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Role and Control of Isocitrate Lyase in *Candida lipolytica*

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Mutants of *Candida lipolytica* that were unable to grow on acetate but able to utilize succinate or glycerol as a sole carbon source were isolated. Amongst the mutants isolated, one strain (Icl^-) was specifically deficient in isocitrate lyase activity, whereas another strain ($Acos^-$) was deficient in acetyl coenzyme A synthetase activity. Since the Icl^- mutant could not grow either on *n*-alkane or its derivatives, such as fatty acid and long-chain dicarboxylic acid, any anaplerotic route other than the glyoxylate pathway was inconceivable as far as growth on these carbon sources was concerned. Acetyl coenzyme A is most likely a metabolic inducer of isocitrate lyase and malate synthase, because the $Acos^-$ mutant was characterized by the least susceptibility to induction of these enzymes by acetate. The structural gene for isocitrate lyase was most probably impaired in the Icl^- mutant, since revertants (Icl^+) produced thermolabile isocitrate lyase. The production of isocitrate from *n*-alkane by the revertants was enhanced in comparison with the parental strain.

Growth of microorganisms on C_2 compounds, such as acetate, or substrates to be converted ultimately to C_2 intermediates requires the glyoxylate pathway as an anaplerotic sequence for the supply of C_4 compounds to the tricarboxylic acid cycle (12). Isolation of mutants unable to utilize acetate as a sole carbon source due to a deficiency in isocitrate lyase or malate synthase activity permits an assessment of the anaplerotic function of these enzymes. Studies along this line have been made in various microorganisms, including bacteria (*Escherichia coli* [4]) and filamentous fungi (*Neurospora crassa* [9], *Aspergillus nidulans* [3], *Coprinus cinereus* [11]).

Candida lipolytica can grow on acetate as well as *n*-alkane as a sole carbon source. The ability of this yeast to assimilate the latter carbon source stimulates an interest in the role and control of the glyoxylate pathway enzymes. It is thought that *n*-alkane is first converted to fatty acid by terminal oxidation, and the latter is degraded to yield acetyl coenzyme A (acetyl-CoA) via β-oxidation. In this case, the glyoxylate pathway is an indispensable anaplerotic route for growth on *n*-alkane. However, if a diterminal oxidation of *n*-alkane with an even number of carbons occurs (15), the long-chain dicarboxylic acid thus produced might lead directly to succinic acid as an intermediate of the tricarboxylic acid cycle after successive removal of C_2 units via β-oxidation. In the latter case, the yeast might not require the glyoxylate pathway. The problem of whether or not the glyoxylate pathway is indispensable for growth on *n*-alkane remains to be resolved.

Evidence that acetyl-CoA functions as an inducer for isocitrate lyase and malate synthase in this yeast will be presented. The syntheses of these anaplerotic enzymes in procaryotes and eucaryotes in terms of "negative control" have been revealed genetically by Brice and Kornberg (5) and other workers; it is claimed that the metabolite responsible for the control is phosphoenolpyruvate for *E. coli* (12) or acetyl-CoA for *A. nidulans* (3) and *C. cinereus* (6).

C. lipolytica, used in this study, produces citrate and isocitrate from *n*-alkane (1). The ratio of these tricarboxylic acids excreted into the culture medium might be affected by the relative amounts of enzymes in the tricarboxylic acid cycle and its related metabolic pathways. Akiyama et al. (2) isolated a mutant of *C. lipolytica* that produced citrate almost exclusively and found that the aconitate hydratase activity of this mutant was lowered considerably. By the same token, it is worthwhile to examine the possibility of enhancing isocitrate production by a mutant defective or leaky in isocitrate lyase activity. This paper describes how revertants obtained from a mutant deficient in isocitrate lyase activity have been used for this purpose.

MATERIALS AND METHODS

Strains. A citrate-producing yeast strain, *C. lipolytica* MT4, stocked in our laboratory was used throughout as a wild-type parent (1). Mutants and their revertants were derived from this parent as described below.

Media. The minimal medium and complete medium (yeast extract-peptone-dextrose [YEPD]) used

were essentially those of Fink (8). The minimal medium containing 20 g of glucose and 1 g of $(NH_4)_2SO_4$ per liter is designated hereafter as MM-glucose. For minimal media other than MM-glucose, glucose was replaced by 0.1 M potassium acetate, pH 6.5 (MM-acetate); 0.1 M potassium succinate, pH 6.5 (MM-succinate); 2% (vol/vol) glycerol (MM-glycerol); or 5% (vol/vol) *n*-alkane mixture (MM-alkane) (for composition of the mixture, see reference 1). Media except for MM-alkane were solidified by 2% (wt/vol) agar, when necessary. Nitrogen-free minimal medium was prepared by omitting $(NH_4)_2SO_4$ from the medium. Each medium was autoclaved at 120°C for 20 min.

Mutagenesis. All the following procedures, unless otherwise stated, were carried out at 30°C. Cells subjected to mutagenesis were prepared from an overnight culture in liquid YEPD. Ethyl methane sulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was used as a mutagen. The procedure of mutagenesis with ethyl methane sulfonate was basically that of Fink (8) except that cells were treated with ethyl methane sulfonate in 0.1 M sodium phosphate buffer (pH 8.0) containing 2% (wt/vol) glucose. For *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis, a cell suspension in 0.1 M Tris-maleate buffer (pH 7.0) was shaken with 400 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml. Each mutagenesis was conducted in such a way that the survival of cells was around 10%. The mutagen-treated cells were washed three times by centrifuging and resuspending them in sterile water and distributed into several flasks containing MM-succinate or MM-glycerol. The flasks were incubated for 2 to 3 days on a rotary shaker. The surviving cells which had grown were treated with nystatin to enrich for acetate-non-utilizing (Acu^-) mutants (see below). Only one Acu^- mutant was taken independently from each subdivided flask.

When revertants of the Acu^- mutant were required, cells of the Acu^- mutant were treated with ethyl methane sulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described above, but portions of the cell suspension after mutagenesis and washing were directly spread onto MM-acetate plates. Revertant colonies which appeared on the plates within 1 week of incubation were obtained.

Nystatin enrichment. The nystatin enrichment derived by Snow (16) was adopted to facilitate the selection of Acu^- mutants. Cells grown in MM-succinate or MM-glycerol were washed twice with water, suspended in nitrogen-free MM-acetate or MM-glycerol, and shaken for at least 6 h in order to expose cells to nitrogen starvation. Then the cells were washed twice with water, transferred into MM-acetate at about 10^7 cells per ml, and incubated on a rotary shaker overnight. Taking into account the facts that the pH of the culture in MM-acetate increases with the cellular growth and that nystatin is not effective in alkali, a 1 N HCl solution was carefully dropped into the culture to adjust the pH to around 4. To this acidified culture nystatin dissolved freshly in ethanol was added to a final concentration of 20 μg/ml, and the culture was incubated for 1 h with shaking. The survival of cells after nystatin treatment was confirmed to be about 0.1%. Nystatin-treated cells were washed three times, spread on YEPD plates, and incubated for 2 days. Colonies which appeared on the plate were replicated onto an MM-acetate plate, and those unable to grow on the MM-acetate plate were purified by single-colony isolation. Mutants (Acu^-) were finally obtained after reexamining them for growth on an MM-glucose plate and nongrowth on an MM-acetate plate.

Preparation of cell extracts. Cells grown in MM-glucose for 18 h were washed, transferred into MM-acetate, and incubated for 6 h to induce enzymes for acetate metabolism before the cells were recovered by centrifugation. When *n*-alkane was used as the carbon source, cells grown in MM-alkane were harvested by filtration on a membrane filter (pore size, 8.0 μm; Millipore Corp., Bedford, Mass.) and stored, if necessary, at −20°C.

A cell-free crude extract was prepared by disintegrating the cells with a French press (400 to 600 kg/cm^2) at 4 to 10°C, followed by centrifugation at 35,000 × *g* for 15 min. The supernatant liquid was used for the following assays.

Enzyme assays. For enzyme assays, a double-beam spectrophotometer (model 124; Hitachi Works, Tokyo, Japan) equipped with a temperature controller was used at 30°C.

Isocitrate lyase (*threo*-D$_s$-isocitrate glyoxylate-lyase, EC 4.1.3.1) and malate synthase (L-malate glyoxylate-lyase [CoA-acetylating], EC 4.1.3.2) were assayed by the method of Dixon and Kornberg (G. H. Dixon and H. L. Kornberg, Biochem. J., **72**:3P, 1959, and reference 7, respectively) with the slight modification that concentrations of isocitrate, acetyl-CoA, and sodium glyoxylate in the reaction mixture were made twofold those in the original method.

Acetyl-CoA synthetase (acetate:CoA ligase [adenosine 5'-monophosphate-forming], EC 6.2.1.1) was assayed by the method of Webster (17) except that $NiCl_2$ was omitted from the reaction mixture.

One unit of enzyme activity was defined as that amount which consumes 1 μmol of substrate per min. Specific activities were expressed as milliunits per milligram of protein. Protein was assayed by the method of Lowry et al. (14).

Heat stability of isocitrate lyase. Cells grown in MM-acetate were suspended in an assay buffer (80 mM potassium phosphate buffer, pH 6.85, containing 6 mM $MgCl_2$ and 2.4 mM cysteine hydrochloride) and were disrupted as described above. The supernatant after centrifugation was used to examine the heat stability of isocitrate lyase at 35 and 40°C.

Production of citrate and isocitrate. MM-alkane, 100 ml in a 1-liter Erlenmeyer flask, was inoculated with a preculture grown in YEPD medium and incubated on a rotary shaker. Sterile $CaCO_3$ was added after 1 day.

Cell density and citrate and isocitrate in the broth were determined as described elsewhere (1).

Chemicals. Tetradecane-1,14-dicarboxylic acid was purchased from ICN-K & K Laboratories Inc., New York, N.Y. Ethyl methane sulfonate and nystatin were from Sigma Chemical Co., St. Louis, Mo. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was purchased from Wako Pure Chemical Industries, Osaka, Japan. The *n*-alkane mixture used was obtained from Nikko Petrochemical Co., Tokyo, Japan.

RESULTS

Isolation and characterization of Acu⁻ mutants. Thirty-one Acu⁻ mutants were isolated independently. All mutants could grow on glucose, glycerol, and succinate as sole carbon sources, but some of them failed to grow on *n*-alkane. Figure 1 shows the specific activity of malate synthase versus that of isocitrate lyase in acetate-induced Acu⁻ mutants. When the wild-type cells used as the control in each run of the enzyme assay were transferred from MM-glucose to MM-acetate and incubated successively for at least 6 h, both isocitrate lyase and malate synthase activities reached the maximal levels. However, these enzyme activities in the wild-type cells exhibited deviations of ±20% from run to run even under the same culture conditions. Accordingly, the ordinate and abscissa of Fig. 1 represent malate synthase and isocitrate lyase specific activities in relative scale, respectively, taking each specific activity of the wild-type cells in the same run as unity.

The positive correlation between isocitrate lyase and malate synthase specific activities in Fig. 1 suggests the existence of a common control mechanism for both enzymes. It is interesting to note that one group of Acu⁻ mutants capable of growing in MM-alkane (open symbols in Fig. 1) exhibited, by and large, lower inducibilities of the glyoxylate pathway enzymes than those of the wild-type parent, whereas the other group of Acu⁻ mutants, which failed to grow in MM-alkane (closed symbols in Fig. 1), showed mostly higher inducibilities of these enzymes by acetate. This correlation between inducibility and ability to utilize *n*-alkane in Acu⁻ mutants would be worth reassessing in more detail once the common inducer for the glyoxylate-pathway enzymes has been identified (see below).

Role of isocitrate lyase for growth on *n*-alkane. A specific mutant which was obtained from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis showed an extremely low level of isocitrate lyase activity, exhibiting a high activity of malate synthase. The activities of isocitrate lyase in this strain both before and after the induction by acetate were far below the uninduced level of glucose-grown wild-type cells (basal level) (Table 1), and thus the strain was designated an isocitrate lyase-deficient (Icl⁻) mutant.

The Icl⁻ strain was also unable to utilize *n*-alkane. No growth was detected after 1 week in a minimal medium containing *n*-hexadecane, *n*-pentadecane, palmitic acid, or tetradecane-1,14-dicarboxylic acid as the sole carbon source, whereas the wild-type parent could grow on these carbon sources (data not shown). Thus, it was inferred that isocitrate lyase was indispensable for assimilation of these carbon sources.

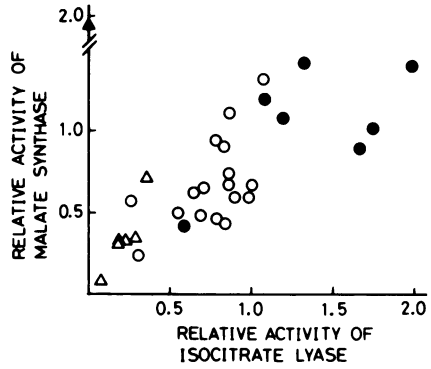

FIG. 1. *Malate synthase versus isocitrate lyase activities of Acu⁻ mutants. Glucose-grown cells were induced by acetate for 6 h. The Acu⁻ mutants were obtained from succinate-grown cells (circles) or glycerol-grown cells (triangles) after the mutagenesis. Open and closed symbols represent the ability and the inability to utilize n-alkane, respectively. Activities are shown in relative scale, taking each specific activity of the wild-type parent as unity (mean values for isocitrate lyase and malate synthase in the parent cells were 152 and 235 mU/mg of protein, respectively).*

Acetyl-CoA as a possible inducer. As mentioned above, Acu⁻ mutants that could grow on *n*-alkane were poorly induced for the glyoxylate pathway enzymes by acetate. In addition, such mutants, when grown on *n*-alkane, were found to have isocitrate lyase and malate synthase activities comparable to those of the wild-type cells (data not shown). These findings imply the presence of a specific metabolic inducer that does not come from acetate but does come from *n*-alkane. One of the possible enzyme lesions in such Acu⁻ mutants might be in the activity of acetyl-CoA synthetase. In fact, measurements of this enzyme activity in Acu⁻ mutants that could utilize *n*-alkane revealed one specific mutant which was deficient in acetyl-CoA synthetase activity (Acos⁻).

Table 1 summarizes the activities of isocitrate lyase, malate synthase, and acetyl-CoA synthetase in glucose-grown, acetate-induced, and *n*-alkane-grown cells of wild-type, Acos⁻, and Icl⁻ strains. When induced by acetate, the Acos⁻ strain exhibited the least inducibility of the glyoxylate pathway enzymes among all Acu⁻ mutants, and the levels of these enzymes did not increase appreciably above basal levels. Since the Acos⁻ strain could not convert acetate into acetyl-CoA, this fact strongly suggests the possibility that acetyl-CoA serves as an inducer of the glyoxylate pathway enzymes. Moreover, the

TABLE 1. *Specific activities of isocitrate lyase, malate synthase, and acetyl-CoA synthetase in glucose-grown, acetate-induced, and n-alkane-grown cells*

Strain	Sp act[a] in:						
	Glucose-grown cells		Acetate-induced cells			*n*-Alkane-grown cells	
	Isocitrate lyase	Malate synthase	Isocitrate lyase	Malate synthase	Acetyl-CoA synthetase	Isocitrate lyase	Malate synthase
Wild type	15	19	177	221	39	105	32
MX8-1 ($Acos^-$)	14	12	12	17	0	104	45
MX9-11 (Icl^-)	0.8	17	0.6	429	61	—[b]	—

[a] Specific activities are expressed in milliunits per milligram of protein as mean values of duplicate measurements.

[b] —, No growth was observed.

Icl^- strain possessed a marked activity of acetyl-CoA synthetase and was able to induce malate synthase more markedly than was the wild-type parent.

The above interpretation was confirmed by an observation that the *n*-alkane-grown $Acos^-$ strain, in contrast to the acetate-induced one, showed activities of isocitrate lyase and malate synthase that were commensurable to those in the wild-type parent (see also the data for *n*-alkane-grown cells in Table 1). In view of the fact that acetyl-CoA originates from *n*-alkane via β-oxidation of fatty acid, acetyl-CoA thus produced might have induced the glyoxylate pathway enzymes.

Properties of revertants from the Icl^- strain. The Icl^- strain, when smeared on MM-acetate agar, did not revert spontaneously. Hence, revertants of the Icl^- strain (284 in total) capable of growing on an MM-acetate plate at 20°C were isolated by ethyl methane sulfonate mutagenesis. When these Acu^+ revertants were transferred onto MM-acetate at 30°C, all grew normally, i.e., as did the wild-type parent. Thus, a temperature-sensitive Acu^+ phenotype was not observed among these revertants. Forty-two revertants among them were examined for their isocitrate lyase activities when grown in MM-alkane. The results showed that every revertant from the Icl^- strain regained, more or less, the activity of isocitrate lyase, whose recovery ranged from 30 to 100% of that in the wild-type parent. This fact is indicative that isocitrate lyase protein in these revertants had been altered.

Properties of three representative revertants are shown in Table 2. It was confirmed that the recovery of isocitrate lyase activity was rather independent of culture conditions used, since a revertant (MX9-11 R3) grown on *n*-alkane or induced by acetate exhibited nearly identical isocitrate lyase activities.

Table 2 also demonstrates the effect of temperature on the residual activities of isocitrate lyase when the cell-free crude extracts from acetate-grown revertants were heated at 35 or 40°C for 10 min. The heat stabilities in two of these isocitrate lyase-defective strains (MX9-11 R3 and MX9-11 R4) considerably deteriorated in vitro. The deterioration of heat stability of isocitrate lyase seemed to be correlated with the specific activity of isocitrate lyase in the revertant; the less isocitrate lyase activity recovered, the more the heat stability deteriorated. In addition, when the crude extracts from the wild-type parent and the revertant (MX9-11 R3) were mixed, the heat stability of isocitrate lyase in this mixture was deteriorated as expected from a premise that isocitrate lyase in each extract was inactivated independently. These observations for the revertants are evidence that the locus of the *icl* mutation is in the structural gene for isocitrate lyase.

TABLE 2. *Specific activities and heat stabilities of isocitrate lyase in wild-type cells and Acu^+ revertants*

Strain	Relative activity (%)[a]		Remaining activity (%) after 10 min at:	
	n-Alkane-grown	Acetate-induced	35°C	40°C
Wild type	100	100	100	99
MX9-11 R3	28	38	91	23
MX9-11 R4	26	—[b]	89	28
MX9-11 R34	72	—	101	97

[a] Specific activities for *n*-alkane-grown and acetate-induced cells of the wild-type strain were 157 and 221 mU/mg of protein, respectively. Mean values of at least duplicate determinations are shown.

[b] —, Not determined.

Citrate and isocitrate production by isocitrate lyase-defective revertants. Revertants from the Icl^- strain, with the wild-type parent as a control, were cultivated in MM-alkane as described in Materials and Methods. The revertant (MX9-11 R3) whose specific activity of isocitrate lyase was about 30% of the parent's (Table 2) accumulated more isocitrate

than did the parent, whereas the production ratio of citrate and isocitrate in the revertant (MX9-11 R34) which exhibited 72% of the isocitrate lyase activity of the parent remained unchanged (Fig. 2). Thus, the production ratio of these tricarboxylic acids was rather sensitive to the change in isocitrate lyase activity.

However, the amount of total acids produced (citrate plus isocitrate) did not significantly change among these isocitrate lyase-defective strains. This fact suggests that the isocitrate lyase activity of the parent cells might not have been rate limiting for the production of these acids.

DISCUSSION

The performance of an Icl$^-$ strain of *C. lipolytica* unable to grow on *n*-alkane and its derivatives clearly supported the hypothesis that glyoxylate pathway is indispensable for growth on these carbon sources. However, this argument does not necessarily exclude the possibility that C_4 compounds, such as succinate, are directly supplied via diterminal oxidation of *n*-alkane. Since only one-fourth of the carbons of *n*-hexadecane or its corresponding dicarboxylic acid are to be used directly as C_4 compounds, the amount of this C_4 supply is expected to be insufficient for the carbon requirement for growth of the Icl$^-$ strain. Thus, any anaplerotic route via diterminal oxidation of *n*-alkane, if it exists, could be regarded only as supplementary.

As to the control of the glyoxylate pathway enzymes, it has been claimed that acetyl-CoA is an inducer in *Aspergillus* (3) and *Coprinus* (11) in view of the fact that relevant enzymes (isocitrate lyase or malate synthase or both) in Acos$^-$ mutants of these fungi were poorly induced by acetate, albeit acetyl-CoA as an inducer was not clearly referred to in another fungus, *Neurospora* (10). The observation here that the glyoxylate pathway enzymes were least susceptible to induction by acetate in an Acos$^-$ mutant of *C. lipolytica* is evidence that acetyl-CoA functions as an inducer in this yeast also. In addition, this interpretation was justified by the fact that the Acos$^-$ strain was able to induce the glyoxylate pathway enzymes when another source of acetyl-CoA is available, for instance, from *n*-alkane (Table 1).

The majority of Acu$^-$ mutants isolated in this work, except for Icl$^-$ and Acos$^-$ mutants, have not been identified yet for their enzyme lesions. Armitt et al. (3) reported that the enzyme lesions in Acu$^-$ mutants of *A. nidulans* identified out of 12 *acu* loci were acetyl-CoA synthetase, isocitrate lyase, malate synthase, phosphoenolpyruvate carboxykinase (adenosine 5'-triphosphate),

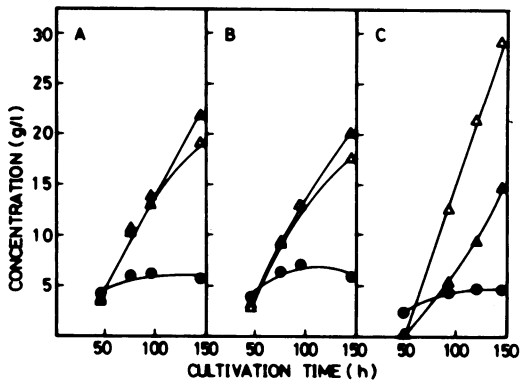

FIG. 2. *Citrate and isocitrate production by the wild-type strain (A) and isocitrate lyase-defective revertants MX9-11 R34 (B) and MX9-11 R3 (C). Symbols: ●, dry cell mass; ▲, citrate; △, isocitrate.*

fructose-bisphosphatase, and malic enzyme. However, mutants deficient in activities of the last three enzymes should be excluded here in this work, since every Acu$^-$ mutant that could grow on glycerol was proved to be able to utilize succinate, too, and vice versa.

Taking it for granted that acetyl-CoA is an inducer of isocitrate lyase and malate synthase, the fact that the inducibilities of these enzymes by acetate scattered considerably in Acu$^-$ mutants (Fig. 1) could be ascribed to the different intracellular levels of acetyl-CoA. Thus, a group of Acu$^-$ mutants which were characterized by their abilities to utilize *n*-alkane and their poor inducibilities of the glyoxylate pathway enzymes are deemed to have been impaired in the ability to produce acetyl-CoA from acetate. This interpretation could be justified by the fact that they (especially the Acos$^-$ strain), when grown on *n*-alkane and hence when acetyl-CoA was made available, bypassing their metabolic lesions, recovered activities of the glyoxylate pathway enzymes comparable to those of the wild-type parent.

In contrast, the intracellular levels of acetyl-CoA in a group of Acu$^-$ mutants unable to grow on *n*-alkane might have been higher than that in the wild-type parent, presumably because they could produce acetyl-CoA from acetate but were malfunctioning in utilization of acetyl-CoA for biosynthesis. This situation is more apparent when we consider the increased level of malate synthase activity in the acetate-induced Icl$^-$ strain (Table 1): i.e., acetyl-CoA gratuitously derived from acetate could not have been metabolized further due to the lack of isocitrate lyase activity; thus, the higher level of acetyl-CoA accumulated might have enhanced the induction of malate synthase.

Although the temperature-sensitive revertants of the Icl$^-$ strain were obtained in *E. coli* (4), *N. crassa* (13), and *C. cinereus* (11), such a revertant could not be isolated from the Icl$^-$ strain of *C. lipolytica* in this work. However, the increased heat sensitivities of isocitrate lyase in some of the revertants were at least indicative that the structural gene for isocitrate lyase was impaired in the Icl$^-$ mutant.

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