed as μg-atoms of O consumed per minute per milligram; NT, not tested. Details of assays are given under Experimental Procedures.

Southern hybridization (Southern, 1979) was also used to verify that the cloned DNA fragments carried by pIY1 and pIY2 are unrelated. The insert carried by pIY1 was purified by gel electrophoresis and labeled by nick translation (see Experimental Procedures). The plasmid pIY2 and its parent plasmid, pGM706, were similarly labeled. The three different labeled DNA probes were hybridized against a *Hin*dIII digest of *E. coli* chromosomal DNA. With the plasmid pIY2 as the hybridization probe, the strongest band corresponded to the size of the cloned *Hin*dIII fragment possessed by this plasmid, as expected. In contrast, no hybridization band was obtained in this region when the DNA insert carried by pIY1 was used as a probe, thus verifying that the two fragments are unrelated. Similarly, no band was obtained when pGM706, the parent plasmid of pIY2, was used as the hybridization probe, indicating that the hybridization observed with pIY2 was due to the insert. As a further control, it was shown that no DNA hybridizing with any of the three probes was detectable in the *Hin*dIII preparation used.

Respiratory Activities in Strains Carrying the Hybrid Plasmids. Since the restriction analysis of pIY2 indicated that it did not carry the *ndh* gene, it was not clear how pIY2 was able to complement the *ndh* mutant. Membranes were prepared from strain IY36 (*ndh*), which carries pIY2, and respiratory activities determined. The very low NADH oxidase activity confirmed that there was no restoration of NADH dehydrogenase by pIY2 and that this hybrid plasmid did not carry the *ndh* gene. The levels of succinate oxidase were normal. There was, however, a significant elevation of the respiratory D-lactate oxidase and D-lactate dehydrogenase (Table I). The amplified dehydrogenase was shown to be specific for D(−)-lactate rather than for L(+)-lactate, and greater than 95% of the activity was present in the membrane fraction. No amplification of D-lactate dehydrogenase was observed in cells carrying the parental plasmid pGM706, indicating that the elevation was due to the cloned DNA fragment.

The elevation of D-lactate oxidase in strains carrying pIY2 suggests that in the wild-type situation the activity of the D-lactate dehydrogenase limits the overall oxidase rate.

Table II: Cytochromes and Quinone Levels in Wild-Type and Plasmid-Containing Strains

strain	plasmid	cytochromes (nmol/mg of membrane protein)			quinones[b] (nmol/g wet wt)		
		b[a]	*d*	*o*	UQ	MK	DMK
IY36(*ndh*)	pIY2	0.137	0.087	0.085	180	26.0	42.9
IY13(*ndh*$^+$)		0.159	0.053	0.087	189	11.5	35.1

[a] Dithionite-reduced difference spectra at 77 K failed to reveal any significant differences in the cytochrome b_{556}, b_{558}, and b_{562} content of IY13 and IY36 membranes. [b] Abbreviations: UQ, ubiquinone; MK, menaquinone; DMK, demethylmenaquinone.

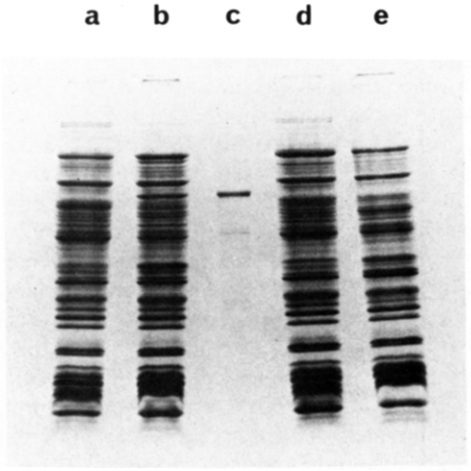

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of membranes prepared from wild-type and plasmid-containing cells. The direction of running is from top to bottom. Samples: (a) IY12 membranes; (b) IY36 membranes (pIY2); (c) purified D-lactate dehydrogenase; (d) IY65 membranes (pGM706); (e) IY13 membranes.

Measurement of cytochrome and quinone levels in the membranes from cells carrying pIY2 (Table II) established that the levels of these components were within normal limits. The measurement of cytochrome spectra at 77 K showed that cytochromes b_{556}, b_{558}, and b_{562} were present in similar concentrations to the wild type in both cases. This suggests that the elevated oxidase levels are not due to an amplification of the other components in the respiratory chain. A similar elevation of NADH oxidase was obtained when NADH dehydrogenase levels were amplified with pIY1 (Young et al., 1978), and again no elevation in other components of the respiratory chain was detected (Jaworowski, 1980).

The respiratory D-lactate dehydrogenase has been purified from *E. coli* by three different laboratories (Futai, 1973; Kohn & Kaback, 1973; Pratt et al., 1979). $NaDodSO_4$–polyacrylamide gel electrophoresis of membrane preparations from cells containing pIY2 regularly showed significant elevation of a band corresponding in mobility to purified D-lactate dehydrogenase (Figure 2). This polypeptide was not elevated in membranes from cells carrying the parent plasmid pGM706 (Figure 2).

The most likely explanation for the above results is that pIY2 carries the structural gene for the respiratory D-lactate dehydrogenase. Verification that the structural gene has been cloned comes from the recent demonstration that pIY2 can be used as a template for the synthesis of D-lactate dehydrogenase in an in vitro transcription/translation system (Santos et al., 1982).

Mechanism of Complementation of the ndh Mutant by pIY2. One of the major roles of the respiratory NADH dehydrogenase in cell metabolism is the reoxidation of NADH generated by the tricarboxylic acid cycle and glycolysis. The properties of *ndh* mutants, which lack the respiratory enzyme, have been described elsewhere (Young & Wallace, 1976). When culture supernatants from *ndh* mutants were examined by gas chromatography, high levels of lactic acid were detected whereas the corresponding *ndh*$^+$ strain did not accumulate this compound (Table III). The lactic acid levels were accurately measured enzymically, and it was verified that the lactate accumulated was solely D(−)-lactate. The most likely explanation for the accumulation of D-lactate is that NAD^+ is being regenerated in the *ndh* mutant by the reduction of pyruvate catalyzed by the soluble, pyridine nucleotide linked D-lactate dehydrogenase, EC 1.1.1.28 (Tarmy & Kaplan, 1968).

Table III: Products of Mannitol Catabolism by Various Strains

strain	plasmid	concn (mM) in culture supernatant[a]		
		ethanol	acetate	D(−)-lactate
IY12(*ndh*)		0.7	3.9	20
IY13(*ndh*$^+$)		0.5	6.9	<0.2
IY36(*ndh*)	pIY2	0.4	5.4	<0.2
IY35(*ndh*)	pIY1	0.3	3.6	<0.2

[a] Supernatants of cultures grown to late logarithmic phase on mannitol mineral salt medium supplemented with 0.1% casamino acids were examined for volatile and nonvolatile products by gas chromatography as described previously (Wallace & Young, 1977b). Only ethanol, acetate, and lactate were detected. The levels of ethanol and acetate were quantitated by gas chromatography, and that of D(−)-lactate was quantitated by enzymic assay with D-lactate dehydrogenase (see Experimental Procedures). No L(+)-lactate was detectable by specific assay with L-lactate dehydrogenase.

In principle, the D-lactate oxidase of the respiratory chain, which is normal in the *ndh* mutants (Young & Wallace, 1976), converts the D-lactate back to pyruvate. This would provide a cyclic system for the reoxidation of NADH, which does not involve the respiratory NADH dehydrogenase. However, the levels of D-lactate oxidase are apparently too low in the wild-type cell for this to occur to any major extent. In the case of the *ndh* mutant carrying pIY2 (strain IY36) no D-(−)-lactate was accumulated under comparable conditions. Thus the elevated levels of D-lactate oxidase are apparently high enough to allow for NADH oxidation to occur via the alternative mechanism described above.

Measurement of growth rates on mannitol-minimal medium was made for the various strains to gauge some idea of the efficiency of the alternative pathway for NADH oxidation (Table III). Under the conditions used, the *ndh* mutant was unable to grow whereas the isogenic *ndh*$^+$ strain grew with a mean generation time of 1.3 h. When complemented by pIY1 (strain IY35), the *ndh* mutant grew at essentially the same rate as the wild type (mean generation time 1.2 h), indicating that the high levels of NADH dehydrogenase generated by the plasmid provide adequate NADH oxidation and do not inhibit growth. Complementation by pIY2 (strain IY36), however, resulted in slower growth (mean generation time 2.0 h), suggesting that the rate of oxidation of NADH by the alternative pathway could be limiting with growth on mannitol. The slower growth could not be attributed to an effect of the parental plasmid since the presence of pGM706 in the *ndh*$^+$ strain did not affect its growth rate on mannitol.

Discussion

The selective amplification of the respiratory D-lactate dehydrogenase of *E. coli* by gene cloning provides another ex-

ample of the considerable elevation in levels of a membrane protein that can be achieved by this technique. There are a number of similarities between the amplification of this respiratory enzyme and that of the NADH dehydrogenase reported elsewhere (Young et al., 1978; Jaworowski et al., 1981). In both cases the enzymes are present in relatively small amounts in wild-type membranes. As a result of gene cloning onto relaxed plasmids, considerable elevation of the enzymes in the membrane has been achieved with no significant amounts of activity detectable in the supernatant fraction. In both cases the increase in dehydrogenase levels apparently results in considerable elevations of the respective oxidases, implying that the activity of the dehydrogenases is limiting their respective oxidase activities in the wild-type situation. The increased oxidase levels also suggest that at least a portion of the overproduced enzyme can function normally in electron transport and is therefore inserted correctly into the membrane. The degree of overproduction in both cases is consistent with the gene copy number expected due to relaxed replication of the plasmid vector. In the case of IY36, however, it has sometimes been possible to obtain higher levels of amplification of the D-lactate dehydrogenase (I. G. Young, unpublished results). The reasons for this observation are not understood, but it may be that the slower growth of this strain on mannitol compared to the wild-type and IY35 strains can cause an increase in the plasmid copy number of the cell analogous to that obtained under amino acid starvation conditions or inhibition of protein synthesis by chloramphenicol (Young et al., 1978).

That the NADH dehydrogenase defect was complemented by overproduction of the membrane-bound D-lactate dehydrogenase highlights the fact that the considerable elevation of particular enzymes, which can be achieved by gene cloning, can give rise to new metabolic routes that are not significant in the wild type. These results suggest that the modulation of metabolic pathways using gene cloning provides a new approach to studying such aspects as regulation and rate limitation in metabolism as was pointed out elsewhere (Raetz et al., 1977). In this context, since the level of NADH is believed to be important in the regulation of many areas of intermediary metabolism, the plasmids pIY1 and pIY2 may provide interesting insights into the maintenance of the $NADH/NAD^+$ ratio in the cell.

The respiratory D-lactate dehydrogenase appears to play an important role in generating the energy used for the active transport of various sugars and amino acids in *E. coli* (Kaback, 1974). The cloning of the structural gene for D-lactate dehydrogenase makes possible several new approaches to the study of this enzyme such as the determination of its primary structure via DNA sequencing, studies on gene expression in vitro, and the use of site-directed mutagenesis to probe structure–function relationships.

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