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L-Serine Degradation in *Escherichia coli* K-12: Directly Isolated *ssd* Mutants and Their Intragenic Revertants

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Two methods for the direct isolation of spontaneous *ssd* mutants of *Escherichia coli* K-12 strains are described; (i) by growth with L-serine as the carbon source, and (ii) by low-level kanamycin resistance. A newly isolated mutant had the same phenotype as the mutant described previously, including inefficient use of glucose, inability to grow with succinate, altered transport characteristics, and altered resistance to certain growth effectors. Succinate-utilizing derivatives which appear to be intragenic are characterized in detail. The relation between the mutants isolated here and mutants which are thought to have impairments in a system of coupling respiratory energy to active transport (*ecfB* mutants) is discussed.

Many strains of *Escherichia coli* K-12 do not grow with L-serine as carbon source (12). We have described previously a very pleiotropic mutation, *ssd*, which allows the strain to grow with L-serine as sole carbon source (12). *ssd* mutants have considerably elevated levels of the enzyme which converts L-serine to pyruvate, L-serine deaminase (L-SD). However, they also have many other changes in cell metabolism. The characteristics of these mutants include slow and inefficient use of glucose as carbon source, inability to use succinate and related compounds as carbon source, inability to grow anaerobically, susceptibility to fluoride inhibition, resistance to certain antibiotics and a colicin, and defects in amino acid transport (12).

Progress in understanding these mutants has been hindered by uncertainty as to how they were isolated (11). Therefore, we describe here the direct selection of spontaneous mutants which are able to grow with L-serine as sole carbon source. These fall into at least three classes in terms of phenotype and genotype. We report in detail on mutants of one of these classes, *ssd*. The newly isolated *ssd* mutants show the entire phenotype previously described for *ssd* (12). We also describe here the physiology of succinate-utilizing revertants of a newly isolated *ssd* mutant and show that they differ subtly from both the mutant and its parent and seem to carry two mutations within the *ssd* gene.

MATERIALS AND METHODS

Cultures. The *E. coli* K-12 strains used are described in Table 1. L-Serine-utilizing mutants were isolated by growing individual colonies of strain CU1008 or W4977 in 4 to 5 ml of Luria broth (LB), centrifuging, and plating the pelleted cells on previously described (12) minimal medium, pH 7 to 7.3, with 2 mg of L-serine, 100 μg of isoleucine, and 100 μg of valine per ml at 37°C. L-Serine must be filtered or autoclaved separately from the rest of the medium. The best results were obtained when the medium was made with ion-exchange-treated, glass-distilled water. Even with these precautions, however, colonies arose on these plates in only about one-third of the experiments. In successful isolations, 50 to 300 colonies per plate were visible in 5 to 7 days. In unsuccessful attempts, no colonies were seen in up to 16 days. The reason for this remains obscure.

Determination of antibiotic sensitivity. Antibiotic sensitivity was determined by streaking exponential-phase cultures of the strain of interest onto either LB or minimal-glucose plates, placing a commercial sensitivity-testing disk in the middle, and determining the diameter of the inhibition ring after 18 h of incubation at 37°C.

Assay of L-SD activity. L-Serine deaminase (L-SD) was assayed in two ways. One way, involving centrifuged cells resuspended in phosphate buffer and treated with toluene, has been previously described (4, 14). For screening high and low values in numbers of transductants, a rapid assay based on the assay of beta-galactosidase (10) was used. For this assay, 0.3-ml samples of an exponential-phase culture were pipetted directly into assay tubes, L-serine and toluene were added, and the assay was continued as previously described (4). Results of this assay are reported as micromoles of pyruvate formed by 0.1 ml of a culture of 100 optical density units (Klett 420 filter) in 35 min. In both assays, 1 unit of enzyme is the amount of enzyme which forms 1 μmol of substrate in the assay. All results quoted refer to the standard assay unless the rapid assay is specifically noted. Any strain with <0.01 unit of enzyme activity is described as having low L-SD activity.

All other methods and enzyme assays have been previously described (12).

TABLE 1. *E. coli* strains used and their relevant characteristics

Strain	Genotype[a] and/or relevant characteristics	Source/ reference
CU1008	K-12	L. S. Williams
W4977	K-12	R. L. Soffer
KEC1 to KEC20	CU1008 derivatives able to use L-serine as carbon source	This work
KEC21 to KEC30	W4977 derivatives able to use L-serine as carbon source	This work
KEC3-DC, -DE, -DF, -DG	Intragenic succinate-utilizing revertants of KEC3	This work
WES1	*metB* derivative of CU1008	12
WES2	CU1008 with *lac* deletion	This work

[a] Abbreviations as in reference 1.

Caution on growth of *ssd* strains. We wish to note that the *ssd* mutant is a "sick" strain and quickly accumulates revertants and suppressors. We find it necessary to store it as a transducing phage and retransduce it regularly. It is therefore imperative to check at least part of the phenotype of every culture used for biochemical or genetic purposes in every experiment, a precaution which has been taken in our work on *ssd* mutants.

RESULTS

Isolation of mutants by using L-serine as carbon and nitrogen source. Strains CU1008 and W4977 were both able to grow with L-serine as nitrogen source, but not with L-serine as carbon or carbon and nitrogen source. Spontaneous mutants which can use L-serine as the carbon source were isolated readily from both strains by plating between 5×10^8 and 5×10^9 cells on minimal medium with L-serine (2 mg/ml) and isoleucine and valine (100 μg/ml each). Isoleucine is required by strain CU1008 but not by strain W4977. However, L-serine is known to be toxic to *E. coli* K-12, and this toxicity is reversed by addition of isoleucine and valine (2). Therefore, isoleucine and valine were added in both cases. Colonies were seen after 5 to 8 days.

Twenty such mutants (KEC1 to KEC20) have been isolated from strain CU1008, and 10 (KEC21 to KEC30) have been isolated from strain W4977. To compare these isolates with the *ssd* mutant described previously, we tested their ability to grow on succinate-minimal medium and on nutrient agar supplemented with 40 mM sodium fluoride, conditions under which the *ssd* mutants do not grow (12). Two phenotypes were found: succinate utilizing, fluoride resistant; and succinate nonutilizing, fluoride sensitive.

To see whether these strains carried the *ssd* mutation, which is closely linked to *metB* (11), phage P1 grown on each of the strains was used to transduce strain WES1 *metB* to methionine independence. One hundred transductants from each cross were screened for ability to use L-serine or succinate as carbon source.

The genetic classification indicated that mutations resulting in the succinate-nonutilizing fluoride-sensitive phenotype can occur at at least two loci. One of these is linked to *metB* and so is considered to be *ssd*. The other does not show linkage to *metB*. Mutations resulting in the group of strains able to use succinate and fluoride resistant were also unlinked to *metB*.

There are, then, at least three classes of mutations which confer the ability to grow with L-serine as carbon source. This is, of course, a minimum estimate, and the classes may be subdivided later. The remainder of this paper deals with one of the newly isolated *ssd* mutants, strain KEC3. Characteristics of the other classes will be reported later.

Comparison of strain KEC3 with a previously described *ssd* mutant. We have previously described the characteristics of an *ssd* mutant in considerable detail (12). Because the origin of this mutant is unclear, we have characterized strain KEC3 in the same way, comparing it with its parent, strain CU1008. Since its phenotype is substantially the same as that previously described the data are not presented in detail (Table 2).

We described earlier that the *ssd* mutant has considerably lower levels of phosphoglycerate dehydrogenase, the first enzyme in serine bio-

TABLE 2. Comparison of strain KEC3 with another *ssd* mutant[a]

Strain	L-SD activity	Response to:			Yield on glucose	Anaerobic growth
		Fluoride	Kanamycin, neomycin	Strain 28 colicin		
CU1008	Low	R	S	S		+
KEC3	High	S	R	R	Low	−
VE2-2 *ssd*	High	S	R	R	Low	−

[a] Strain CU1008 is the reference strain with respect to growth yields; the other strains are compared to it. S indicates that a strain was sensitive and R indicates that it was resistant, as judged by the area of lysis around sensitivity disks.

synthesis, than does its parent (12). Strain KEC3 has slightly lower levels than its parent strain CU1008, but this difference is much smaller than that described previously (12). This appears to be an effect of the genetic background, since the *ssd* mutation described earlier also caused less change in this enzyme in the background of strain CU1008 than when in the MN-2 background (data not shown).

Strain KEC3 has a decreased ability to transport both proline and arginine. The mutant described earlier showed a decreased ability to transport proline, but arginine transport was not tested.

Genetic characterization of succinate-utilizing derivatives of strain KEC-3. Succinate-utilizing revertants of an *ssd* mutant have already been characterized (12), although not in great detail. Since strain KEC3 is a spontaneous mutant of clear origin, it seemed worthwhile to characterize its revertants in somewhat greater detail. Therefore, seven independent spontaneous succinate-utilizing mutants were isolated by plating 10^8 to 10^9 cells on succinate-minimal medium; these isolates were named KEC3-DA through KEC3-DG.

The acquisition of the ability to use succinate may be due to a change at the same locus as the original mutation or to a suppressor mutation at another locus. To determine which was the case, phage P1 was grown on each of the revertants and used to transduce strain WES1 (*metB*) to methionine independence. Transductants were then tested for ability to use succinate and L-serine as the carbon source. Three of the strains tested (KEC3-DA, KEC3-DB, and KEC3-DD) transferred a mutation resulting in inability to use succinate and ability to use serine. This mutation was linked to *metB* to about the same extent as *ssd* (44 of 100 KEC3-DA, 41 of 100 KEC3-DB, and 35 of 100 KEC3-DD). These three strains were therefore considered to carry *ssd* and to be externally suppressed and will not be further discussed here.

In 100 transductants each from the other four strains, no succinate-nonutilizing clone was observed. It seems, then, that the second mutation in strains KEC3-DC,-DE,-DF, and −DG is at the *ssd* locus and that these are, by this definition, true revertants. However, the possibility that one or all of these carries a second mutation in a gene tightly linked to *ssd* cannot be excluded.

Physiological characterization of succinate-utilizing intragenic revertants. The selection method described above assures that the revertants are able to use succinate. Revertants were also tested for several of the characteristics affected by the *ssd* mutation, including L-SD activity and sensitivity to antibiotics and fluoride.

All revertants had much lower L-SD activity than the mutant (Table 3) and were unable to grow with L-serine as carbon source. Otherwise, the phenotypes of the revertants do not follow any well-defined pattern. Indeed, a strain in which the *ssd* function is restored well enough to permit growth on succinate may still retain several of the altered responses to growth inhibitors. Two revertants were resistant to kanamycin but sensitive to colicin; these differed in their fluoride sensitivity. A third revertant was sensitive to both kanamycin and colicin, and the fourth was resistant to both.

Physiological characterization of transductants. The preceding results indicate that the *ssd* mutation affects a cell structure and that this is only partially restored by a second mutation within the same gene. The restoration was good enough to permit growth on succinate and make growth on L-serine impossible. However, it had various effects on antibiotic inhibition, so that the cells remained at least somewhat antibiotic resistant.

All of the transductants from the backcross of the revertants to WES1 *metB* were succinate utilizing. However, if the revertants do carry a doubly mutated *ssd* gene, one would expect that some of these succinate-utilizing transductants would carry the doubly mutated *ssd* gene. These

TABLE 3. Characteristics of succinate-utilizing revertants of strain KEC3[a]

Strain	Response to:				L-SD activity (U)
	Neomycin	Kanamycin	Colicin	Fluoride	
WES1	S	S	S	S	0.02
KEC3	R	R	R	R	0.19
KEC3-DC	S	R	S	S	0.022, 0.013
KEC3-DE	S	R	S	R	0.007, 0.007
KEC3-DF	S	S	S	S	0.007, 0.013
KEC3-DG	S	R	R	S	0.03, 0.05

[a] The antibiotic inhibition assays were performed with commercial sensitivity disks on LB plates supplemented with 0.2% glucose. S indicates that a strain was sensitive as compared with other strains (noted as R), with a lysis zone 2 mm smaller. Colicin sensitivity was tested as in reference 13. Fluoride resistance (R) refers to the ability to grow on LB plates supplemented with 40 mM sodium fluoride.

might be more antibiotic resistant than the original strain, but less so than the mutant. Now antibiotic resistance tests are most often carried out on rich medium, e.g., LB plates, as was done above (Table 3). To look for more subtle differences, the transductants in the following experiment were tested on glucose-minimal plates. Fifty of the transductants from each cross (WES1 × KEC3-DC, WES1 × KEC3-DE, WES1 × KEC3-DF, and WES1 × KEC3-DG) were tested for kanamycin resistance on glucose-minimal medium with sensitivity disks. All four strains transferred kanamycin resistance in linkage to *metB* (25 of 50 in KEC3-DC, 12 of 25 in KEC3-DE, 13 of 23 in KEC3-DF, and 46 of 100 in KEC3-DG). This is true even for strain KEC3-DF, which appears kanamycin sensitive on LB-glucose plates. The linkage seen is similar to that (36 of 80) previously reported for the linkage of *ssd* to *metB* (11).

If the resistance to kanamycin was due to a mutated, partially functional *ssd* product, the kanamycin-resistant strains should also be neomycin resistant. To check this, five kanamycin-resistant and five kanamycin-sensitive colonies from each cross were tested with neomycin sensitivity disks on glucose-minimal medium. In all cases, strains resistant to kanamycin were also resistant to neomycin, and sensitive strains were sensitive to both.

It is clear, then, that all revertants, even those which are phenotypically antibiotic sensitive, can transfer, by linkage to *metB*, a gene conferring antibiotic resistance. This would seem to mean that the phenotype of the revertant with respect to antibiotic resistance is influenced by something else in addition to the two mutations linked to *metB*, whereas the transductants carry only the alterations near *metB*. Since revertants are selected through several single-colony isolations on a relatively poor growth substrate, and since the starting strain is itself a sick strain (see Materials and Methods), it is likely that the revertants carry more than one alteration. The backcross transductants, however, would carry only the area near *metB* and therefore allow examination of the function of the *ssd* gene.

In any case, it can be seen that the extent of antibiotic resistance varied in the four revertants, and this quantitative variation also is transduced in linkage to *metB*. Transductants carrying the *ssd* allele of strains KEC3-DF and KEC3-DG showed virtually no inhibition zone around the antibiotic-containing disks on glucose-minimal plates; those carrying the allele from KEC3-DC showed a zone of 1 to 2 mm compared with 3 to 4 mm for the host strain. Resistant colonies from the cross with KEC3-DE were only slightly more resistant than the host but could be visually distinguished. The zone of inhibition around sensitive colonies was the same in all cases.

The *ssd* gene can revert in such a way as to restore serine deaminase and succinate utilization to normal, without restoring (totally) antibiotic sensitivity. In the case of one revertant, KEC3-DG, even L-SD activity was not restored to the parental level, although it still had much lower activity than the mutant strain. To be sure that this was also due to an alteration in *ssd*, i.e., an alteration linked to *metB*, several kanamycin-resistant and kanamycin-sensitive transductants from the WES1 × KEC3-DG cross were tested for L-SD activity. In all cases, the kanamycin-resistant strains had slightly elevated L-SD (0.014, 0.015, 0.017, 0.021 U; average, 0.017 U) and the sensitive strains had lower activity (0.005, 0.007, and 0.008 U; average, 0.0067 U), about the same as the original parent, strain CU1008 (0.005 U).

Selection of *ssd* mutants by kanamycin resistance. Since the *ssd* mutant is kanamycin resistant, one would expect to be able to isolate it by selection for kanamycin-resistant derivatives. To test this, strain WES2 was plated on LB plates containing 6 μg of kanamycin per ml, and resistant colonies were isolated and screened for inability to grow on succinate. Three kanamycin-resistant, succinate-negative strains were assayed for L-SD and shown to have high activity (0.23, 0.13, and 0.08 μmol of pyruvate formed per 100 Klett units of cells in the standard assay) compared with the parental activity of 0.02 μmol of pyruvate formed.

Since there are at least two classes of succinate-nonutilizing mutants, the preceding data are not sufficient to show that *ssd* mutants can be isolated by kanamycin resistance. To show that one of these strains is likely to be an *ssd* mutant, phage were grown on the mutant strain WES2 KR3 and used to transduce strain WES1 *metB*. Of 74 methionine-independent transductants, 14 were kanamycin resistant and also unable to grow with succinate. Seven of these kanamycin-resistant, succinate-nonutilizing transductants were assayed by the rapid 1-SD assay and showed high enzymatic activities (0.27, 0.27, 0.28, 0.28, 0.29, 0.30, and 0.32 U; average, 0.29 U). By the criteria of inability to use succinate and elevated L-SD WES2 KR3 is likely to be an *ssd* mutant.

DISCUSSION

We have shown in this paper that an exceedingly pleiotropic mutation, *ssd*, can be isolated from *E. coli* K-12 by direct selection with L-serine as carbon source. This mutation has a broad effect on cell metabolism, resulting in a lowered efficiency of glucose utilization, inability to grow on succinate or related compounds,

inability to grow anaerobically, antibiotic resistance, fluoride sensitivity, and a deficiency in proline and arginine uptake. We have described this mutation previously (11, 12). However, this is the first report of direct isolation of spontaneous mutants. This seemed important in establishing that the *ssd* phenotype results from a single mutation, in view of the complex phenotype and the uncertain origin of the strain previously described.

We have suggested earlier that the *ssd* mutation is identical to *ecfB* (11, 12). That this is likely is shown here by the selection of an *ssd* mutant by low-level resistance to kanamycin, the selection used for *ecfB* mutants (13, 15). All such kanamycin-resistant mutants tested so far have shown high L-SD activity. Only one of the kanamycin-resistant mutants has been actually mapped; it is linked to *metB* to the same extent as *ssd*. We therefore think that the two mutations are at the same locus.

The *ecf* mutations have been interpreted as involving an energy transduction factor which participates in or regulates the coupling of the proton motive force to nutrient transport and to ATP generation (2, 6–8, 13, 15). In such a view, the uptake of proline, arginine, kanamycin, neomycin, and perhaps fluoride would be in some way coupled to, or regulated by, the proton motive force via the *ssd* product. It is unclear why the yield on glucose should be lowered. Perhaps the generation of the proton motive force from glucose also involves the *ssd* product. Alternatively, the mutated form may be inefficiently coupled, dissipating energy available from glucose oxidation and causing a decreased yield on glucose. The only evidence directly bearing on this is that growing mutant cells show the same proton motive force as their parent (5).

Kell et al. have recently made an interesting, and much more detailed, proposal as to the nature of *ecf* mutations (6). In their view, "the energy-transducing membrane systems normally contain a number of proteinaceous components whose role is to act co-operatively as conformationally switchable proton conductors" (6). The *ecf* gene product (and thus the *ssd* gene product) would then be one of these protein components in the cytoplasmic membrane and would allow for transfer of protons laterally through the membrane.

Succinate-using derivatives of the *ssd* mutant have been shown to occur as a result of either external suppression or a second intragenic mutation. We have compared four derivatives which carry independently isolated suppressor mutations which we believe to be intragenic, although the possibility that the suppressor is in another gene tightly linked to *ssd* cannot at present be excluded. The discussion here is based on the assumption that these strains constitute a set of isogenic strains, each carrying the same primary mutation in the *ssd* gene and varying from each other only in the location of a second mutation, also within the *ssd* gene. They thus contain doubly mutated *ssd* products, the second mutation restoring succinate utilization and partial function to the *ssd* product.

The extent of antibiotic resistance varied greatly from revertant to revertant. None of the four revertants are completely restored to parental antibiotic sensitivity; neither are they all similar. This suggests that the second mutation in these strains took place at different points within the gene, resulting in a gene product of slightly different structure (and perhaps conformation), and that these differences in structure are closely reflected in function. This variation of function with subtle changes of conformation would of course fit very nicely with Kell's formulation. Moreover, it should be possible to alter the product to a functionally quite different form by selecting for antibiotic sensitivity or fluoride resistance rather than succinate utilization.

As a result of the selection used, all of these different structures are functional with respect to succinate use; the differences between them show only with respect to antibiotics. Uptake of kanamycin, neomycin, and colicin involves binding to receptors, followed by an energy-dependent transfer into the cell (9). Whatever the mechanism of the mutation may be, if the energization of this transfer were deficient to varying degrees, the strains could clearly be antibiotic resistant, also to varying degrees.

None of the preceding considerations suggest why a change in the *ssd* gene product should affect L-SD levels. One might suppose that this is a secondary effect, the more so since L-SD levels are restored to low levels in all of the succinate-utilizing revertants. However, in at least one case, the revertant has higher than normal L-SD and that alteration transduces with the *ssd* gene. It seems then that even the level of L-SD activity depends on the details of the structure of the *ssd* gene product.

This entire discussion is based on the idea that the *ssd* mutation is identical to the *ecfB* mutation and affects some factor in energy transduction. It should be noted, however, that other interpretations of the data are also possible (12).

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