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Anaerobic growth defects resulting from gene fusions affecting succinyl-CoA synthetase in *Escherichia coli* K12

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Summary. Insertion of the fusion-generating phage Mu*dI* (Ap, *lacZ*) yielded two similar isolates, DC511 and DC512, which were unable to grow aerobically on acetate or alpha-ketoglutarate but which could use succinate, malate, fumarate, glycerol, and various sugars. These mutants were unable to grow anaerobically on most sugars unless provided with methionine, lysine, and delta-aminolevulinic acid, all of which require succinyl-CoA for their synthesis. The insertions of both mutants mapped at 17 min, in the *suc* operon. Enzyme assays indicated a lack of succinyl-CoA synthetase; however, full activity of the alpha-ketoglutarate dehydrogenase was retained. Beta-galactosidase expression by strains containing these gene fusions was reduced under anaerobic conditions. In aerobically grown cultures, both fusions were induced about fivefold in the presence of acetate. This type of regulation would be expected of a Krebs cycle enzyme.

Key words: Fermentation – Acetate – Succinate – Alpha-ketoglutarate – Krebs cycle

Introduction

The facultative anaerobe *Escherichia coli* can grow either in the presence or absence of oxygen. Under aerobic conditions acetyl-CoA is oxidized to CO_2 by the Krebs cycle. Under anaerobic conditions, synthesis of the enzymes of the Krebs cycle is either reduced or in some cases completely abolished. In particular the alpha-ketoglutarate dehydrogenase (KGDH) complex is absent (Amarasingham and Davis 1965) and consequently the cycle is reduced to two disconnected branches whose enzymes are retained at a low level for the purpose of biosynthesis. The operation of the branch from citrate to alpha-ketoglutarate is required for the synthesis of glutamate and proline. The other branch is needed both for the synthesis of aspartate from oxaloacetate and also for the synthesis of methionine, lysine and delta-aminolevulinic acid which requires succinyl-CoA (Zubay 1988). Aerobically, succinyl-CoA is made from alpha-ketoglutarate by KGDH. Anaerobically succinyl-CoA must be made from succinate, by succinyl-CoA synthetase (SCS; Gottschalk 1986). However, both KGDH and SCS are coded for by the *suc* operon at 17 min on the *E. coli* chromosome (Bachmann 1983). The two promoter proximal genes *sucA* and *B* code for KGDH (Darlison et al. 1984) whereas the distal genes *sucC* and *D* encode SCS (Buck et al. 1986). The complete absence of KGDH under anaerobic conditions despite the presence of low but significant levels of SCS is thus paradoxical. We report here the isolation of gene fusions in *sucCD* which result in the loss of SCS activity. As expected these mutants are auxotrophic for lysine, methionine, and delta-aminolevulinic acid under anaerobic conditions. The regulation of the two halves of the *suc* operon is discussed with respect to previous work.

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Materials and methods

Bacterial strains and media. All strains used are derivatives of *E. coli* K12 and are listed in Table 1. The Tn*10* insertions of strains FD222 and FD322 are close to the *sucCD* mutation of DC511 and were isolated by Frank Dailey. Minimal medium was medium M9 (Miller 1972). Carbon sources were added to 0.4% (w/v) together with amino acids (50 mg/l) and vitamins (5 mg/l) where appropriate. Rich broth contained, 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract per liter. Solid media contained Bacto-agar (1.5% w/v). Anaerobic growth was performed in Oxoid anaerobic jars under a H_2/CO_2 atmosphere generated by means of Oxoid Gas-Paks. All media used for anaerobic growth were supplemented with the trace elements Fe, Se, and Mo as described previously (Winkelman and Clark 1986). Growth was followed turbido metrically using a Klett-Sumerson colorimeter equipped with a green (540 nm) filter. Anaerobic liquid cultures were grown without agitation in tubes inside an anaerobic jar or in milk dilution bottles filled to overflowing before sealing. Resazurin indicators were used to ensure anaerobic conditions. Cultures were grown at 33° C in various media to approximately 5×10^8 cells/ml before beta-galactosidase assay. Tetrazolium indicator plates were made according to Bochner and Savageau (1977), except that the buffer used was M9 salts.

Isolation of gene fusions. Gene fusions were selected as before in strain MC4100 using the phage Mu*dI* (Ap^r *lac*) prepared by heat induction of the double lysogen MAL103 (Clark 1981). Adsorption was performed at an m.o.i. of approximately 1 in rich broth containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$ for 20 min at 30° C. After a 1 in 10 dilution into rich broth, the culture was grown for 30 min at 30° C to allow expression of ampicillin resistance. Samples were then plated onto lactose MacConkey agar containing

Table 1. Strains of *Escherichia coli* K12

Strain	Relevant characteristics	Source/reference
DC511	*Φ (sucCD-lacZ)* of MC4100	See text
DC512	*Φ (sucCD-lacZ)* of MC4100	See text
DC859	*purF*:: Tn*10* of DC511	P1 (NK6035) × DC511
DC869	*fadR*:: Tn*10* of DC511	P1 (RW11) × DC511
DC870	*fadR*:: Tn*10* of DC512	P1 (RW11) × DC512
DC873	*Δ (sucCD-lacZ)* of DC511	See text
DC876	*pur*$^+$*facA* of DC859	P1 (JRG1078) × DC859
DC879	*pur*$^+$*Δ (pta ackA)* of DC859	P1 (TA3516) × DC859
DC966	*zbg2*:: Tn*10* of W1485	P1 (FD222) × W1485
DC967	*zbg3*:: Tn*10* of W1485	P1 (FD332) × W1485
DC977	"Revertant" of DC512	See text
DC980	"Revertant" of DC873	See text
FD222	*zbg2*:: Tn*10* (near *suc*)	F. Dailey
FD332	*zbg3*:: Tn*10* (near *suc*)	F. Dailey
TA3516	*Δ (pta ackA)*	G. Ames
NK6035	*purF*:: Tn*10*	N. Kleckner
JRG1003	*sdh trp*	J. Guest
JRG1078	*facA*	J. Guest
MAL103	Mu*cts d1* (Ap^r/*lac*), Mu*cts Δ (pro-lac) XIII rpsL*	M. Casabadan
MC4100	*araD139 Δ (argF-lac) U169 rpsL thiA relA ptsF*	M. Casabadan
N43	*acrA gal*	B. Bachmann
NK6033	*nadA*:: Tn*10*	N. Kleckner
N3030	*gal*:: Tn*10*	B. Bachmann
RW11	*fadR*:: Tn*10*	W. Nunn
W1485	Wild-type	B. Bachmann

ampicillin (50 mg/l). Ampicillin-resistant colonies showing some Lac$^+$ response were picked and screened for their growth properties.

Enzyme assays. Beta-galactosidase was assayed as previously described (Winkelman and Clark 1986) except that cultures were grown to mid-exponential phase (approx. 5×10^8 cells/ml) before assay in a variety of media, under both aerobic and anaerobic conditions. The units of beta-galactosidase activity are micromoles of *o*-nitrophenyl galactoside (ONPG) hydrolysed per 10^9 cells per hour, at 37° C. Acetate kinase (ACK) and phosphotransacetylase (PTA) were assayed as described by Guest (1979) and acetyl-CoA synthetase (ACS) was assayed by the method of Brown et al. (1977). SCS was assayed by following the formation of succinyl-CoA at 230 nm as described by the method of Bridger et al. (1969). KGDH was assayed by following NADH formation at 340 nm as described by Hager and Kornberg (1961).

Results

Isolation and growth properties of acetate negative fusion strains

Approximately 5000 randomly generated gene fusion strains made by the insertion of Mu*d1* into MC4100 (as described in Materials and methods) were screened for their ability to grow aerobically on a variety of carbon sources. Eight insertion mutants were found which were unable to grow on acetate (i.e., Ace$^-$) but grew well on succinate, malate, lactate, or glucose. Two of these Ace$^-$ mutants (DC511 and DC512) grew well on oleate and retained wild-type levels of the two glyoxylate cycle enyzmes, malate synthase, and isocitrate lyase (data not shown). Neither DC511 and DC512 grew anaerobically on glucose.

Table 2. Aerobic growth properties of succinyl-CoA synthetase mutants[a]

Strain[b]	Genotype	Carbon source[c]			
		ACE	OLE	KG	SUC
MC4100	Wild-type	++	++	++	++
DC511	*sucCD*:: *lacZ*	−−	++	−−	++
DC512	*sucCD*:: *lacZ*	−−	++	−−	++
DC873	*Δ (sucCD-lacZ)*	−−	++	−−	++
DC977	Revertant of DC512	++	++	++	++
DC980	Revertant of DC873	+	++	++	++
DC876	*sucCD*:: *lacZ facA*	−−	+/−	−−	++
DC879	*sucCD*:: *lacZ Δ (pta/ack)*	−−	+/−	−−	++
JRG1078	*facA*	+/−	+	+	++
TA3516	*Δ (pta/ack)*	+/−	+	+	++

[a] Growth was tested on M9 minimal agar containing the indicated carbon source, under aerobic conditions
[b] All strains grew on malate, glycerol or a variety of sugars
[c] ACE, acetate; OLE, oleate; SUC, succinate; KG, alpha-ketoglutarate

Starting with DC511 we selected colonies able to grow at 42° C. Some of these were Lac$^-$ and had lost ampicillin resistance, and are therefore deletions which have lost the temperature-sensitive Mu*d1* (Ap *lac*) phage. Several independent deletion derivatives showed similar growth properties to DC511 and showed similar enzyme defects. Strain DC873 is representative of these deletion derivatives (see Table 2).

The parent strain MC4100, and the mutants DC511 and DC512 were tested on a variety of media under aerobic and anaerobic conditions. As shown in Table 2, the novel Ace$^-$ mutants failed completely to grow on acetate aerobically whereas *facA* and *pta/ack* mutants grew slowly. All strains grew aerobically on oleate, malate, succinate, glucose, and a variety of other carbon sources. However neither DC511 nor DC512 was able to grow aerobically on alpha-ketoglutarate whereas wild-type and *pta/ack* strains could use this substrate.

DC511, DC512, *pta/ack* and *facA* strains all failed to grow anaerobically on most hexoses and hexitols (Table 3). However, DC511 and DC512 grew on the deoxysugar L-fucose and on glycerol plus fumarate whereas *pta/ack* and *facA* strains did not. Derivatives of DC511 and DC512 also carrying *pta/ack* or *facA* mutations showed the more severe growth defects characteristic of the latter mutations (not shown). All strains grew anaerobically on glycerol plus nitrate. We found that supplementation with metabolites derived from succinate via succinyl-CoA, i.e. methionine, lysine, and delta-aminolevulinic acid overcame the anaerobic growth defect of DC511 and DC512 whereas succinate itself had no effect (Table 3). This argued that DC511 and DC512 were not defective in fermentation per se, but were

Table 3. Anaerobic growth tests[a]

Strain[b]	Genotype	Growth medium[c]				
		GLC	GLC + SUC	GLC + MKD	FUC	GRL + FUM
MC4100	Wild-type	++	++	++	++	++
DC511	*sucCD::lacZ*	−−	−−	++	++	++
DC512	*sucCD::lacZ*	−−	−−	++	++	++
DC873	*Δ (sucCD-lacZ)*	−−	−−	++	++	++
DC977	Revertant of DC512	++	++	++	++	++
DC980	Revertant of DC873	++	++	++	++	++
JRG1078	*facA*	−−	−−	−−	−−	−−
TA3516	*Δ (pta/ack)*	−−	−−	−−	−−	−−

[a] Growth was tested on M9 minimal agar containing the carbon source and supplements indicated. Incubation was for 3 days at 33° C under anaerobic conditions

[b] All strains grew on glycerol plus nitrate. Strains with succinyl-CoA synthetase (SCS) defects grew on glucuronic acid without supplementation (as for fucose) whereas mannose, sorbitol, gluconate, galactose and xylose gave results equivalent to glucose

[c] GLC, glucose; SUC, succinate; MKD, methionine plus lysine plus delta-aminolevulinic acid; FUC, fucose; GRL, glycerol; FUM, fumarate

Table 4. Succinyl-CoA synthetase (SCS) and alpha-ketoglutarate dehydrogenase (KGDH) activities[a]

Strain	Medium additive[b]	Air	SCS[c]	KGDH[d]
W1485 wild type	−	+	0.333	0.34
MC4100 wild type	−	+	0.240	0.39
DC511 *sucCD*	−	+	0.009	0.21
DC512 *sucCD*	−	+	0.024	0.13
DC873 *Δ sucCD*	−	+	0.000	0.21
DC980 revertant	−	+	0.004	0.33
DC977 revertant	−	+	0.150	0.18
MC4100 wild type	ACE	+	0.542	0.58
MC4100 wild type	GLC	+	0.019	0.26
MC4100 wild type	GAL	−	0.099	0.21
MC4100 wild type	GLC	−	0.027	0.05
MC4100 wild type	GRL/FUM	−	0.184	0.02

[a] Cultures were grown at 33° C in rich broth with additives indicated

[b] ACE, acetate; GLC, glucose; GAL, galactose; GRL/FUM, glycerol plus fumarate

[c] SCS activities are in micromoles of succinyl-CoA formed per minute per milligram protein

[d] KGDH activities are in micromoles of NADH formed per minute per milligram protein

unable to generate succinyl-CoA anaerobically. Aerobic growth ability on acetate or alpha-ketoglutarate was not regained by supplementation with these amino acids.

The dye alizarin yellow has been shown to inhibit *pta/ack* mutants selectively although the basis for its action is unknown (Levine et al. 1980). We found that 0.25% alizarin yellow inhibited the growth of JRG1078 (*facA*), TA3516 (*pta/ack*), and also of DC511 and DC512 on minimal agar with succinate plus casamino acids. Wild-type strains such as MC4100 and mutants defective in the glyoxylate cycle were not inhibited. We also tested Martius yellow, a nitroaromatic dye of simpler structure and found that DC511 and DC512 were selectively inhibited by 25 mg/l, whereas both wild-type and *pta/ack* strains were relatively resistant (killed by 125 mg/l).

Assay of enzymes involved in Krebs cycle and acetate metabolism

The fusion strains DC511, DC512, their parent MC4100, and several other wild-type strains of *E. coli* K12 were assayed for the enzymes ACK, PTA and ACS. All of these strains had approximately the same levels of these enzymes under most growth conditions. These data have already appeared in a preliminary account of these mutants (Clark et al. 1988).

The parent MC4100 and the mutants DC511 and DC512 were assayed for SCS. Both MC4100 and the wild-type strain W1485 had high and comparable levels of SCS. However, no significant activity was found in DC511 or several derivatives of this strain such as DC873 or DC980 (Table 4). In contrast DC512 had some activity remaining which suggested that the insertion in this strain may not actually be in either of the structural genes for SCS (i.e., *sucC* or *sucD*).

The SCS activity of the wild-type strain MC4100 was induced two- to threefold by acetate, repressed about tenfold by glucose and substantially reduced under anaerobic conditions in the presence of sugars (Table 4). These effects are similar to those previously noted by Buck et al. (1986). All strains were also assayed for KGDH. The SCS-negative mutants all retained full KGDH activity (Table 4).

Selection of second site revertants

The fusion strains DC511 and DC512 form white colonies on tetrazolium/acetate indicator plates. However, cultures of DC512 showed occasional red derivatives on such plates. One such isolate DC977 was purified and tested. It showed the growth properties of the wild type (Tables 2 and 3) yet retained the Mu*dI* insertion, suggesting a mutation at a second site, rather than true reversion. We also selected derivatives of the SCS mutants which had regained resistance to the dye Martius yellow. One such derivative, DC980 was derived from the deletion mutant DC873. Strain DC980 also regained the ability to grow aerobically on alpha-ketoglutarate or acetate and to grow anaerobically without supplementation with methionine, lysine, and delta-aminolevulinic acid. Since this "revertant" was selected from a deletion derivative of DC511 it confirms that second site mutations somewhere else on the chromosome can cure the growth defects due to a defect in SCS.

The revertants DC977 and DC980 were assayed for SCS. Strain DC977 (which is a derivative of DC512) had regained substantial SCS activity whereas DC980 (a derivative of DC511) had no significant SCS activity yet is nonetheless able to grow on acetate or alpha-ketoglutarate. It has been shown that mammalian tissues contain distinct ATP- and GTP-linked SCSs (Jenkins and Williams 1988). We therefore re-assayed the revertant strains using GTP

Table 5. Beta-galactosidase expression by *sucCD* fusion strains

Strain	Additional mutation	Beta-galactosidase activity[a]		
		GLC Air	GLC Ana	ACE Air
DC511	– –	13880	2190	84800
DC869	*fadR*	11800	3180	17720
DC875	– –	11270	1980	23210
DC876	*facA*	9340	7290	26910
DC879	*pta/ack*	16380	7230	5750
DC512	– –	5150	1210	25300
DC870	*fadR*	15030	3530	21230

[a] Cultures were grown aerobically (Air) or anaerobically (Ana) at 33° C in rich broth plus 0.4% of the indicated substrate. GLC, glucose; ACE, acetate

instead of ATP, in case the appearance of a GTP-linked SCS activity was responsible for their ability to grow on alpha-ketoglutarate. No significant GTP-linked activity was found in either the SCS mutants or the revertant DC980. In wild-type strains, SCS activity with GTP was less than 1% of the activity seen with ATP (data not shown).

Regulation of beta-galactosidase

The expression of beta-galactosidase by the fusion strains DC511 and DC512 and a variety of derivatives containing *fadR*, *facA*, and *pta/ack* mutations was examined both aerobically and anaerobically (Table 5). Strain DC511 and its *fadR*, *facA*, and *pta/ack* derivatives all produced approximately equivalent amounts of beta-galactosidase activity, aerobically with rich broth plus glucose. Under anaerobic conditions expression was reduced about fivefold for DC511 and DC869 *fadR*. However, the *facA* and *pta/ack* derivatives both showed little decrease. Strain DC512 showed much the same pattern, except that its level of expression was lower than that of DC511 and was increased about threefold by introduction of a *fadR* mutation. The *fadR* mutation is known to derepress both the enzymes of fatty acid degradation (Clark 1981) and of the glyoxylate cycle (Maloy and Nunn 1982). The parental fusions DC511 and DC512 were also examined after anaerobic growth in rich broth plus galactose, a poorly catabolite-repressing sugar and both expressed approximately 5000 units of activity, i.e., about threefold greater than with glucose. Under anaerobic conditions, with glycerol plus fumarate beta-galactosidase levels were higher still (11000 for DC511 and 8000 for DC512). Under aerobic conditions, a moderate induction of around three- to fourfold was observed in the presence of acetate.

Genetic mapping

Preliminary mapping was carried out by conjugation and indicated a location in the 15 to 20 min region of the chromosome (data not shown). More detailed mapping was performed by cotransduction with Tn*10* insertions in the region of interest (Table 6). The *nadA*:: Tn*10* insertion of NK6033, located at 16.9 min showed the highest contransduction frequency with the mutations of DC511, DC512, and the corresponding deletion of DC873. The two Tn*10* insertions which were isolated as being near to the mutations of DC511 and DC512 were from 30% to 80% cotransducible with genetic markers in the 17 min region (i.e., *sdh* and *gal*). The Tn*10* insertions of FD222 and FD322 were therefore designated respectively *zbg2*:: Tn*10*, and *zbg3*:: Tn*10*. A map of the 17 min region showing cotransduction frequencies appears in Fig. 1.

Table 6. Cotransductional mapping of acetate defect

P1 donor		Recipient[a]		Colonies scored	% Co-transduction
DC966	*zbg2*:: Tn*10*	DC511	*sucCD*	200	86
DC966	*zbg2*:: Tn*10*	DC512	*sucCD*	100	82
DC966	*zbg2*:: Tn*10*	JRG1003	*sdh*	100	68
DC967	*zbg3*:: Tn*10*	DC511	*sucCD*	100	32
DC967	*zbg3*:: Tn*10*	DC512	*sucCD*	100	40
DC967	*zbg3*:: Tn*10*	DC873	*Δ sucCD*	100	34
DC967	*zbg3*:: Tn*10*	N43	*gal*	100	4
DC967	*zbg3*:: Tn*10*	JRG1003	*sdh*	140	56
N3030	*gal*:: Tn*10*	DC511	*sucCD*	61	57
N3030	*gal*:: Tn*10*	JRG1003	*sdh*	100	43
NK6033	*nadA*:: Tn*10*	DC511	*sucCD*	100	88
NK6033	*nadA*:: Tn*10*	DC512	*sucCD*	100	90
NK6033	*nadA*:: Tn*10*	DC873	*Δ sucCD*	100	92
NK6033	*nadA*:: Tn*10*	JRG1003	*sdh*	100	75

[a] Transductants were selected for resistance to tetracyline and scored for the markers of the recipient by testing for growth on acetate (*sucCD*), succinate (*sdh*), or galactose (*gal*)

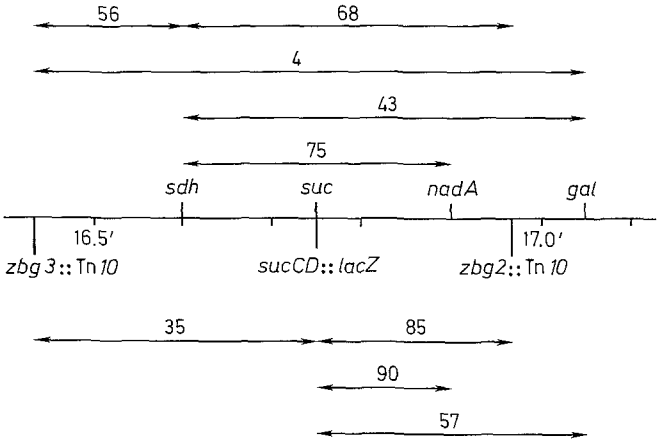

Fig. 1. Map of 17 min region. The gene order is according to Bachmann (1983) and Buck et al. (1986). Since the fusions of DC511 and DC512 inactivate the succinyl-CoA synthetase but not the alpha-ketoglutarate dehydrogenase activity of the *suc* operon they have been indicated as *sucCD*:: *lacZ*. The *zbg2*:: Tn*10* insertion is that of DC966 and *zbg3*:: Tn*10* is that from DC967. Cotransduction frequencies shown are averages from our own experiments

Discussion

The two mutants, DC511 and DC512, lack SCS. These fusions mapped at 17 min and are therefore probably insertions in the *sucABCD* operon, which encodes both KGDH

and SCS (also known as succinate thiokinase). Mutants defective in *sucAB* which encode KGDH cannot convert alpha-ketoglutarate to succinyl-CoA and hence are succinate auxotrophs, at least under aerobic conditions (Creaghan and Guest 1972), KGDH is considered to be inactive anaerobically (Amarasingham and Davis 1965) and succinate and succinyl-CoA must be synthesized by another route, presumably from fumarate (Langley and Guest 1978). Our mutants grow aerobically on glucose minimal medium and retain KGDH, suggesting that the fusion point is in the rear half of the operon. This accords with the gene order of Buck et al. (1986), in which *sucAB* coding for KGDH precede *sucCD* which encode SCS. A defect in SCS should prevent aerobic growth on acetate and ketoglutarate but should still allow growth on Krebs cycle intermediates beyond the block (e.g., succinate, malate, etc). Aerobically SCS mutants make succinyl-CoA from alpha-ketoglutarate via the KGDH and succinate from fumarate by reversing the succinate dehydrogenase reaction. They are therefore not auxotrophs in air. However, succinate is made anaerobically from fumarate by fumarate reductase and converted by SCS to succinyl-CoA the precursor of lysine, methionine, and delta-aminolevulinic acid. This is necessary since KGDH is inactive anaerobically (Amarasingham and Davis 1965). Thus SCS mutants are auxotrophic, under anaerobic conditions, for metabolites derived from succinyl-CoA while succinate itself is ineffective.

One curious observation was that DC511 and DC512 grew well on oleic acid which is converted to acetyl-CoA by the beta-oxidation pathway. Initially this seemed incompatible with a Krebs cycle defect. However energy can be produced from the NADH produced during beta-oxidation and the acetyl-CoA produced can be excreted as acetic acid via PTA and ACK. This interpretation is supported by the finding that whereas both separate SCS and *pta/ack* mutants can grow on long chain fatty acids, double mutants grew very poorly (Table 2). The fact that DC511 and DC512 can grow anaerobically on the deoxysugar fucose or on glucuronic acid without any amino acid supplements suggests that an alternative pathway for the synthesis of succinyl-CoA exists under these conditions. Perhaps second site revertants such as DC980 have deregulated this pathway and hence can synthesize succinyl-CoA without using SCS.

The beta-galactosidase measurements indicated lower gene expression under anaerobic conditions and some induction by acetate. This seems reasonable for a Krebs cycle gene, and agrees with previous observations by Buck et al. (1986). However, under anaerobic conditions there is still substantial expression of the *sucCD-lacZ* fusions. This is reasonable for SCS, which is required under these conditions for biosynthesis, however KGDH, coded by the proximal genes of the same operon, is essentially absent. Perhaps it is the lack under anaerobic conditions of the lipoamide dehydrogenase component, coded by *lpd* in the *aceEFlpd* operon (Langley and Guest 1978) which accounts for this. Alternatively Guest has suggested the possibility of a secondary promoter within the *suc* operon (Buck et al. 1986), which results in transcription of only the *sucCD* genes.

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