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Three Overlapping *lct* Genes Involved in L-Lactate Utilization by *Escherichia coli*

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In *Escherichia coli*, the *lct* locus at min 80 on the chromosome map is associated with ability to grow on L-lactate and to synthesize a substrate-inducible flavin-linked dehydrogenase. Similar to that of the *glpD*-encoded aerobic glycerol-3-phosphate dehydrogenase, the level of induced enzyme activity is elevated by aerobiosis. Both of these controls are mediated by the two-component signal transduction system ArcB/ArcA, although sensitivity to the control is much more striking for L-lactate dehydrogenase. This study disclosed that the *lct* locus contained three overlapping genes in the clockwise order of *lctD* (encoding a flavin mononucleotide-dependent dehydrogenase), *lctR* (encoding a putative regulator), and *lctP* (encoding a permease) on the chromosomal map. These genes, however, are transcribed in the counterclockwise direction. No homology in amino acid sequence was found between aerobic glycerol-3-phosphate dehydrogenase and L-lactate dehydrogenase. A Φ(*lctD-lac*) mutant was inducible by L-lactate but not D-lactate. Although the mutant lost the ability to grow on L-lactate, growth on D-lactate, known to depend on a different enzyme, remained normal.

Growth of *Escherichia coli* on L-lactate results in the induction of a flavin-linked L-lactate dehydrogenase (9, 13, 29), and this trait is associated with the *lct* locus at min 80.8 on the chromosome map (3, 19, 31). Since L-lactate is oxidized to the central metabolite pyruvate, three proteins (or protein complexes) should suffice for the pathway: a specific permease, L-lactate dehydrogenase, and a specific regulator protein. It is not yet known whether the genes involved are situated at the same locus. Because substrate induction of L-lactate dehydrogenase is highly sensitive to modulation by the respiratory growth condition (about 30-fold) and because this modulation is attributable to the two-component regulatory system ArcB/ArcA (16), we were prompted to identify the structural gene for the dehydrogenase so that subsequently its ArcA-binding site(s) in the promoter region could be identified. Also, the physiological function of L-lactate dehydrogenase seems analogous to that of the aerobic glycerol-3-phosphate dehydrogenase encoded by *glpD*: both enzymes can function in conducting electrons to O_2 or nitrate (22, 32). Obtaining the sequence of the gene for L-lactate dehydrogenase should tell us whether significant homology exists between the two dehydrogenases.

MATERIALS AND METHODS

Materials. 3-(*N*-Morpholine)propanesulfonic acid (MOPS) and 2,3,5-triphenyltetrazolium chloride (TTC) were obtained from Sigma Chemical Co., St. Louis, Mo. MacConkey agar base, tryptone, and yeast extract were obtained from Difco Laboratories, Detroit, Mich. The 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) used was from Bachem, Inc., Torrance, Calif. Vitamin-free casein acid hydrolysate (CAA) was obtained from ICN Nutritional Biochemicals, Cleveland, Ohio. Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs, Beverly, Mass. L-[U-^{14}C]lactate (177 mCi/nmol), D-[U-^{14}C]lactate (89 mCi/nmol), and L-[^{35}S] methionine (1,000 μCi/nmol) were obtained from Amersham Life Sciences, Arlington Heights, Ill. Biofluor and nylon membranes were obtained from Du Pont NEN, Boston, Mass. All other reagents used in this study were commercial products of the highest grade available.

Bacterial strains, phages, and plasmids. All strains used were *E. coli* K-12 derivatives. The genotypes and sources of the bacterial strains, phages, and plasmids are given in Table 1.

Growth conditions. Overnight cultures were grown in LB medium (28). Minimal agar media (38) were supplemented with thiamine. When used, the following compounds were added to the media at the following concentrations unless otherwise specified: D- or L-lactate, 20 mM; D-xylose, 10 mM; CAA, 1%; thiamine, 2 μg/ml; kanamycin (kan), 40 μg/ml; and ampicillin, 100 μg/ml. Mannitol (1%)-MacConkey agar plates were used for screening the inheritance of the *mtl* allele.

Cultures for enzyme and permease assays were grown in 300-ml flasks containing 10 ml of MOPS (0.1 M) mineral medium at pH 7.6 with appropriate supplements (15). Ample aeration was ensured by vigorous agitation, and cells were harvested when growth reached the mid-exponential phase.

Enzyme and permease assays. For L- and D-lactate dehydrogenase assays, cells were harvested by centrifugation at 5,000 × *g* for 15 min and washed once in cold 10 mM potassium phosphate buffer (pH 7.0). The pellet was weighed and suspended in 4 volumes of the same buffer. The suspended cells were lysed for 1 min/ml in a model 60 W ultrasonic disintegrator (MSE) at 1.5 A while being chilled in a dry ice-ethanol bath. Lysates were cleared by centrifugation for 30 min at 10,000 × *g*. Enzyme assays were performed in a manner similar to that for glycerol-3-phosphate dehydrogenase by measuring the reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide mediated by phenazine methosulfate (20), but with 0.1 M L- or D-lactate as a substrate. Specific activity of L- or D-lactate dehydrogenase was expressed in nanomoles per minute per milligram of protein at 30°C. Protein concentrations were calculated by comparison with bovine serum albumin (26).

β-Galactosidase activity was assayed by measuring the rate of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis (28) in whole cells rendered permeable by the addition of 2 drops of chloroform and 1 drop of 0.1% sodium dodecyl sulfate (SDS).

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TABLE 1. *E. coli* K-12 strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Genotype or phenotype	Source, reference, or construction
LG1	F⁻ *thi-1 hisG1 argG6 metB1 tonA2 supE44 rps-1104 lacY1 galT6 gatR49 gatA50 gutP049 gutA50 (mtlA')'AD*⁺	J. W. Lengeler
LCB482	F⁻ *thr-1 leuB6 fhuA2 lacY1 supE44 rfbD1 rpsL175 lct-2 mt-110 thi-1*	31
ECL116	F⁻ *endA hsdR* Δ(*argF-lac*)*U169 thi*	8
ECL525	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 flb-5301 ptsF15 frd-101*	16
ECL901	Φ(*lctA-lac*) (otherwise as in ECL525)	This study
ECL903	Φ(*lctD-lac*) (otherwise as in ECL116)	P1(ECL901) × ECL116
pBR322	Ap^r Tc^r	
pLCT1	*lctD*⁺ *lctT*⁺	This study
pLCT2	*lctD*⁺ *lctT*⁺	This study
λp*lac*Mu9		4

Specific activity of β-galactosidase was expressed in Miller units at 30°C.

For permease assays, 100 μl of chloramphenicol (2 mg/ml) was added to 5 ml of cell culture in mid-exponential phase. The cells were washed twice and suspended in 0.1 M MOPS (pH 7.0) to give an A_{600} of about 0.5. The rate of L-lactate uptake was assayed by diluting the substrate 10-fold with the cell suspension to give a final L-lactate concentration of 2.8 μM. Samples of 100 μl were taken at 15-s intervals and filtered through 0.45-μm-pore-size filters. The filters were washed with 5 ml of the same MOPS buffer, dried, and counted in a Biofluor scintillator. The specific activity of the permease was expressed in nanomoles per minute per milligram (dry weight) of cells at 30°C.

Construction of plasmids. Standard molecular cloning procedures were followed for transformations, restriction enzyme digests, and the preparation of plasmid DNA and competent cells (27). Digested DNA samples were run in horizontal agarose gels (0.7%) in Tris-borate buffer. The sizes of DNA fragments were estimated by comparison with λ*Bst*EII and λ*Hin*dIII markers.

To facilitate cloning of the *lct* genes, the Φ(*lct-lac*) fusion of ECL901 (see Results) was transduced into an efficiently transformable host, ECL116 [*lct*⁺Δ(*argF-lac*)*U169* (see reference 8)], by selecting and screening on kan–X-Gal–LB agar. A blue colony, ECL903, that lost the ability to grow on L-lactate minimal agar was isolated for transformation by a pBR322 genomic library of strain ECL116 purified by cesium chloride gradient centrifugation (27). Transformants were selected on ampicillin-LB and screened on L-lactate–X-Gal–kan agar for growth. An Lct⁺ transformant bearing a plasmid with a 10.6-kb insert, pLCT1, was used for further studies of the *lct* genes.

To create subclones of pLCT1, DNA from single restriction enzyme digests was electrophoresed in low-melting-point agarose gels (0.7%) in Tris-acetate buffer. The appropriate bands were excised and self-ligated with T4 ligase (37). The ligated DNA was then transformed into strain ECL903. The inserts were examined by restriction analysis.

DNA sequencing. The *lct* region was subcloned into plasmid pBluescript for sequencing by the dideoxynucleotide method (39). Each cloned insert was first sequenced by using T3 and T7 primers and then by synthetic primers to walk through the remaining region. To avoid sequencing errors, both strands were sequenced by regular dGTP as well as dITP reactions. The entire nucleotide sequence obtained was analyzed by the program DNASIS to generate a restriction map and to reveal possible open reading frames (ORFs). To explore for protein homologies, a combined data base search was performed with the programs AUTOSEARCH and MAILFASTA. The homologous proteins were aligned by the program PILEUP.

Northern blot analysis of *lct* mRNAs. Wild-type ECL525 cells were grown in MOPS-CAA-thiamine-xylose medium in the presence or absence of 10 mM L- or D-lactate to 100 to 150 Klett units. Cells were pelleted and resuspended in 150 mM Tris-HCl (pH 8.0), 0.45 M sucrose, 8 mM EDTA, and 0.4 mg of lysozyme per ml. After 15 min of incubation at 4°C, the cells were extracted with 50% guanidine isothiocyanate–0.5% *N*-lauroylsarcosine and the extracts were loaded on a CsCl gradient (5.7 M CsCl, 1 mM EDTA, and 24 mM sodium acetate at pH 5.2). The samples were centrifuged overnight at 36,000 rpm with an SW50.1 swinging rotor. The supernatant fractions were carefully removed, and the RNA pellets were dissolved in DEPC-H_2O. The RNA was then precipitated by ethanol, dried, and resuspended in DEPC-H_2O. For Northern (RNA) blot analysis (35), 10 μg of total cellular RNA was electrophoresed, transferred to a nylon membrane, and fixed by UV light. The nylon membrane was prehybridized for 7 to 8 h at 65°C in 10× Denhardt's solution plus 1 M NaCl, 1% SDS, 50 μg of salmon sperm DNA per ml, 50 mM Tris-HCl (pH 7.5), 0.1% sodium PP_i, and 0.1% dextran sulfate. Hybridization was carried out by adding [α-^{32}P]dATP-labelled probe at 10^6 cpm/ml and incubating the membrane at 65°C overnight. The membrane was washed in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 1% SDS, dried, and exposed to X-ray film.

Nucleotide sequence accession number. The insert between

TABLE 2. Specific activities of L-lactate dehydrogenase and β-galactosidase and L-lactate uptake of aerobically grown parent and Φ(*lct-lac*) strains[a]

Strain	Sp act				L-Lactate uptake[b]	
	L-Lactate dehydrogenase[b]		β-Galactosidase[c] (U)			
	None	L-Lactate	None	L-Lactate	None	L-Lactate
ECL525 (wild type)	13	44	0	0	3.5	13
ECL901 [Φ(*lct-lac*)]	6	7	290	880	2.2	1.8

[a] Specific activities and L-lactate uptake are expressed as described in Materials and Methods.

[b] Cells were grown on CAA-MOPS medium with or without L-lactate as an inducer.

[c] Cells were grown on xylose–0.03% CAA–MOPS medium with or without L-lactate as an inducer.

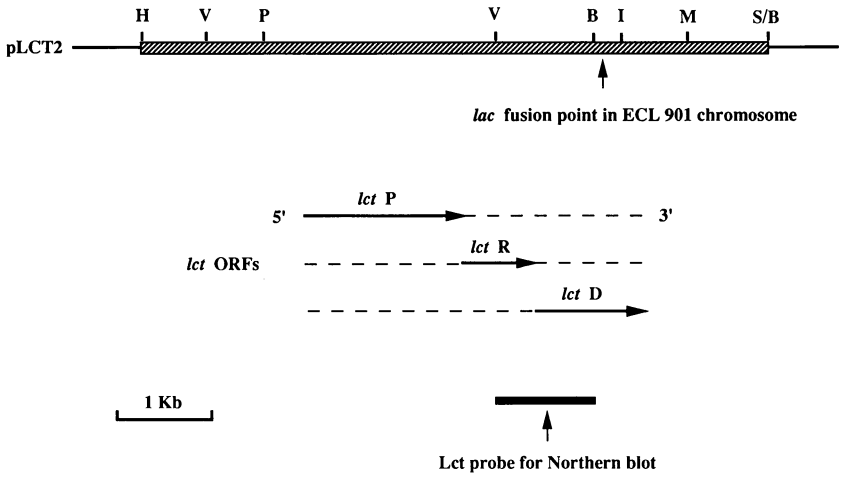

FIG. 1. Restriction map of pLCT2. Three ORFs (horizontal arrows) are identified: ORF1 of *lctP*, probably encoding L-lactate permease, ORF2 of *lctR*, probably encoding a regulator, and ORF3 of *lctD*, encoding L-lactate dehydrogenase. The junction between *lct* and *lacZ* in strain ECL901 is indicated by an arrow. The fragment from *Eco*RV to *Bam*HI was used for Northern blot analysis. Restriction enzyme symbols: H, *Hin*dIII; V, *Eco*RV; P, *Pst*I; B, *Bam*HI; I, *Eco*RI; M, *Mlu*I; and S, *Sau*III. The complete nucleotide sequence (6,633 bp) was submitted to GenBank under the accession number L13970.

the *Hin*dIII site and the junction of *Sau*III-*Bam*HI of pLCT2 was sequenced as described above. This interval of 6,633 bp (see Fig. 1) was deposited in GenBank (accession number L13970).

RESULTS AND DISCUSSION

Mutant isolation. Cells of strain ECL525 subjected to insertion mutagenesis by λp*lac*Mu9(*kan*) were plated on TTC–L-lactate–X-Gal–kan agar (4). Colonies of essentially four different colors were found: white (*lacZ* not expressed and L-lactate negative), red (*lacZ* not expressed and L-lactate positive), purple (*lacZ* expressed and L-lactate positive), and blue (*lacZ* expressed and L-lactate negative). Of the more than 37,000 colonies examined, 937 appeared blue. Only one blue colony, upon further screening, was found to have lost the ability to grow on L-lactate (but retained the ability to grow on glucose, succinate, D-lactate, or pyruvate) as the sole carbon and energy source. The insertion mutation was transduced back into the parent strain, ECL525, to ensure a clean genetic background. The transductants were selected on X-Gal–kan–LB agar, and a blue colony, ECL901, with the expected phenotype was picked.

L-Lactate dehydrogenase and β-galactosidase activities of the parent ECL525 and fusion ECL901 strains. In the parent strain ECL525 [Δ(*arg-lac*)] the level of L-lactate dehydrogenase activity was induced by the substrate more than threefold when the cells were grown aerobically on CAA (Table 2). However, in the fusion strain ECL901, the level of the dehydrogenase activity was barely detectable but the β-galactosidase activity was induced about threefold (from 290 to 880 U). In contrast, under anaerobic conditions of growth, the β-galactosidase activity levels in strain ECL901 were barely inducible (from 46 to 55 U [data not shown]). Thus substrate induction of Φ(*lct-lac*) was highly dependent on the respiratory growth condition.

Contrary to previous reports that L-lactate dehydrogenase was induced by either L- or D-lactate (18, 30), we did not find any inducing effect of Φ(*lct-lac*) by D-lactate in cells grown on xylose minimal medium (data not shown).

Transport activities for L-lactate in parent ECL525 and fusion ECL901 strains. Since genes encoding enzymes in a catabolic pathway are often linked to a gene encoding the corresponding permease, we examined the uptake rates of labelled L-lactate under noninducing and inducing conditions in the parent and the fusion strains grown aerobically. In strain ECL525, the transport of L-lactate was induced threefold by the substrate (Table 2). In strain ECL901, only a low basal activity remained.

Mapping of the *lac* insertion. To test whether the *lac* insertion occurred in the expected *lct* region, linkage of the fusion to *mtl* was analyzed (3, 23, 31). It should be noted parenthetically here that in the standard *E. coli* genetic map this *lct* locus is erroneously attributed to the gene for the NAD-linked D-lactate oxidoreductase (3). The presumptive Φ(*lct-lac*) in strain ECL901 was first transduced into strain ECL116 to give strain ECL903. P1 particles prepared from strain ECL903 were then used to infect strain LG1 (Δ*mtlA*). Transductants were selected on kan-LB and scored on mannitol-MacConkey agar. The cotransduction frequency between the fusion and *mtl* was about 37%, indicating that the fusion was at the known *lct* locus.

Cloning of the *lct* region. Transformation of strain LCB482, bearing the *lct2* allele in the min 80.8 region (31), by plasmid

TABLE 3. Specific activities of L-lactate permease and dehydrogenase of aerobically grown parental and fusion strains bearing various plasmids[a]

Strain	Sp act (nmol/min/mg of protein)			
	L-Lactate uptake		L-Lactate dehydrogenase	
	Basal	Induced	Basal	Induced
ECL116 (wild type)/pBR322	2.9	11	10	37
ECL903 (Φ[*lctD-lac*])/pBR322	2.6	2.7	4	3
ECL903 (Φ[*lctD-lac*])/pLCT2	4.6	16	42	190

[a] Cells were grown on CAA-MOPS medium with or without L-lactate as an inducer.

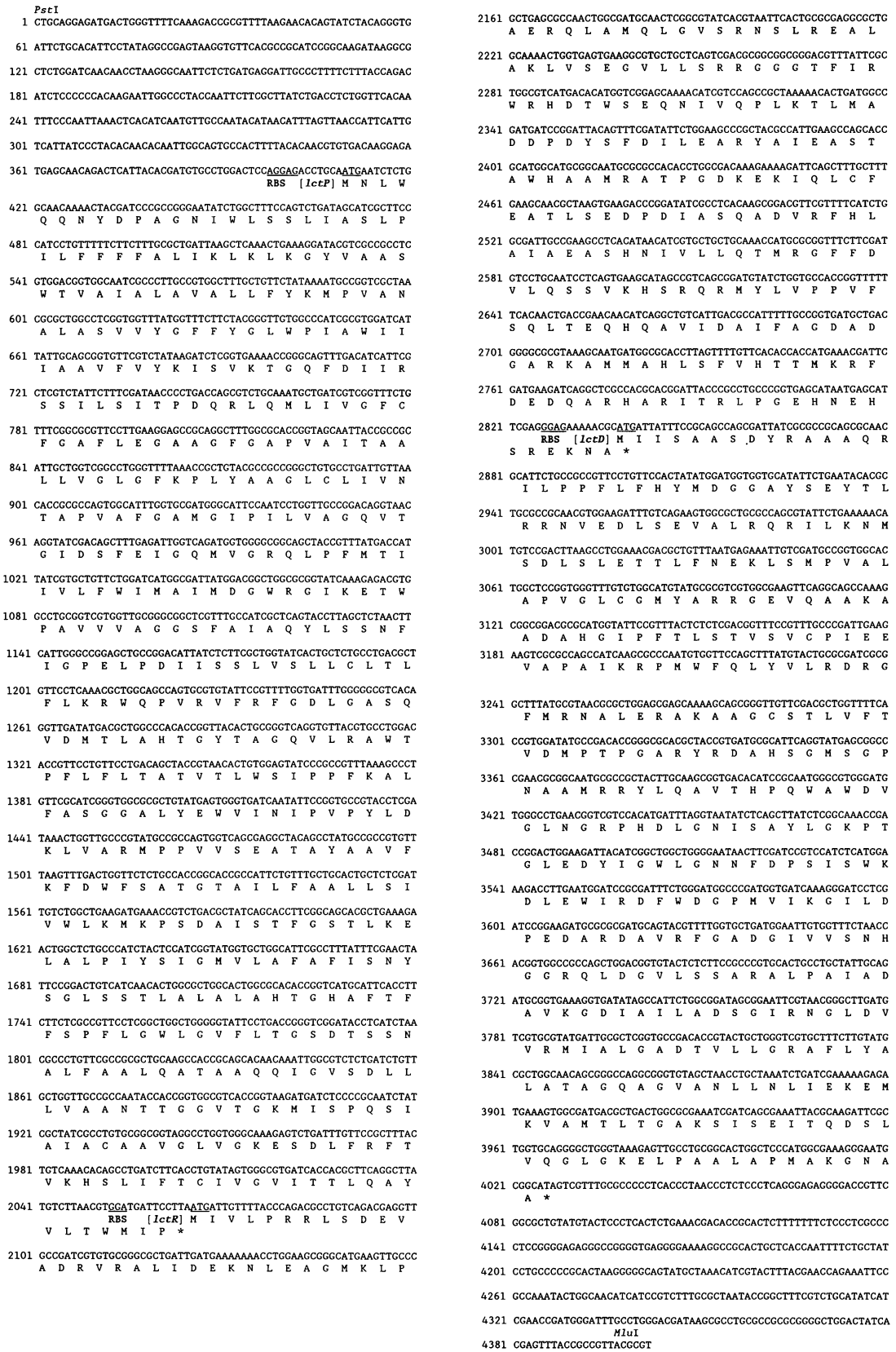

FIG. 2. Nucleotide sequences of the *lct* genes and their deduced amino acid sequences. The DNA sequence presented here starts from the *Pst*I site and ends at the *Mlu*I site (Fig. 1). The putative ribosomal binding site (RBS) for each ORF is underlined. The three genes, *lctP*, *lctR*, and *lctD*, overlap within stop and start codons.

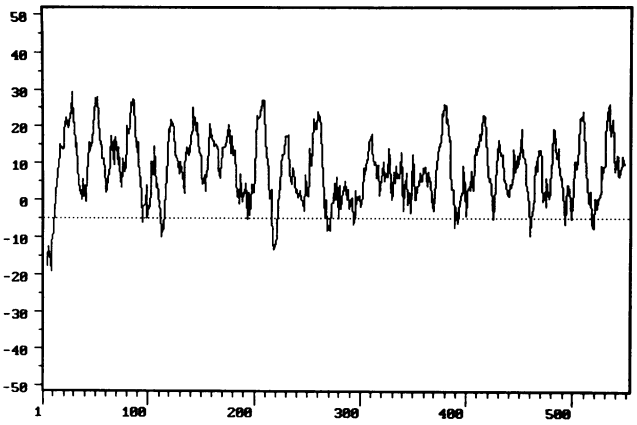

FIG. 3. Hydrophobicity plot of LctP. The *lctP* gene encodes 551 amino acids. The hydropathic index of the protein was calculated by the program SOAP. By using 9-amino-acid windows, a total of 12 transmembrane regions were identified.

pLCT1 with the 10.6-kb insert restored the L-lactate growth ability of the mutants. This confirmed that the correct region was cloned. The insert was precisely located at min 80.8 by comparison of the positions of the single *Bam*HI, *Eco*RI, *Kpn*I, *Eco*RV, and *Pst*I sites, the double *Hin*dIII sites, and the triple *Pvu*II sites (data not shown) with a Kohara fragment (19).

The insert of pLCT1 was shortened to about 6.6 kb and religated to give pLCT2 (Fig. 1). Strains ECL116 (*lct*$^+$)/pBR322, ECL903 [Φ(*lctD-lac*)]/pBR322, and ECL903 [Φ(*lctD-lac*)]/pLCT2 were grown aerobically to compare their basal and induced specific activities of L-lactate permease and dehydrogenase. ECL116 (*lct*$^+$)/pBR322 showed three- to fourfold induction ratios for both activities, whereas ECL903 [Φ(*lctD-lac*)]/pBR322 showed only low noninducible activities. The plasmid pLCT2 conferred to strain ECL903 elevated both basal and inducible activities of the permease and the dehydrogenase (Table 3). The insert therefore should contain at least the structural genes *lctD* for L-lactate dehydrogenase and

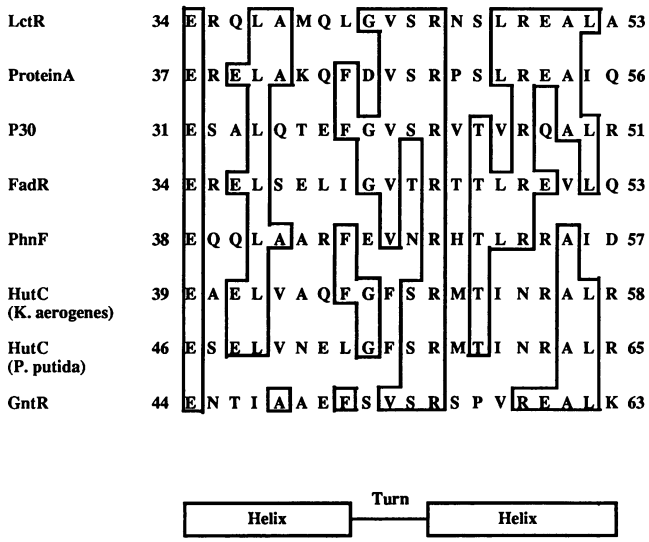

FIG. 4. Conserved helix-turn-helix DNA binding domain of LctR. The *lctR* gene encodes 258 amino acids. The N-terminal region contains a conserved helix-turn-helix DNA binding domain shared by several bacterial transcription factors, including protein A (a hypothetical transcription factor), P30 (a 30-kDa hypothetical transcription factor), FadR (a regulator for fatty acid metabolism), and PhnF (a hypothetical transcription factor) of *E. coli*; HutC (histidine utilization repressor) of *K. aerogenes* and *P. putida*; and GntR (a repressor protein) of *B. subtilis*.

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                                        *
LCTD      1   MiIsaasDYr AaAQrILPpf lFhYmdgGAy sEYTLRrNve dLseVaLrqr
GOX       1   MeItNvnEYE AiAkQkLPkM VYdYYasGAE DqWTLaENrn AFsRILFrPr
MDH       3   qNlfNveDYr klrQkrLPkM VYdYlegGAE DEYgvkhNrd vFqqwrFkPk
LOX      22   tlpmsyaDWE AhAQQaLPpg VLsYvagGsg DEhTqRaNve AFkhwgLmPr
B2-S     120  dNIINlyDFE ylAsQtLtkq aWaYYssGAn DEvThRENhn AYhRIFFkPk
B2-H     185  sqmINlhDFE tiArQILPpp aLaYYcsaAD DEvTLRENhn AYhRIFFnPk
Consensus     MNIIN--DYE A-AQQILP-M V--YY--GAE DEYTLREN-- AF-RIFF-P-

LCTD     51   ILknmsDlsL eTtLFneKlS MPvalAPvgL CgMyar.rGE VqaAkAAdAh
GOX      51   ILIDVtnIDM tTtiLGfKIS MPimIAPTaM qKMaHP.EGE yAtARAAsAA
MDH      53   rLVDVsrrsL qaEvLGkrqS MPLLIgPTgL ngalwP.kGD lAlARAAtkA
LOX      72   mLmaatErDL SvELWGktwa aPMFfAPigv iaLc.aqDGh gdaAsAqasA
B2-S     170  ILVDVrkVDi STDMLGshVd vPFYVsaTaL CKLgnPlEGE kdvARgcGqg
B2-h     235  ILIDVkDVDi STEFFGeKtS aPFYIsaTaL aKLgHP.EGE VAiAkgAGre
Consensus     IL-DV-DVDL STELLG-K-S MPFYIAPT-L CKL-HP-EGE VA-ARAAGAA

                                             *
LCTD     100  ..GIPFtLST vsvCpIEEVA ...PAikrpm WFQLYVlrDR gfmrnaleRA
GOX      100  gTimt..LSs wAtsSVEEVA st...Gpgir FFQLYVykDR nVvaqLVRRA
MDH      102  ..GIPFvLST asnmSIEDlA ...rgcdgdl WFQLYVih.R EIaqgMVlkA
LOX      121  rTGVPYitST LAvsSlEDI. .rkhAGdtpa YFQLYypeDR DlaEsFIRRA
B2-S     220  vTkVPqmiST LAsCSpEEIi EAaPsdkqiq WYQLYVNsDR kItDdLVknv
B2-H     284  ..dVvqmiST LAsCSfDEIA DAripGqq.q WYQLYVNaDR sItEkaVRhA
Consensus     -TGVPF-LST LA-CSIEEIA -A-PAG---- WFQLYVN-DR -I-E-LVRRA

                *
LCTD     145  kaAGCstLVf TVDmPtpGaR yRDaHsgmsg Pna.amrrYL qavtHPqWaw
GOX      145  ErAGFKaial TVDtPrLGRR EaDi..... ..Kn..... ......rFvL
MDH      146  lhtGYttLVl TtDvavnGYR ERDLHnrfki PmsySakvvL dGclHPrWsL
LOX      169  EEAGYdgLVi TlDtwiFGWR pRDL..... ..tiSnfpFL rGlcltnYvt
B2-S     270  EklGvKaLfv TVDaPsLGqR EkDM..... ..KL..... ......kFsn
B2-H     331  EErGmKgLfi TVDaPsLGRR EkDM..... ..KM..... ......kFea
Consensus     EEAGYK-LV- TVD-P-LGRR ERD-H----- P-K-S----L -G--HP-F-L

LCTD     194  DvgLngrpHD LgnIsAYlgk pt.gLedyig WLgnnfDPSi SWKDlEWiRd
GOX      175  pPfLtlKnfE gidlg...km dkAndsgLSS YvaGQIDrSL SWKDVaWlqt
MDH      196  DfV....rHg MpqlanFvsS QtsSLemqaa LMsrQmDaSF nWealrWlRd
LOX      211  DPVFqkKfka hsgVEA..Eg lrdnpRlaad FwhGlfghSv tWeDIDWvRs
B2-S     300  tk.agpKamk ktnVE...ES QgAS.RaLSk F....IDPSL tWKDIEelkk
B2-H     361  Ds.dvqg..D dedID...rS QgAS.RaLSS F....IDPSL SWKDIaFiks
Consensus     DPVL--K-HD ----EA--ES Q-ASLR-LSS F--GQIDPSL SWKDIEW-R-

                *                                  * *
LCTD     243  fwdgPmVIKG IldpEDARdA VrfGADGIVv SNHGGRQLDg VlssaraLp.
GOX      222  ITsLPIlVKG VitaEDARlA VqHGAaGIIv SNHGaRQLDY VpAtImaLeE
MDH      242  lwphkllVKG llsaEDAdrc IaeGADGVIL SNHGGRQLDC ais...pMe.
LOX      259  ITKMPVIlKG IQhpDDARrA VDsGvDGIyc SNHGGRQang glpalDcLpE
B2-S     341  kTKLPIVIKG VQRtEDvikA aEiGvsGVVL SNHGGRQLDF srAPIqvLaE
B2-H     400  ITKMPIVIKG VQRkEDvllA aEHGlqGVVL SNHGGRQLDY trAPVEvLaE
Consensus     ITK-PIVIKG VQR-EDAR-A VEHGADG-VL SNHGGRQLDY V-API--L-E

LCTD     292  ...aiAdaV. KGdIaILaDs GIRnGlDVvr miALGAdtVl LGRaFLYALA
GOX      272  Vv.....kaA qGrIPVFlDg GVRRGTDVfK ALALGAaGVf iGRPvvFsLA
MDH      288  ...VLAqsVA KtgkPVLIDs GfRRGsDIvK ALALGAeaVl LGRatLYgLA
LOX      309  Vv.....kA sGdtPVLfDs GIRtGaDVvK ALAMGAsaVg iGRPYaWgaA
B2-S     391  tmPILeqRnl KdkleVFVDg GVRRGTDVlK ALcLGAkGVg LGePFLYAns
B2-H     450  VmPILkeRgl dqkIdIFVDg GVRRGTDVlK ALcLGAkGVg LGRPFLYAMs
Consensus     V-PILA-RVA KG-IPV-VD- GVRRGTDV-K ALALGA-GV- LGRPFLYALA

LCTD     338  taGqaGVanl LnLiekEmkv aMtLtGakSI sEiTqDsLvq glgKelpaAl
GOX      317  AeGEaGVkKV LQMMrDEfEL TMALsGCRSl KEisrshiaa DWdgpssrAV
MDH      335  ArGEtGVDeV LtLLkaDIDr TLAqiGCpdI tsLsPDyLqn EgVtNtApvd
LOX      353  lgGskGIEhV arsLlaEaDL iMAvdGYRnl KELTiDaLrp tR........
B2-S     441  cYGrnGVEKa ieiLrDEIEM sMrLLGvtSI aELkPDLLDl stlKaRtVgV
B2-H     500  sYGDkGVtKa iQLLkDEIEM nMrLLGvnkI eELTPELLDt rsIhNRAVpV
Consensus     AYGE-GVEKV LQLL-DEIE- TMALLGCRSI KELTPDLLD- ---KNRAVAV

LCTD     388  ApmakgNaA. .......... ....
GOX      367  Arl..... .......... ....
MDH      385  hligkgthA. .......... ....
LOX           .......... .......... ....
B2-S     491  pndvlyNevY egptltEFed a...
B2-H     550  AkdylyeqnY qrmsgaEFrp gied
Consensus     A-----N-AY ------EF-- ----
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FIG. 5. Alignment of LctD to other FMN-specific flavoproteins. The *lctD* gene encodes 396 amino acids whose sequence is highly homologous to several FMN-specific dehydrogenases or oxidases (program PILEUP). The six conserved amino acids required for FMN-binding and enzymatic catalysis are indicated by asterisks. The consensus sequence is shown on the bottom line of each section. Symbols: GOX, glycolate oxidase of spinach; MDH, (*S*)-mandelate dehydrogenase of *P. putida*; LOX, lactate monooxygenase of *M. smegmatis*; B2-S, flavocytochrome b_2 (L-lactate:cytochrome *c* oxidoreductase) of *S. cerevisiae*; and B2-H, flavocytochrome b_2 (L-lactate: cytochrome *c* oxidoreductase) of *H. anomala*.

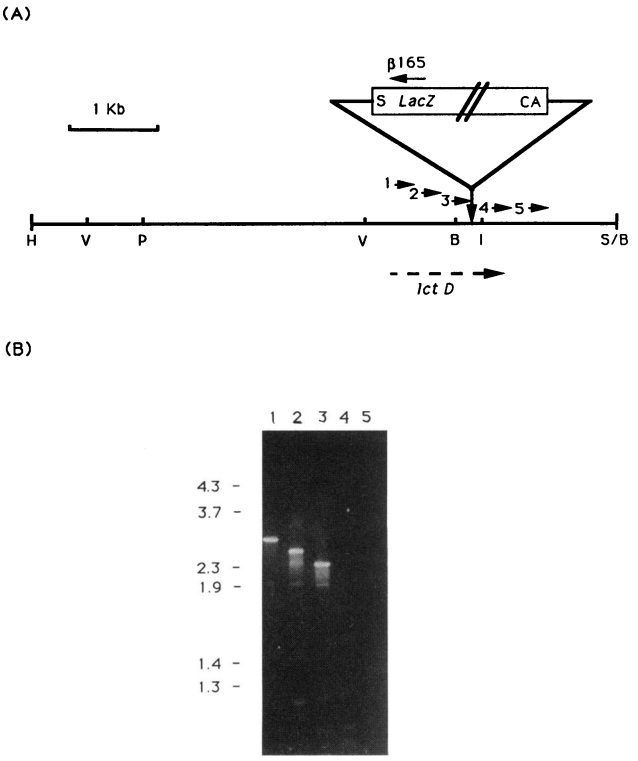

FIG. 6. Localization of the *lacZ* insertion in the *lctD* gene. (A) Primer β165, spanning nucleotides from 165 to 149 in *lacZ*, was paired with each of the *lct* primers, 1 through 5. Approximate positions of primers used in the polymerase chain reaction (solid arrows) and the position, size, and transcriptional direction of the *lctD* gene (dashed arrow) are indicated. (B) The polymerase chain reaction products were electrophoretically separated in agarose gel. Lanes 1 to 5 show the products respectively generated by primers 1 to 5. Sizes (in kilobases) of standard markers are indicated on the left border of the gel. Abbreviations: H, *Hin*dIII; V, *Eco*RV; P, *Pst*I; B, *Bam*HI; I, *Eco*RI; S, *Sau*III; CA, the carboxyl end of λ; S, the S end of λ; *lctD*, the coding region for L-lactate dehydrogenase.

lctP for L-lactate permease (see the section below for additional evidence). As a control experiment, D-lactate dehydrogenase specific activity (specified by *dld* at min 47) was also assayed in strains ECL116, ECL903, and ECL903/pLCT2. As expected, all three showed similarly high constitutive specific activities (data not shown).

Sequence analysis of the *lct* genes. The insert between the *Hin*dIII site and the junction of *Sau*III-*Bam*HI of pLCT2 was sequenced as described in Materials and Methods. Three ORFs were identified: ORF1 (*lctP*) of 1,653 bp (from nucleotides 1699 to 3354), ORF2 (*lctR*) of 774 bp (from nucleotides 3354 to 4128), and ORF3 (*lctD*) of 1,107 bp (from nucleotides 4127 to 5317). The deduced amino acid sequences contained within the span of the *Pst*I and *Mlu*I sites are shown in Fig. 2. The stop codon of the upstream gene overlaps the start codon of the downstream gene in such a way that the three coding regions are arranged in three different reading frames.

The *lctP*-encoded protein has 551 amino acid residues. A hydrophobicity plot indicates that LctP is a transmembrane protein (Fig. 3). Although no homology between the gene product and known bacterial permeases was found by AUTOSEARCH, the role of LctP as a transport protein was supported by a complementation test. Whereas pLCT2 conferred both transport and dehydrogenase activities (Table 3), a plasmid containing the downstream gene conferred only the dehydrogenase activity (data not shown). The upstream *lctP* gene, therefore, most likely specifies the permease.

The *lctR*-encoded protein has 258 amino acid residues. The N-terminal helix-turn-helix motif was found, and that motif was homologous to those of a number of bacterial transcriptional factors (Fig. 4), including protein A (36), P30 (5), FadR (11), and PhnF (7) of *E. coli*; HutC of *Klebsiella aerogenes* (34) and *Pseudomonas putida* (1), and GntR of *Bacillus subtilis* (12).

The *lctD*-encoded protein has 369 amino acid residues. Significant homology was found between LctD and several flavin mononucleotide (FMN)-dependent enzymes (Fig. 5), including the glycolate oxidase of spinach (41), the (*S*)-mandelate dehydrogenase of *P. putida* (40), the L-lactate 2-monooxygenase of *Mycobacterium smegmatis* (14), and flavocytochromes b_2 (L-lactate dehydrogenases) of *Saccharomyces cerevisiae* (21) and *Hansenula anomala* (33). These proteins probably all contain an eight-stranded α/β barrel with conserved sites for FMN-substrate binding and catalysis (24, 25, 42). To confirm the coding region for L-lactate dehydrogenase, we subcloned the insert of pLCT2 from the *Eco*RV site to the *Sau*III-*Bam*HI junction (Fig. 1). This fragment restored the enzyme activity in strain ECL901 (data not shown).

Although homology was found between L-lactate dehydrogenase and a number of FMN-dependent enzymes from both prokaryotes and eukaryotes, no significant homology was found by pairwise alignment (program PILEUP) with several flavodehydrogenases of *E. coli* itself. These include the *dld*-encoded D-lactate dehydrogenase (6), the *glpD*-encoded aerobic glycerol-3-phosphate dehydrogenase (2), and the *glpACB*-encoded anaerobic glycerol-3-phosphate dehydrogenase (10).

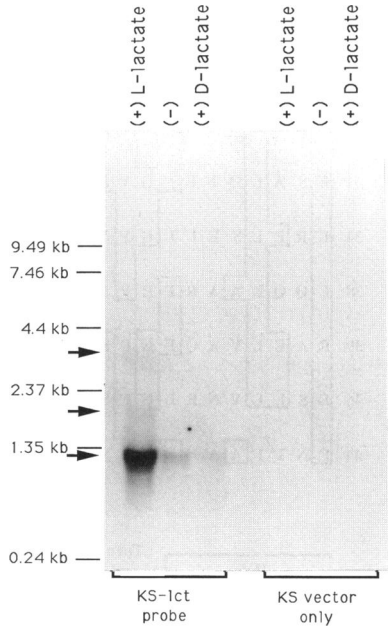

FIG. 7. Stimulation of *lct* transcription. Wild-type strain ECL525 (*lct*$^+$) was grown aerobically in the presence (+) or absence (−) of 10 mM L- or D-lactate. Total RNA was prepared for Northern blotting as described in Materials and Methods. The left panel was blotted with the specific ^{32}P-labelled Lct probe shown in Fig. 1. The positions of three Lct mRNAs are indicated by arrows. The right panel was blotted with the labelled vector only. The standard RNA markers are shown on the left side of the blot.

Lack of homology with the FAD-dependent GlpA protein is especially noteworthy, because in vitro assay of its catalytic activity can be stimulated up to 10-fold by FMN (17). The solitary status of *lctD* might reflect its origin by horizontal gene transfer.

Localization of the *lacZ* insertion in *lctD* by polymerase chain reaction. From the sequence of the *lct* genes, we designed primers to determine the position of the λp*lac*Mu*9* insertion in strain ECL901 by polymerase chain reaction and found that the insertion was in *lctD* (Fig. 6). A puzzling feature of *lct* transcription is the loss of permease activity encoded by the upstream *lctP* as a result of the λp*lac*Mu*9* fusion in the downstream *lctD*. The possibility that permease activity required the presence of the dehydrogenase protein was excluded by failure of an *lctD*-expressing plasmid to restore transport activity in the fusion strain. Among other possibilities is the stabilization of the *lct* transcript by a downstream *cis* element which was disrupted by the insertion.

Stimulation of *lct* transcriptions by L-lactate. To display the expression pattern of the *lct* genes, Northern blot analysis was performed with strain ECL525 (*lct*$^+$), using the specific probe shown in Fig. 1. Three Lct mRNAs were revealed (Fig. 7): the most abundant and shortest messenger had the expected size for *lctD* (around 1.2 kb); the other two mRNA bands, about 2.0 kb and 3.6 kb, were faint but reproducible. The total length of the three *lct* genes was expected to be about 3.6 kb. The amounts of all three transcripts were increased aerobically by L-lactate but not by D-lactate. Whether the appearance of multiple transcripts resulted from mRNA processing and/or the presence of several start sites remains to be resolved. One approach would be to carry out sequential deletions from the upstream end to see the extent of loss of *lctD* expression. The use of the Φ(*lctD-lac*) protein fusion should also facilitate the definition of the promoter(s).

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