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Escherichia coli utilizes methanesulfonate and L-cysteate as sole sulfur sources for growth

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Abstract

Twenty-three *Escherichia coli* strains were tested for their ability to use taurine, methanesulfonate, L-cysteate and other alkanesulfonates as sole sulfur sources for growth. One strain was unable to use any of the alkanesulfonates offered as sole sulfur sources for growth but grew with sulfate. Seven strains (class I) used alkanesulfonates for this purpose, but not methanesulfonate or L-cysteate. A further seven strains (class II) grew with all compounds tested, except with L-cysteate, and eight strains (class III) utilized all compounds tested as sulfur sources. Sulfur assimilation from methanesulfonate and L-cysteate was absolutely dependent on the *ssuEADCB* operon that encodes an alkanesulfonate uptake system (SsuABC) and a two-component monooxygenase (SsuDE) involved in the release of sulfite from alkanesulfonates. Long-term exposure of class I strains to methanesulfonate and of class II strains to L-cysteate selected for derivatives that utilized these two sulfur sources as efficiently as sulfate. The nucleotide sequence of the *ssuEADCB* operon in the methanesulfonate- and L-cysteate-utilizing derivative EC1250Me^{+} was identical to that in the class I wild-type EC1250. Gain of the ability to utilize methanesulfonate and L-cysteate as sulfur sources thus appears to result from increased expression of *ssu* genes rather than from a change in the quality of one or several of the Ssu proteins. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

When cysteine and sulfate are not available, *Escherichia coli* expresses two operons whose products enable assimilation of sulfur from alkanesulfonates. The expressed systems are responsible for the release from taurine and other alkanesulfonates of sulfite, which then serves as a source of sulfur for cysteine synthesis. The *tauABCD* operon encodes an ABC-type transport system (TauABC) and the α-ketoglutarate-dependent taurine dioxygenase TauD, which are responsible for the uptake [1] and desulfonation [2] of taurine. The *ssuEADCB* operon encodes an ABC-type uptake system (SsuABC) and a two-component monooxygenase system (SsuDE) responsible for sulfite release from alkanesulfonates other than taurine [1,3]. The biochemistry of sulfonate-sulfur assimilation and its regulation in *E. coli* have been reviewed recently [4]. In vitro the TauD and SsuD enzymes are required for the desulfonation of as many as 20 different alkanesulfonates. The assimilation of sulfur from methanesulfonate and L-cysteate has been poorly investigated and there are conflicting reports in the literature as to the ability of *E. coli* to use these compounds as sulfur sources. Early studies showed that *E. coli* K-12 [5] and B [6] utilized methanesulfonate and/or L-cysteate as sulfur sources for growth, whereas a fecal isolate did not [5]. In contrast, recent work reported on the inability of *E. coli* K-12 strains to assimilate sulfur from these two compounds [7,8]. We now demonstrate that the use of methanesulfonate and L-cysteate as sole sulfur sources is widespread among *E. coli* strains including an *E. coli* K-12 derivative, and provide evidence that the gene products of *ssuEADCB* are absolutely required for the uptake and desulfonation of methanesulfonate and L-cysteate, although the desulfonation of methanesulfonate and L-cysteate by the $FMNH_2$-dependent alkanesulfonate monooxygenase SsuD is not detectable in vitro ([3], this study).

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2. Materials and Methods

2.1. *E. coli* strains and growth conditions

A collection of *E. coli* strains was analyzed for the ability to use alkanesulfonates as sulfur sources for growth. It included the laboratory strains EC1250 [9], BL21(DE3) [10], B [11], C [12], TA4315 [13], strains SG60, SG61, SG63, SG65, SG80 isolated from Swiss cheeses [14], the clinical isolates USZ1, USZ2, USZ3, USZ4, USZ5, and strains ECOR05, ECOR10, ECOR30, ECOR31, ECOR41, ECOR51, ECOR58, ECOR66 from the *E. coli* reference strain collection [15].

The ability of these strains to use alkanesulfonates as sulfur sources was investigated by growth experiments in a sulfur-free M63 minimal medium supplemented with 4 μg ml^{-1} tryptophan [1] and one of the following sulfur sources at 250 μM: sulfate, taurine, isethionate, 3-aminopropanesulfonate, sulfoacetate, methanesulfonate, butanesulfonate, hexanesulfonate, octanesulfonate, decanesulfonate, *N*-phenyltaurine, 2-(4-pyridyl)ethanesulfonate and L-cysteate, of highest purity grade available. Growth curves were determined in microtiter plates with 150 μl of culture by using a SPECTRAmax Plus microtiter plate reader with SOFTmax PRO software (Molecular Devices). Cells from overnight cultures grown in sulfur-free M63 minimal medium supplemented with sulfate as a sulfur source were collected, washed, diluted 100-fold in sulfur-free M63 minimal medium and used to inoculate the microtiter plate as described previously [1]. Standard growth experiments were carried out for 24 h; the optical densities at the stationary phase ranged from 0.3 to 0.6 and corresponded to optical densities of 1.5–2.0 when measured with a 1-cm light path in a Uvikon P-810 spectrophotometer.

The identity of derivative strains generated by long-term exposure to methanesulfonate and/or L-cysteate was investigated using the BBL™ Enterotube™ II for the identification of *Enterobacteriaceae* from Becton Dickinson as indicated by the manufacturer.

2.2. Construction of chromosomal *ssuD* in-frame deletion mutants

Chromosomally located in-frame deletions of the alkanesulfonate monooxygenase encoding gene *ssuD* were constructed in *E. coli* B, SG65 and EC1250Me^{+} (a methanesulfonate-utilizing EC1250 derivative obtained by long-term exposure of wild-type EC1250 to methanesulfonate) with plasmid pME4723 as described previously [1]. Complementation of the in-frame deletions was carried out by analyzing growth after transformation of the deletion strains either with a pUC19-based plasmid containing the *ssuD* gene or with plasmid pME4729, which carries the *ssu* promoter region and the *ssuEAD* genes from *E. coli* EC1250 [1].

2.3. PCR amplification and cloning of *ssu* genes for sequence analysis

Genomic DNA was prepared as described elsewhere [16] from *E. coli* B, SG65 and EC1250Me^{+}. This DNA was used as a template for PCR amplification of the *ssuEADCB* genes using the Expand High-Fidelity PCR system (Roche Molecular Biochemicals). The following primers were used for the PCR amplification of *ssuE* (EE71 (5′-ggaattttcatatgtctccg-3′) and EE23 [1]), *ssuA* (EE45 [1] and EE60 (5′-gcaccgcccatatgttcagg-3′)), *ssuD* (EE8 and EE10 [3]), *ssuC* (EE63 (5′-tatcccccatatgtcgcgc-3′) and EE64 (5′-attgagcaagcttggcgtgc-3′)), *ssuB* (EE29 and EE32 [1]). The *ssuD* genes amplified from *E. coli* B, SG65 and EC1250Me^{+} were cloned into plasmid pET24a(+) (Novagen) to generate plasmids pME4735, pME4736 and pME4737, respectively. The *E. coli* strain DH5α used for cloning purposes was grown in either liquid or solid LB medium supplemented when appropriate with kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), isopropyl-β-D-1-thiogalactopyranoside (0.5 mM), 5-bromo-4-chloro-3-indolyl galactoside (80 μg ml^{-1}). Standard procedures [16] were used for plasmid isolation, restriction enzyme digestion and transformation of *E. coli*. DNA for sequencing was prepared using the QIAprep Spin Miniprep kit (Qiagen). The *ssuD* genes were sequenced from plasmids pME4735, pME4736 and pME4737, whereas the *ssuA*, *ssuB*, *ssuC* and *ssuE* PCR products were directly sequenced using internal primers after gel extraction with the QIAquick gel extraction kit (Qiagen). All sequencing work was done at Microsynth (Balgach, Switzerland).

2.4. Protein production, SDS–PAGE and SsuD activity staining

Desulfonation of methanesulfonate and L-cysteate was investigated in crude extracts from cells of *E. coli* BL21(DE3) that overproduced the SsuD proteins of *E. coli* B, SG65 or EC1250Me^{+}. Protein production was carried out as described previously [3] using plasmids pME4735, pME4736 and pME4737, as were the preparation of crude extracts and measurement of SsuD activity. SDS–PAGE on a Mini-PROTEAN II system (Bio-Rad) with 12% polyacrylamide gels under denaturing conditions and silver stains were performed as described elsewhere [16]. Protein concentrations were measured with Bio-Rad reagent dye concentrate, following the manufacturer's instructions. Bovine serum albumin was used as a standard.

3. Results and discussion

Twenty-three *E. coli* strains of diverse origins were surveyed for their ability to use alkanesulfonates including taurine, methanesulfonate and L-cysteate as sole sulfur

Table 1
Utilization of alkanesulfonates as sole sulfur sources for growth by *E. coli* strains[a]

Strain	Sulfur source				Class
	SO_4^{2-}	*N*-Phenyltaurine	C_1-SO_3H	L-Cysteate	
USZ1, USZ2, USZ3, USZ4, USZ5, SG63, EC1250	+	+	−	−	I
B, BL21(DE3), C, SG60, SG61, ECOR30, ECOR31	+	+	+	−	II
TA4315, SG65, SG80, ECOR05, ECOR10, ECOR51, ECOR58, ECOR66	+	+	+	+	III

[a]Strains USZ are clinical isolates, strains SG were isolated from typical Swiss cheeses, ECOR strains are members of the *E. coli* reference strain collection. The growth phenotypes obtained with *N*-phenyltaurine (SsuD substrate), methanesulfonate (C_1-SO_3H) and L-cysteate are shown. −: no growth, +: growth with a particular sulfur source, with typical growth profiles shown in Figs. 1 and 2. Growth experiments were carried out for 24 h in a microtiter plate (150-μl culture) as described in Section 2.

sources for growth. Strain ECOR41 was the only strain that grew with sulfate but showed no growth with any of the 12 alkanesulfonates offered as sole sulfur sources. PCR amplification of the *tauD* and *ssuD* genes from this strain remained unsuccessful, suggesting that the *tau* and *ssu* genes are missing. The growth phenotypes of the 22 alkanesulfonate-utilizing strains as observed in 24-h growth experiments are summarized in Table 1. Seven strains (class I) showed no growth when methanesulfonate or L-cysteate was the sole sulfur source (e.g. the tryptophan auxotrophic *E. coli* K-12 derivative EC1250, Fig. 1A). In this class, strains USZ1, USZ3 and USZ5 were unable to use taurine as a source of sulfur, although they utilized SsuD substrates (e.g. *N*-phenyltaurine). A further seven strains (class II) used all alkanesulfonates except L-cysteate as sulfur sources for growth. The eight remaining strains (class III) utilized all alkanesulfonates tested including methanesulfonate and L-cysteate as sole sulfur sources. With class II and class III strains, methanesulfonate and/or L-cysteate allowed for growth at somewhat reduced rates, but final cell yields were in most cases identical to those obtained with sulfate. We did not find strains that utilized L-cysteate but were unable to use methanesulfonate as sole sulfur source.

Class I and class II strains were subjected to long-term exposure to methanesulfonate and/or L-cysteate as sole sulfur source (5–20 days, 5-ml cultures, 180 rpm, 37°C). Biomass production with class I strains supplied with methanesulfonate or L-cysteate required 2–7 days, except with strains USZ1, USZ2, USZ3 and USZ5, which were permanently unable to grow with either substrate. Class II strains grew well with methanesulfonate in standard growth experiments, and required 3–10 days for growth when supplied with L-cysteate as sole sulfur source. Low cell yields were obtained in most cases.

Cells from long-term growth experiments were collected, washed and used to inoculate fresh sulfur-free M63 minimal medium supplemented with methanesulfonate or L-cysteate. These cultures reached stationary growth phase within 10–15 h only as shown for strain EC1250Me$^+$, the methanesulfonate-utilizing derivative of the tryptophan auxotrophic strain EC1250 generated by long-term exposure of strain EC1250 to methanesulfonate (Fig. 1B). The ability of strain EC1250Me$^+$ to assimilate sulfur from methanesulfonate and L-cysteate was maintained after eight successive growth cycles (16 h, 37°C) on LB medium agar plates. The identity of this strain was confirmed by tracing its auxotrophy for tryptophan (Fig. 1B). The identity of the other derivative strains obtained in long-term growth experiments was established as *E. coli* using the

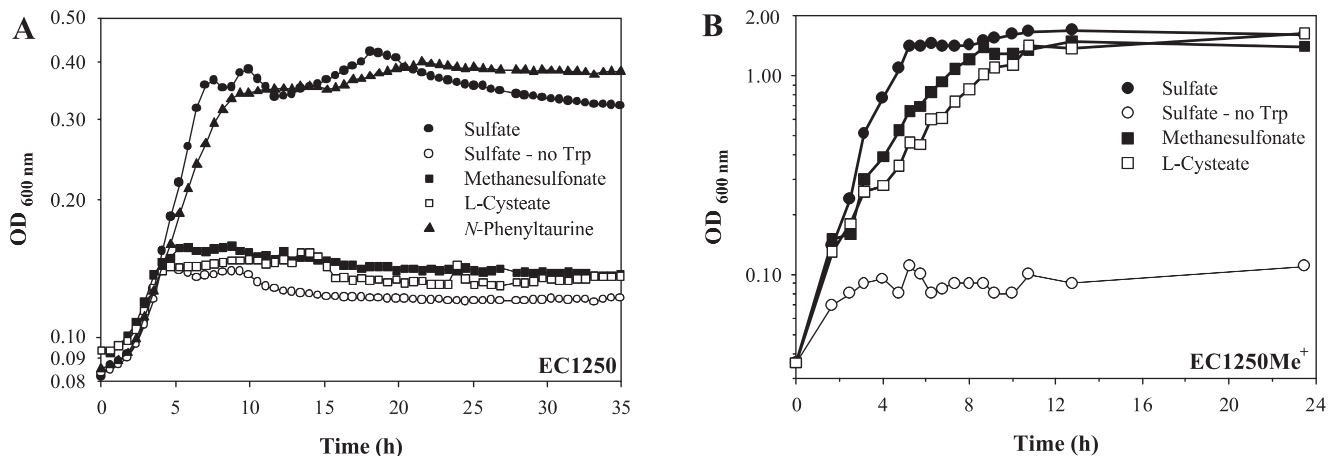

Fig. 1. *E. coli* EC1250 gives rise to spontaneous derivatives showing improved growth with methanesulfonate and L-cysteate as sulfur sources. A: EC1250 cells were grown in a microtiter plate as described in Section 2 (150-μl culture). The optical density at 600 nm ($OD_{600\,nm}$) was recorded with a microtiter plate reader. B: The methanesulfonate-utilizing strain EC1250Me$^+$ was grown in 5 ml medium at 37°C on a rotary shaker (180 rpm). Trp: tryptophan.

BBL™ Enterotube™ II for the identification of *Enterobacteriaceae*.

The following conclusions can be drawn from the long-term growth experiments. Among 22 alkanesulfonate-utilizing strains (Table 1), only the clinical isolates USZ1, USZ2, USZ3 and USZ5 were permanently unable to use methanesulfonate and L-cysteate as sulfur sources for growth. The remaining class I and class II strains showed no growth with methanesulfonate and/or L-cysteate in standard growth experiments but grew with these sulfur sources when sufficient time was given. Long-term exposure of class I strains to methanesulfonate and of class II strains to L-cysteate selected for derivatives that utilized these two sulfur sources as efficiently as sulfate (e.g. EC1250Me$^+$, Fig. 1B). Moreover, all class I derivative strains obtained after exposure to methanesulfonate were able to use L-cysteate as sole sulfur source, and vice versa. Growth behavior with methanesulfonate and L-cysteate was invariably reproduced in long-term growth experiments using wild-type strains, as was the generation of derivative strains with improved growth rates. The present results demonstrate that both methanesulfonate and L-cysteate belong to the range of alkanesulfonates that are used as sulfur sources by 18 of the 22 alkanesulfonate-utilizing *E. coli* strains, including the *E. coli* K-12 derivative EC1250.

A representative of each strain class (EC1250, B, SG65) plus a derivative strain generated in a long-term growth experiment (EC1250Me$^+$) were used to investigate the requirement for the *ssu* genes for sulfur assimilation from methanesulfonate and L-cysteate.

Chromosomally located in-frame deletions of the *ssuD* gene were constructed in these strains, and growth with methanesulfonate and L-cysteate was analyzed. The deletion strains had lost the ability to use SsuD substrates [3] as sulfur sources. As expected, they were still able to grow with taurine and other TauD substrates [2]. The deletion strains had also lost the ability to use methanesulfonate and L-cysteate as sulfur sources as shown for *E. coli* SG65 (Fig. 2). This defect was successfully complemented when the *ssuD* in-frame deletion mutants were transformed either with a plasmid containing a copy of the *ssuD* gene or with plasmid pME4729 [1] containing the *ssu* promoter region and the *ssuEAD* genes. Long-term exposure to methanesulfonate or L-cysteate of an EC1250 strain that was in-frame deleted for *ssuD* [1] did not give rise to derivatives utilizing these compounds as sulfur sources. Alkanesulfonate monooxygenase SsuD thus is absolutely required for the use of methanesulfonate and L-cysteate as sulfur sources for growth.

Growth with methanesulfonate and L-cysteate as sulfur sources was also analyzed with EC1250 strains in-frame deleted for genes encoding components of the SsuABC transport system [1]. EC1250 strains missing either the periplasmic substrate-binding protein SsuA or the membrane component SsuCB, or the whole SsuABC transporter, were reproducibly unable to grow with methanesulfonate and L-cysteate (long-term growth experiments). These deletion strains were, however, able to grow with *N*-phenyltaurine, an alkanesulfonate that is also transported by TauABC, but is desulfonated exclusively by SsuD. This demonstrates that methanesulfonate and L-cysteate enter the cells exclusively via the SsuABC transport system.

From growth studies with deletion strains we conclude that uptake and desulfonation of methanesulfonate and L-cysteate by *E. coli* depend absolutely on the presence of the *ssuEADCB* gene products.

In the previous in vitro characterization of the EC1250 SsuD enzyme [3] methanesulfonate and L-cysteate were not considered substrates of the enzyme. This contrasts with the present in vivo study. It was therefore hypothesized that sulfur assimilation from these two substrates by *E. coli* could be due to SsuD proteins with extended substrate range and differing in sequence from EC1250 SsuD. To confirm or refute this hypothesis, in vitro desulfonation with crude extracts of *E. coli* BL21(DE3) cells overproducing SsuD from *E. coli* EC1250Me$^+$, B and SG65 was examined. With none of the three crude extracts, release of

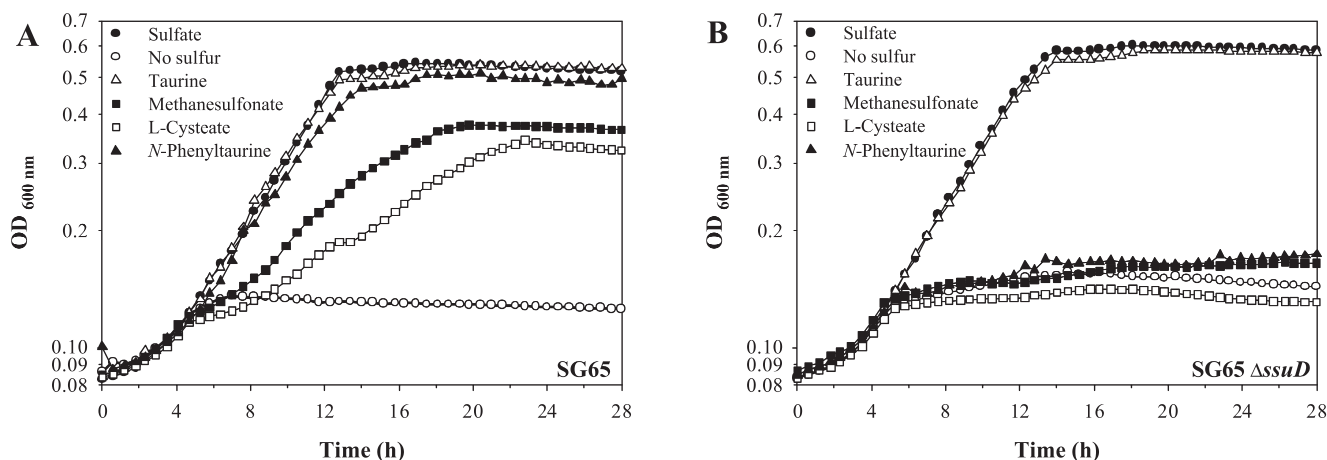

Fig. 2. Growth of *E. coli* SG65 with methanesulfonate and L-cysteate depends on *ssuD*. Cells were grown in a microtiter plate as described in Section 2 (150-μl cultures) and the optical density at 600 nm ($OD_{600\ nm}$) was recorded with a microtiter plate reader. A: *E. coli* SG65. B: *E. coli* SG65 Δ*ssuD*.

sulfite from methanesulfonate and L-cysteate was detected. Sequencing of the *ssuD* genes of these strains obtained from two independent PCR amplifications showed that the *ssuD* gene sequences in all three strains were 100% identical to that of the wild-type EC1250 *ssuD*. We therefore conclude that the ability of *E. coli* to assimilate sulfur from methanesulfonate and L-cysteate is not due to an extension of the SsuD substrate range by mutations in the gene encoding this enzyme, and postulate that alkanesulfonate monooxygenase SsuD is responsible for methanesulfonate and L-cysteate desulfonation in *E. coli*.

When C1 to C8 unsubstituted linear alkanesulfonates were offered as sulfur sources, the doubling time of *E. coli* increased as the chain length of the alkanesulfonate decreased. This correlates well with the in vitro specific activities of pure SsuD [3] and confirmed that methanesulfonate and L-cysteate are least efficiently desulfonated. The minimal desulfonation rates required for growth with these two sulfur sources fall below the limit of detection of the standard enzyme activity test, which is based on the determination of sulfite with Ellman's reagent (DTNB).

Gain of the ability to grow with methanesulfonate and L-cysteate might possibly be due to selection of mutants with an improved uptake system for these two sulfur sources. This hypothesis was refuted by the result of sequence analyses of the *ssuA*, *ssuB*, *ssuC*, and *ssuE* genes that were PCR-amplified from EC1250Me$^+$ genomic DNA (two independent experiments). The *ssuEADCB* sequence of the methanesulfonate- and L-cysteate-utilizing strain EC1250Me$^+$ was 100% identical to the wild-type EC1250 *ssuEADCB*. We conclude that the ability to use methanesulfonate and L-cysteate as sole sulfur source is not due to a qualitative change in the *ssuEADCB* genes that would improve transport or desulfonation of these two compounds.

The present study demonstrates that the use of alkanesulfonates in general and of methanesulfonate and L-cysteate in particular as sole sulfur sources is widespread among *E. coli* strains and confirms that this trait is also present in an *E. coli* K-12 derivative. Only four *E. coli* strains out of 22 alkanesulfonate utilizers were unable to assimilate sulfur from methanesulfonate and L-cysteate. The present in vivo study extends the substrate range of the SsuD enzyme to methanesulfonate and L-cysteate and demonstrates that the use of these two substrates as sole sulfur sources for growth depends absolutely on the presence of the *ssuEADCB* gene products. We strongly suspect that gain of the ability to use methanesulfonate and L-cysteate as sulfur sources is due to mutations leading to increased expression of the *ssu* genes, rather than to mutations altering the substrate range of the SsuEADCB proteins. This view is supported by an increased intensity of the SsuD protein band upon SDS–PAGE analysis of cell extract prepared from the methanesulfonate/L-cysteate utilizer strain EC1250Me$^+$ as compared to extract from its non-utilizing parent strain EC1250.

Acknowledgements

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