sucAB and sucCD are mutually essential genes in *Escherichia coli*

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Abstract

sucAB and sucCD of *Escherichia coli* encode enzymes that generate succinyl-CoA from 2-oxoglutarate and succinate, respectively. Their mutual essentiality was studied. sucAB and sucCD could be deleted individually, but not simultaneously. The mutual essentiality of sucAB and sucCD was further confirmed by the conditional expression of sucABCD, sucAB, and sucCD under the control of a PBAD in *E. coli* MG1655, *E. coli* MG1655 (ΔsucCD), and *E. coli* MG1655 (ΔsucAB), respectively. These strains grew well in Luria–Bertani medium containing 0.1% arabinose, but not in the absence of arabinose unless the medium was supplemented with succinyl-CoA. Our results indicate that either sucAB or sucCD is enough to produce succinyl-CoA that is essential for cell viability.

Introduction

Over the last 10 years, many microbial genomes and the functions of their genes have been studied. With the advancement of microbial genomics research, several genome-wide approaches such as transposon-based mutagenesis (Rappleye & Roth, 1987; Chow & Berg, 1988; Smith et al., 1995; Mushegian & Koonin, 1996; Akerley et al., 1998, 2002; Hutchison et al., 1999; Ross-Macdonald et al., 1999; Judson & Mekalanos, 2000; Sassetti et al., 2001; Gerdes et al., 2003; Jacobs et al., 2003) and the deletion of genes and large genomic regions (Posfai et al., 1999; Datsenko & Wanner, 2000; Kolisnychenko et al., 2002; Yu et al., 2002; Goryshin et al., 2003; Hashimoto et al., 2005) have been explored to identify essential and nonessential genes on a whole-genome scale. Although these global gene knockouts are impressive scale-ups of the analogous early experiments, they have dealt mostly with individual gene disruptions. These analyses also assumed that the removal of individual genes has no effect on the essentiality of the remaining genes.

So far, little research has been carried out into the effect of simultaneous deletion of genes on cell growth, although the function of many genes has been studied individually. Some essential functions of the deleted or knocked out genes may be complemented by paralog(s) or by other gene(s) playing a similar role. These genes should be classified as mutually essential genes because simultaneous deletion of these genes leads to cell death. Mutually essential genes are very important in the study of a minimal genome and in reconstructing metabolic pathways, and may help to estimate accurately the number of genes in the minimal gene set that are needed to form a viable microorganism. Therefore, combinatorial or simultaneous gene deletions are required to identify mutually essential genes that might be involved in multiple pathways leading to synthesis of an essential compound(s).

sucAB and sucCD encode the two tricarboxylic acid (TCA) cycle enzymes in *Escherichia coli*, two components of α-ketoglutarate dehydrogenase and succinyl coenzyme A synthetase to generate succinyl-CoA, respectively. The α-ketoglutarate dehydrogenase catalyzes the oxidative decarboxylation of α-ketoglutarate to generate succinyl-CoA and carbon dioxide, along with the production of NADH and H⁺. The succinyl-CoA synthetase catalyzes the interconversion of succinyl-CoA and succinate, and this interconversion is accomplished by the production or hydrolysis of GTP.

Here, we describe a new approach by which we have found that sucAB and sucCD are mutually essential genes...
involved in producing the essential compound succinyl-CoA in *E. coli*.

**Materials and methods**

**Bacterial strains, plasmids, and chemicals**

Two types of Tn5-inserted *E. coli* MG1655 mutant libraries (MG1655::TnKloxP and MG1655::TnClloxP) (Yu et al., 2002) and pELCre (Yoon et al., 1998) were used for combinatorial deletions as described by Yu et al. (2002). pKD3, pKD4, pKD46, and pCP20, obtained from Datsenko & Wanner (2000), were used for stepwise subdeletions and elimination of selection markers. pNJ17 (Judson & Mekalanos, 2000) was used to construct a DNA cassette for the elimination of selection markers. pKD3, pKD4, pKD46, and pCP20, obtained from Datsenko & Wanner (2000), were used for stepwise subdeletions and elimination of selection markers.

Plasmids were electroporated using a Gene Pulser system (Bio-Rad, Hercules, CA) and propagated in *E. coli* MG1655, MG1655::TnKloxP and MG1655::TnClloxP mutants. All experiments used Luria–Bertani (LB) liquid or agar medium (Sambrook et al., 2001) containing the following antibiotics where appropriate: 50 μg mL<sup>−1</sup> ampicillin (Amp), 25 μg mL<sup>−1</sup> kanamycin (Km), and 17 μg mL<sup>−1</sup> chloramphenicol (Cm).

**Construction of deletion strains**

The subdeletion of a selected genomic region *ybfO–clpA* (b0703–b0882) was performed by the method of Yu et al. (2002). Four deletion strains, *E. coli* MG1655 (Δb0703–b0719), *E. coli* MG1655 (Δb0732–b0774), *E. coli* MG1655 (Δb0774–b0835), and *E. coli* MG1655 (Δb0835–b0882), were constructed. For the region b0719–b0732 that could not be deleted, a stepwise subdeletion of the b0719–b0732 genomic region was performed by the λ-Red recombination system described by Datsenko & Wanner (2000). From this second-round subdeletion, six more deletion strains, *E. coli* MG1655 (Δb0719–b0727), *E. coli* MG1655 (Δb0728–b0732), *E. coli* MG1655 (Δb0730–b0732), *E. coli* MG1655 (ΔsucAB), and *E. coli* MG1655 (ΔsucCD), were obtained (Fig. 2a). The deletion of targeted regions was confirmed by PCR using a pair of primers specific to the end points of each deletion region and two pairs of primers specific to the internal genes of the deletion region with *E. coli* MG1655 and deletion strains as templates (data not shown).

**Construction of Km<sup>R</sup>–araC–P<sub>BAD</sub>-inserted mutants**

A DNA cassette (Km<sup>R</sup>–araC–P<sub>BAD</sub>) containing a kanamycin-resistance gene (Km<sup>R</sup>), an araC gene, and an arabinose-inducible promoter (P<sub>BAD</sub>) was constructed from TnAraOut delivery plasmid pNJ17 (Judson & Mekalanos, 2000) by recombinant PCR, and used for the conditional expression of sucABCD, sucAB, and sucCD in *E. coli* MG1655, *E. coli* MG1655 (ΔsucCD), and *E. coli* MG1655 (ΔsucAB) harboring pKD46 to replace the P<sub>SUC</sub> by homologous recombination, generating *E. coli* MG1655 sucABCD::Km<sup>R</sup>–araC–P<sub>BAD</sub>, *E. coli* MG1655 (ΔsucCD) sucAB::Km<sup>R</sup>–araC–P<sub>BAD</sub>, and *E. coli* MG1655 (ΔsucAB) sucCD::Km<sup>R</sup>–araC–P<sub>BAD</sub> (Fig. 2b). The replacement of the P<sub>SUC</sub> by the Km<sup>R</sup>–araC–P<sub>BAD</sub> cassette was verified by PCR using a pair of primers flanking both end points of the P<sub>SUC</sub> (data not shown).

**Induction of Km<sup>R</sup>–araC–P<sub>BAD</sub>-inserted strains by arabinose**

The Km<sup>R</sup>–araC–P<sub>BAD</sub>-inserted strains were cultivated overnight in LB liquid containing 0.1% [weight in volume (w/v)] arabinose at 37 °C, harvested, washed twice with LB liquid, diluted 1:50 000, and grown in LB liquid containing 0.1% arabinose at 37 °C (Judson & Mekalanos, 2000). These mutants were also grown in LB liquid containing 0.5% (w/v) succinyl-CoA in the absence of arabinose at 37 °C. The cell growth was monitored by measuring the optical density at 600 nm.

**Results**

**Combinatorial deletions of a selected genomic region**

The genomic region *ybfO–clpA* (b0703–b0882) was selected as a subdeletion target for identifying essential genes because this region had not been able to be deleted in a previous experiment (Yu et al., 2002). Using the combinatorial deletion method with two types of Tn5-inserted *E. coli* MG1655 mutant libraries, we attempted to sub-delete the selected genomic region into five different regions to identify essential genes: *E. coli* MG1655 (Δb0703–b0719), *E. coli* MG1655 (Δb0719–b0732), *E. coli* MG1655 (Δb0732–b0774), *E. coli* MG1655 (Δb0774–b0835), and *E. coli* MG1655 (Δb0835–b0882). All of these mutant strains, with the exception of *E. coli* MG1655 (Δb0719–b0732), were obtained. When the b0719–b0732 region of *E. coli* MG1655 that could not be deleted was further divided into two regions, b0719–b0727 and b0728–b0732, both deletion strains *E. coli* MG1655 (Δb0719–b0727) and *E. coli* MG1655 (Δb0728–b0732) were viable, suggesting the presence of mutually essential genes.
Identification of mutually essential genes

Analysis of the genomic region b0719–b0732 using the EcoCyc databases (http://ecocyc.org/) revealed that this region contains a cluster of citric acid cycle genes, including the citrate synthase gene (gltA), the succinate dehydrogenase operon (sdhCDAB), E1 and E2 components of α-ketoglutarate dehydrogenase complex (sucAB), and succinyl coenzyme A synthetase (sucCD).

Among these genes, sucAB and sucCD were selected as possible candidates for mutually essential genes because both sucAB and sucCD are involved independently in the production of succinyl-CoA needed for generating diaminopimelate, an essential compound required for cell wall biosynthesis (Patte, 1996) (Fig. 1).

To identify mutually essential genes present in the genomic region b0719–b0732, the region was further segmented into the three regions b0719–b0725, b0726–b0729, and b0730–b0732 using λ-Red recombination (Datsenko & Wanner, 2000). The region b0726–b0729 was found to contain sucABCD (Fig. 2a). As expected, the genomic regions b0719–b0725 and b0730–b0732 could be deleted, but the region b0726–b0729 containing sucABCD could not be deleted. However, when the genomic region b0726–b0729 containing sucABCD was further divided into two regions, b0726–b0727 (sucAB) and b0728–b0729 (sucCD), both regions could be deleted, generating two deletion strains: E. coli MG1655 (ΔsucAB) and E. coli MG1655 (ΔsucCD). These results clearly indicate that sucAB and sucCD could be deleted individually, but not simultaneously.

Conditional expression of sucABCD, sucAB, and sucCD

The mutual essentiality of sucAB and sucCD was further confirmed by the conditional expression of sucABCD, sucAB, and sucCD in E. coli MG1655, E. coli MG1655 (ΔsucCD), and E. coli MG1655 (ΔsucAB), respectively. For the conditional expression of these genes, the original promoter P_SUC of sucABCD in the above strains was replaced by the Km<sup>R</sup>–araC–P<sub>BAD</sub> cassette, generating E. coli MG1655 sucABCD::Km<sup>R</sup>–araC–P<sub>BAD</sub>, E. coli MG1655 (ΔsucCD) sucAB::Km<sup>R</sup>–araC–P<sub>BAD</sub> and E. coli MG1655 (ΔsucAB) sucCD::Km<sup>R</sup>–araC–P<sub>BAD</sub> which were inducible by 0.1% arabinose (Fig. 2b).

These strains in which the P<sub>SUC</sub> was replaced by the Km<sup>R</sup>–araC–P<sub>BAD</sub> cassette did not grow on LB without arabinose, but did grow well on LB containing 0.1% arabinose. The growth rate of E. coli MG1655 sucABCD::Km<sup>R</sup>–araC–P<sub>BAD</sub> was comparable with that of E. coli MG1655 in the presence of 0.1% arabinose in the medium (Table 1). However, both E. coli MG1655 (ΔsucCD) sucAB::Km<sup>R</sup>–araC–P<sub>BAD</sub> and E. coli MG1655 (ΔsucAB) sucCD::Km<sup>R</sup>–araC–P<sub>BAD</sub> grew slowly, with a longer lag phase under the same condition (Fig. 3). The growth defects of these strains in the absence of arabinose were alleviated by culturing them in LB media containing 0.5% succinyl-CoA (Table 1).

Discussion

In this study, using a combinatorial deletion with a Tn5-mediated-Cre/loxP recombination system and a conditional expression system with a Km<sup>R</sup>–araC–P<sub>BAD</sub> cassette, we have demonstrated that sucAB and sucCD are mutually essential genes in E. coli that can be deleted individually, but not simultaneously. We have also found that succinyl-CoA produced by either sucAB or sucCD or sucABCD is essential for E. coli survival.

A similar case of mutually essential genes, but referred to as mutually exclusive genes, was reported by Smalley et al. (2003), in which nrdAB/nrdDG were introduced as a typical example of mutually exclusive genes because nrdAB and nrdDG code for two distinct enzymes that produce the same compound during aerobic and anaerobic growth.

Fig. 1. sucABCD of tricarboxylic acid cycle and lysine biosynthetic pathway. Succinyl-CoA generated by sucAB and sucCD is used to produce lysine and its immediate precursor, diaminopimelate, which is critically required for cell wall (peptidoglycan) biosynthesis.
E. coli rate of can biosynthesis (Patte, 1996). Therefore, the slow growth generating diaminopimelate that is essential for peptidoglycan biosynthesis pathway, in which succinyl-CoA is used for in many metabolic pathways, a major one being the lysine in the TCA cycle, succinyl-CoA is also used as an intermediate. Their significance in the TCA cycle of E. coli is to convert 2-oxoglutarate and succinate, respectively (Fuchs & Karlstrom, 1973; Garriga et al., 1996).

Similarly, sucAB and sucCD encode two enzymes in the TCA cycle of E. coli, 2-oxoglutarate dehydrogenase complex and succinyl coenzyme A synthetase, that function to generate succinyl-CoA from 2-oxoglutarate and succinate, respectively (Patte, 1996).

Even though E. coli can survive with either sucAB or sucCD only, these cells have a longer lag phase, suggesting their significance in the TCA cycle of E. coli. In addition to the TCA cycle, succinyl-CoA is also used as an intermediate in many metabolic pathways, a major one being the lysine biosynthesis pathway, in which succinyl-CoA is used for generating diaminopimelate that is essential for peptidoglycan biosynthesis (Patte, 1996). Therefore, the slow growth rate of E. coli MG1655 (ΔsucCD) and E. coli MG1655 (ΔsucAB) might be ascribed to the involvement of succinyl-CoA in many multiple metabolic pathways.

Our finding that sucAB and sucCD are mutually essential genes differs from the reports of Integrated genomics (http://www.integratedgenomics.com) and PEC (http://www.shigen.nig.ac.jp/ecoli/pec). Integrated genomics reported that only the sucAB genes are essential, whereas PEC reported that sucABCD are all nonessential. This discrepancy might be the result of using transposon mutagenesis, as the result with transposon mutagenesis will vary depending on the location of the transposon insertions. Thus, if a transposon is inserted into sucA, the transcription of sucBCD downstream of sucA would be inhibited because of transcriptional polarity. Therefore, the mutant strain with a transposon insertion occurs either in sucC or sucD, it would not affect cell survival because sucAB are expressed generating respectively (Fuchs & Karlstrom, 1973; Garriga et al., 1996).

<table>
<thead>
<tr>
<th>Strain*</th>
<th>LB</th>
<th>LB + 0.5% succinyl-CoA</th>
<th>LB + 0.1% arabinose</th>
<th>Growth rate (μg mL⁻¹ h⁻¹)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli MG1655</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.48</td>
</tr>
<tr>
<td>E. coli MG1655 sucABCD::Km⁺–araC–PBAD</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>0.47</td>
</tr>
<tr>
<td>E. coli MG1655 (ΔsucCD) sucAB::Km⁺–araC–PBAD</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>0.35</td>
</tr>
<tr>
<td>E. coli MG1655 (ΔsucAB) sucCD::Km⁺–araC–PBAD</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>0.31</td>
</tr>
</tbody>
</table>

*All strains were streaked on LB media containing the indicated compounds. +, colonies formed; –, no colonies formed.
†All strains were cultivated in a 96-well plate with LB liquid (300 μL) containing 0.1% arabinose at 37°C.

Km⁺, kanamycin-resistance gene; PBAD, arabinose-inducible promoter; LB, Luria–Bertani.
succinyl-CoA, also giving false information that sucC and sucD are not essential genes. Our results clearly show a limitation of Tn-mutagenesis for identifying essential genes. It seems that transposon mutagenesis might underestimate the number of genes needed in a minimal gene set of a microorganism because it does not take into account mutually essential or exclusive genes, or genes playing the same role in a critical pathway.

Combining combinatorial deletions and controlled expressions described in this study would be very useful in the analysis of metabolic pathways for identifying mutually essential genes, especially for subregions that can be deleted individually, but cannot be deleted simultaneously.

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References


Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO & Venter JC (1999) Global transposon Fig. 3. Induction and growth of the Km–araC–Pbad-insertion strains with arabinose. Growth of Escherichia coli MG1655 sucABCD::Km–araC–Pbad (a), E. coli MG1655 (ΔsucCD) sucAB::Km–araC–Pbad (b), E. coli MG1655 (ΔsucAB) sucCD::Km–araC–Pbad (c), and E. coli MG1655 (d) on Luria–Bertani either in the presence (●) or in the absence (○) of 0.1% arabinose. The mutants grew only in the presence of arabinose. Km, kanamycin-resistance gene; Pbad, arabinose-inducible promoter.
mutagenesis and a minimal Mycoplasma genome. *Science* **286**: 2165–2169.


