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Role of the *ssu* and *seu* Genes of *Corynebacterium glutamicum* ATCC 13032 in Utilization of Sulfonates and Sulfonate Esters as Sulfur Sources†

D. J. Koch,[1,2]‡ C. Rückert,[1,2]‡ D. A. Rey,[2] A. Mix,[3] A. Pühler,[4] and J. Kalinowski[2]*

International Graduate School in Bioinformatics & Genome Research, Center for Biotechnology, Universität Bielefeld, Universitätsstr. 25, D-33615 Bielefeld, Germany[1]; Institut für Genomforschung, Universität Bielefeld, Universitätsstr. 25, D-33615 Bielefeld, Germany[2]; Lehrstuhl für Anorganische Chemie III, Universität Bielefeld, Universitätsstr. 25, D-33615 Bielefeld, Germany[3]; and Lehrstuhl für Genetik, Universität Bielefeld, Universitätsstr. 25, D-33615 Bielefeld, Germany[4]

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Corynebacterium glutamicum **ATCC 13032 was found to be able to utilize a broad range of sulfonates and sulfonate esters as sulfur sources. The two gene clusters potentially involved in sulfonate utilization, *ssuD1CBA* and *ssuI-seuABC-ssuD2*, were identified in the genome of *C. glutamicum* ATCC 13032 by similarity searches. While the *ssu* genes encode proteins resembling Ssu proteins from *Escherichia coli* or *Bacillus subtilis*, the *seu* gene products exhibited similarity to the dibenzothiophene-degrading Dsz monooxygenases of *Rhodococcus* strain IGTS8. Growth tests with the *C. glutamicum* wild-type and appropriate mutant strains showed that the clustered genes *ssuC*, *ssuB*, and *ssuA*, putatively encoding the components of an ABC-type transporter system, are required for the utilization of aliphatic sulfonates. In *C. glutamicum* sulfonates are apparently degraded by sulfonatases encoded by *ssuD1* and *ssuD2*. It was also found that the *seu* genes *seuA*, *seuB*, and *seuC* can effectively replace *ssuD1* and *ssuD2* for the degradation of sulfonate esters. The utilization of all sulfonates and sulfonate esters tested is dependent on a novel putative reductase encoded by *ssuI*. Obviously, all monooxygenases encoded by the *ssu* and *seu* genes, including SsuD1, SsuD2, SeuA, SeuB, and SeuC, which are reduced flavin mononucleotide dependent according to sequence similarity, have SsuI as an essential component. Using real-time reverse transcription-PCR, the *ssu* and *seu* gene cluster was found to be expressed considerably more strongly during growth on sulfonates and sulfonate esters than during growth on sulfate.**

Corynebacterium glutamicum, a gram-positive, nonsporulating soil bacterium, was identified in 1957 as an L-glutamic acid producer (19). Some information has been obtained recently about the general sulfur metabolism of this industrially important, amino acid-producing microorganism, especially concerning the biosynthesis of the sulfur-containing amino acids L-cysteine and L-methionine (12, 29). Still, little is known about the sulfur supply of *C. glutamicum*. Among the possible sulfur sources used by microorganisms, sulfate esters and sulfonates play a special role since they represent the most common sulfur sources in soils (2). Of these two groups, sulfate esters can easily be cleaved through hydrolysis by sulfatases (20), enzymes which have been identified in many different species and seem to be ubiquitous (17). For the utilization of sulfonates, a specific uptake and utilization system is needed (4).

Proteins involved in the utilization of organic sulfate esters or sulfonate compounds can be found among the sulfate starvation-induced proteins, which are induced in the absence of inorganic sulfate as a sulfur source, as has been shown, for instance, in experiments with *Pseudomonas aeruginosa* (26). The genetic background for utilization of sulfonates has been identified in the last few years. A cluster of genes involved in sulfonate utilization, the so-called *ssu* genes (sulfonate-sulfur utilization), was described for *Bacillus subtilis* (36), *Escherichia coli* (35), and *Pseudomonas putida* (15). In all species, the *ssu* genes are organized as an operon which is responsible for the specific uptake and utilization of aliphatic sulfonates. The *ssu* operon consists of three genes encoding an ABC-type transporter, *ssuA*, *ssuB*, and *ssuC*, and the *ssuD* gene directing the synthesis of a reduced flavin mononucleotide ($FMNH_2$)-dependent sulfonatase. In many organisms, *ssu* operons also contain an *ssuE* gene encoding an NAD(P)H-dependent reductase that contributes to the cleavage process mediated by the sulfonatase (18).

Besides the main sulfonate utilization system, some organisms are equipped with additional systems which are responsible for the degradation of a certain subgroup of sulfonates, like the *E. coli* Tau system encoded by the *tauABCD* cluster for the utilization of taurine (33) or the *P. aeruginosa* Msu system encoded by the *msuEDC* cluster directing the utilization of methanesulfonate (18).

In the actinobacteria, the only sulfur utilization system which has been studied in detail is the *dsz* system of *Rhodococcus* sp. strain IGTS8 (5, 24). The Dsz system consists of the *dszA*, *dszB*, *dszC*, and *dszD* genes. The first three of these genes encode enzymes that are responsible for the sequential cleavage of the aromatic sulfur compound dibenzothiophene, which leads to

* Corresponding author. Mailing address: Institut für Genomforschung, Universität Bielefeld, Universitätsstr. 25, D-33615 Bielefeld, Germany. Phone: (49) 521 106-4825. Fax: (49) 521 106-5626. E-mail: Joern.Kalinowski@CeBiTec.Uni-Bielefeld.de.

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‡ D.J.K. and C.R. contributed equally to this work.

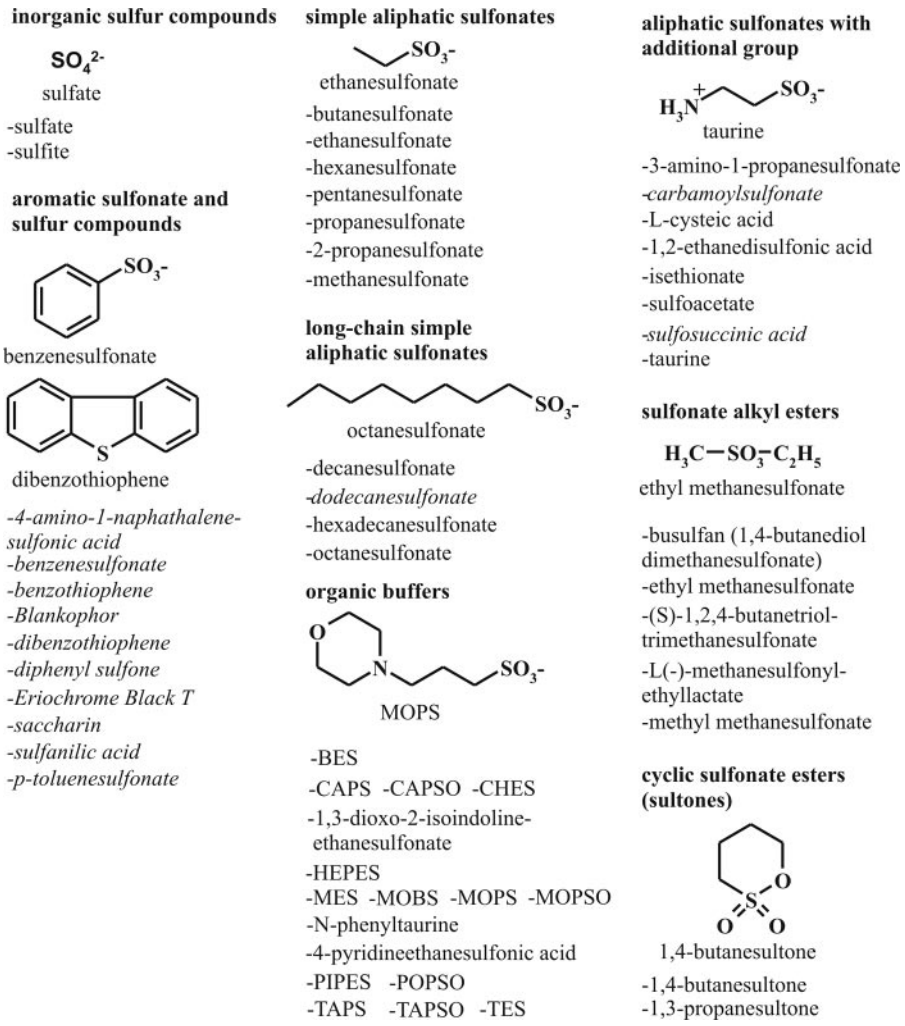

FIG. 1. Sulfur-containing compounds tested as sulfur sources for *C. glutamicum*. The substances are shown in alphabetical order in groups according to their chemical structures. For each group a selected compound and its chemical structure are shown. Compounds that are not utilized as sulfur sources by the *C. glutamicum* wild type are indicated by italics. Abbreviations: BES, *N*,*N*-bis-(2-hydroxyethyl)taurine; CAPS, 3-cyclohexylamino-1-propanesulfonic acid; CAPSO, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonate; CHES, 2-(cyclohexylamine)ethanesulfonic acid; MES, morpholineethanesulfonic acid; MOBS, 4-morpholino-butanesulfonic acid; MOPS, 3-morpholino-propanesulfonic acid; MOPSO, 3-morpholino-2-hydroxypropanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); POPSO, piperazine-*N*,*N*′-bis-(2-hydroxypropanesulfonic acid); TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TAPSO, 3-[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

the release of sulfite. Similar to *ssuD* genes, the *dszA* and *dszC* genes encode $FMNH_2$-dependent monooxygenases whose reactions are essentially dependent on a reductase, which is encoded by *dszD* (22).

In this study we analyzed the utilization of sulfonates and sulfonate esters as sole sulfur sources in *C. glutamicum*. We also characterized *C. glutamicum* genes involved in the utilization of these compounds. In addition, we studied the expression of the genes identified with dependence on different sulfur sources.

MATERIALS AND METHODS

Chemicals. All chemicals used in this work were "for analysis" quality (purity, at least 97%). They were obtained from Sigma-Aldrich, ICN Biomedicals, Roth, Merck, or Acros. The substances tested in growth tests are listed in Fig. 1 or mentioned below. Eriochrome is a Ciba-Geigy trademark, and Blankophor is a Bayer trademark. The stability of the sulfonate esters in water was investigated by proton nuclear magnetic resonance (NMR) spectroscopy. Four-milligram portions of the sulfonate esters were dissolved in 0.6 ml of D_2O at room temperature. The NMR spectra were recorded with a Bruker Advance 600 instrument at a proton resonance frequency of 600.13 MHz with reference to external trimethyl silane.

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *E. coli* strains carrying plasmids were routinely grown on solid antibiotic medium no. 3 (Oxoid, Wesel, Germany) at 37°C. *C. glutamicum* strains were grown on solid brain heart broth (BH) (Merck, Darmstadt, Germany) at 30°C. For analysis of sulfur utilization, *C. glutamicum* strains were grown in sulfur-free minimal medium (MMS) (29) containing 25 g/liter glucose as a carbon source. The medium was prepared with very pure substances containing no sulfur, but no special treatment was used to remove traces of sulfur. Agarose at a concentration of 1.6% (wt/vol) was used for solid media. Sulfur sources were sterilized by filtration and added at a final concentration of 2 mM to autoclaved, cooled MMS medium. A Nephelostar Galaxy nephelometer (BMG Laboratories, Offenburg, Germany) was used to monitor growth of *C. glutamicum* strains in MMES medium (2.5% glucose, 17 mM $NaNH_4HPO_4$, 1

TABLE 1. Bacterial strains used in this study

Strain	Relevant features	Source or reference
C. glutamicum strains		
ATCC 13032	Wild type	ATCC[a]
DK001	Δ*cg1374–80*	This study
DK002	Δ*cg1374*	This study
DK003	Δ*cg1375*	This study
DK004	Δ*ssuD1*	This study
DK005	Δ*ssuC*	This study
DK006	Δ*ssuB*	This study
DK007	Δ*ssuA*	This study
DK008	Δ*ssuI*	This study
DK009	Δ*cg1148*	This study
DK010	Δ*cg1149*	This study
DK011	Δ*cg1150*	This study
DK012	Δ*seuA*	This study
DK013	Δ*seuB*	This study
DK014	Δ*seuC*	This study
DK015	Δ*ssuD2*	This study
DK016	Δ*seuABC*	This study
DK017	Δ*ssuD1* Δ*seuABC ssuD2*	This study
DK018	Δ*ssuD1* Δ*seuA*	This study
DK019	Δ*ssuD1* Δ*seuB*	This study
DK020	Δ*ssuD1* Δ*seuC*	This study
DK021	Δ*ssuD1* Δ*ssuD2*	This study
E. coli DH5αMCR	F$^-$ *endA1 supE44 mcrA thi-1 hsdR17* λ$^-$ *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZ*ΔM15) (*mrr-hsd-RMSmcrBC*)	11

[a] ATCC, American Type Culture Collection, Manassas, Va.

mM $MgCl_2$, 60 mM K_2HPO_4, 10 mM citric acid, 37.5 μM $FeCl_2$, 50 μM $MnCl_2$, 67.5 μM $CaCl_2$, 7.5 μM $ZnCl_2$, 1 μM $CuCl_2$, 0.1 μM $NiCl_2$, 500 μg/liter thiamine, 50 μg/liter biotin) in order to analyze utilization of growth-limiting concentrations of sulfonate esters as sole sulfur sources. Each measurement consisted of two biological replicates, and the assay was conducted with four technical replicates per biological replicate. For clone selection, kanamycin was used at concentrations of 50 μg/ml for *E. coli* and 25 μg/ml for *C. glutamicum* strains.

DNA isolation, manipulation, transfer, and hybridization. Standard procedures were employed for molecular cloning, transformation, and electrophoresis of *E. coli* DH5α, as well as for Southern hybridization of *C. glutamicum* DNA (30). Vector DNA was prepared from *E. coli* by the alkaline lysis technique using a QIAprep Spin miniprep kit according to the manufacturer's protocol (QIAGEN, Hilden, Germany). Transformation of *C. glutamicum* was performed by electroporation using the methods of Tauch et al. (32). Sequence similarity-based searches with nucleotide and amino acid sequences were performed using the basic local alignment search tool (BLAST) described by Altschul et al. (1).

Construction of plasmids. Plasmids pDK001 to pDK017 were constructed using the gene-SOEing method described by Horton et al. (13) with the primers listed in Table S1 in the supplemental material. The appropriate primers were designed using the Sci Ed Central program hub with the Primer Designer 4.2 software (Sci Ed Software). Primers were purchased from SIGMA-ARK (Darmstadt, Germany). The primary products were amplified using *Pwo* DNA polymerase (Roche, Mannheim, Germany). The resulting products were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and then used as templates for the second round of PCR. The final products were digested with restriction enzymes corresponding to cleavage sites which were introduced via the primers and ligated into appropriately digested pK18*mobsacB*. The ligation mixture was used to transform *E. coli* DH5αMCR, and the transformants were selected on antibiotic medium no. 3 plates containing 50 μg/ml kanamycin and 40 mg/liter X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Restriction endonucleases and T4 DNA ligase were obtained from Amersham-Pharmacia (Freiburg, Germany) and Roche-Diagnostics (Mannheim, Germany).

Site-specific gene disruption and gene replacement. Site-specific gene disruption was performed using the nonreplicable integration vector pK18*mobsacB*, which can be used for marker-free deletion of a target gene (31). The resulting plasmids, pDK001 to pDK017, were transformed into *C. glutamicum* ATCC 13032 by electroporation (32). Integration of the introduced plasmids into the chromosome by single crossover was tested by selection on BH plates containing 25 μg/ml kanamycin. For deletion of the target gene, kanamycin-resistant (Km^r) cells were grown overnight in liquid BH and spread on BH plates containing 10% sucrose. Cells growing on these plates were tested for kanamycin sensitivity (Km^s) by parallel picking on BH plates containing either kanamycin or sucrose. Sucrose-resistant and kanamycin-sensitive cells were then tested for the deletion by PCR, and, if necessary, the results were validated by Southern hybridization. Therefore, isolated chromosomal DNA from a putative mutant and the wild type were digested using an appropriate restriction enzyme. An agarose gel containing the digested mutant DNA and similarly digested wild-type DNA was hybridized with a digoxigenin-labeled probe complementary to the genes analyzed (binding outside the deletion region). The probe was generated with a digoxigenin DNA labeling and detection kit (nonradioactive) obtained from Roche (Mannheim, Germany).

RNA preparation and real-time RT-PCR. For real-time reverse transcription (RT)-PCR measurements, cultures were grown in MMS with a sulfur source at a concentration of 2 mM and, in parallel, with 2 mM sulfate as a reference. A total of 1×10^9 *C. glutamicum* cells from a culture in the early logarithmic phase were mixed with killing buffer (10 mM sodium azide, 10 mM Tris, 5 mM $MgCl_2$) at 4°C at a 1:1 ratio and put on ice for 10 min before centrifugation. The cell pellets were dissolved in 700 μl RLT buffer, and total RNA was isolated as described by Hüser et al. (14). The real-time RT-PCR was performed with a LightCycler machine (Roche, Mannheim, Germany) with a QuantiTect SYBR Green RT-PCR kit (QIAGEN). Oligonucleotides used for real-time RT-PCR were constructed to amplify intragenic regions (length, about 150 bp) of the genes analyzed. The primers (Table S2 in the supplemental material) were designed using the Primer Designer 4.2 software (Sci Ed Software) and were purchased from SIGMA-ARK (Darmstadt, Germany). The reverse transcriptase reaction was carried out at 50°C for 20 min, and this was followed by denaturation at 95°C for 15 min, which was used to activate the HotStarTaq DNA polymerase and to inactivate the reverse transcriptase. This was followed by 55 PCR cycles of 10 s at 95°C, 20 s at 55°C, and 12 s at 72°C. The melting curve was recorded over a range from 65 to 95°C with a heating rate of 0.1°C per s by continuous fluorescence measurement, and the reaction mixtures were finally cooled to 40°C. The crossing point (CP) for each gene and condition was determined using the second-derivative maximum data analysis method (LightCycler software, version 3.5). This algorithm measures the CP at the maximum increase or acceleration of fluorescence (27). The CPs obtained with RNA of cultures grown on MMS with sulfate were used as a reference. The nonnormalized relative expression ratios were calculated using the following equation: ratio = $E^{\text{target(CP of control} - \text{CP of sample)}}$ (25), where E is the PCR efficiency (at 100% efficiency, E is 2). Experiments with differentially diluted RNA indicated that the PCR efficiency was 100% (data not shown). Thus, the expression ratios obtained in the experiments can be considered the actual ratios of the mRNAs of the genes analyzed.

Nucleotide and amino acid sequence accession numbers. The nucleotide sequences of *ssuI*, *seuA*, *seuB*, *seuC*, *ssuD2*, *ssuD1*, *ssuC*, *ssuB*, and *ssuA* are available in the GenBank database; the accession number for all these nucleotide sequences is BX927151. The amino acid sequences of the corresponding proteins can be retrieved from the TrEMBL database; the accession numbers are CAF19713 (SsuI), CAF19717 (SeuA), CAF19718 (SeuB), CAF19719 (SeuC), CAF19720 (SsuD2), CAF19924 (SsuD1), CAF19925 (SsuC), CAF19926 (SsuB), and CAF19927 (SsuA).

RESULTS

Sulfur source utilization by *C. glutamicum* ATCC 13032. To analyze the spectrum of sulfonates that could be utilized as sulfur sources by *C. glutamicum*, wild-type strain ATCC 13032 was grown on minimal agar plates containing one of the compounds shown in Fig. 1 as the sole sulfur source at a concentration of 2 mM. For comparison, the wild-type strain was also grown on minimal agar plates containing 2 mM inorganic sulfate, which is a preferred sulfur source for most bacteria (17), as a positive control, or without any added sulfur source as a negative control. This assay yielded information about whether a deleted gene could be considered essential for utilization of the compound tested as a sulfur source and resulted in growth

TABLE 2. *C. glutamicum* candidate coding sequences possibly involved in the utilization of sulfonates and their esters, based on similarity searches

C. glutamicum coding sequence	Similar gene and/or function[a]	Organism	E value	No. of identical amino acids/total no. (%)
cg1147 (*ssuI*)	*actI*, actinorhodin polyketide dimerase[b]	*Streptomyces coelicolor*	8e-11	39/132 (29)
cg1148	Hypothetical protein	No significant hit		
cg1149	Hypothetical protein	No significant hit		
cg1150	Putative reductase[c]	*S. coelicolor*	1e-52	102/184 (55)
cg1151 (*seuA*)	*dszA*, dibenzothiophene desulfurization enzyme A[b]	*Rhodococcus* sp. strain IGTS8	3e-77	173/443 (39)
cg1152 (*seuB*)	*dszC*, dibenzothiophene desulfurization enzyme C[b]	*Rhodococcus* sp. strain IGTS8	3e-13	94/365 (25)
cg1153 (*seuC*)	*dszC*, dibenzothiophene desulfurization enzyme C[b]	*Rhodococcus* sp. strain IGTS8	5e-30	105/384 (27)
cg1156 (*ssuD2*)	*ssuD*, sulfonatase[b]	*B. subtilis*	2e-92	179/359 (49)
cg1376 (*ssuD1*)	*ssuD*, sulfonatase[b]	*B. subtilis*	6e-94	181/358 (50)
cg1377 (*ssuC*)	*ssuC*, transporter (transmembrane)[b]	*E. coli*	8e-53	98/240 (40)
cg1379 (*ssuB*)	*ssuB*, transporter (ATP binding)[b]	*E. coli*	1e-49	115/228 (50)
cg1380 (*ssuA*)	*ssuA*, transporter (periplasmatic)[b]	*B. subtilis*	1e-30	104/328 (31)

[a] Only the genes that represent the best hits for the *C. glutamicum* coding sequences analyzed in similarity searches on the protein level and the corresponding organisms are shown.
[b] Proteins deduced from the coding sequences analyzed were compared with the Swiss-Prot database.
[c] Proteins deduced from the coding sequences analyzed were compared with the NCBI nonredundant database.

of an appropriate mutant comparable to the growth of the negative control.

In total, more than 50 different sulfur sources were tested (Fig. 1), including one amino acid (L-cysteic acid), simple and complex aliphatic sulfonates (like ethanesulfonate and taurine), organic buffers (like morpholinepropanesulfonic acid [MOPS]), sulfonates with one or more esterifications, cyclic sultones, aromatic sulfonates (benzenesulfonate), aromatic sulfides (e.g., benzothiophene), and, in addition, the inorganic sulfur sources sulfate and sulfite. Of the 36 aliphatic sulfonates tested, 33 could be utilized by the *C. glutamicum* wild type (Fig. 1). Carbamoylsulfonate, sulfosuccinate, and dodecanesulfonate could not be utilized. All sulfonate alkyl esters tested could be utilized, but none of the aromatic sulfonates tested (with a sulfonate group linked directly to an aromatic ring), none of the aromatic sulfonate derivatives (saccharin), and none of the aromatic sulfides (benzo- and dibenzothiophene) could be utilized (Fig. 1).

Identification of genes potentially involved in sulfonate utilization. The recent availability of the complete *C. glutamicum* ATCC 13032 genome sequence (16) allowed identification of possible candidate genes for sulfonate utilization by similarity searches. To identify possible homologues of known *ssu* genes, the sequences of the encoded proteins of *B. subtilis*, *E. coli*, and *P. putida* were retrieved from the Swiss-Prot protein database (3). With these sequences, similarity-based searches were performed with the program BLASTP (1) and the amino acid sequences predicted from the *C. glutamicum* genome sequence. This approach resulted in a cluster of coding sequences (CDS) whose gene products showed highly significant hits to SsuA, SsuB, SsuC, or SsuD (Table 2). Additionally, a second CDS whose gene product exhibited a high level of similarity to SsuD was found at a different genomic locus. A candidate gene encoding SsuE was not detected in the *C. glutamicum* genome by similarity searches. Proteins similar to a transport system for aromatic sulfonates, the AsfC/AtsR-AtsB-AtsC system of *P. putida* (37), were also not detected in the amino acid sequences deduced from the *C. glutamicum* genome.

To verify the proposed functions, reverse searches with the Ssu proteins predicted from the *C. glutamicum* genome were carried out with the Swiss-Prot protein sequence database. Additionally, proteins predicted from the neighboring CDS were analyzed (Table 2). No further CDS with potential importance for sulfur metabolism were found near the *ssuD1CBA* gene cluster. In contrast, upstream of the CDS designated *ssuD2*, a number of CDS were found to encode proteins possibly involved in sulfur utilization. Thus, the similarity searches revealed two clusters of interest consisting of the CDS *ssuD1CBA* (*cg1376* to *cg1380*) and *ssuI seuABC ssuD2* (*cg1147* and *cg1151* to *cg1156*) (Fig. 2). For these CDS, the following functions in utilization of sulfur sources could be proposed. The *ssuC*, *ssuB*, and *ssuA* genes encode proteins with similarity to the ABC-type sulfonate transporter SsuABC from *E. coli* or *B. subtilis*. The *ssuD1* and *ssuD2* genes both encode proteins exhibiting high levels of similarity to known SsuD sulfonatases, which are $FMNH_2$-dependent monooxygenases. The CDS designated *seuA*, *seuB*, and *seuC* encode proteins similar to the Dsz monooxygenases from *Rhodococcus* sp. strain IGTS8, where "*seu*" indicates sulfonate ester utilization. The involvement of the *seu* genes in sulfonate ester utilization is described below. While SeuA resembles DszA, the proteins encoded by *seuB* and *seuC* exhibit levels of similarity of 25% and 27%, respectively, to the DszC protein. Searches using the NCBI nonredundant protein database also revealed similarities for both deduced proteins to a putative dehydrogenase from *Pseudomonas syringae*. The protein encoded by *ssuI* exhibits similarity to a putative oxidoreductase from *Mycobacterium tuberculosis* and thus is a possible functional replacement for SsuE.

The deduced protein sequences of the genes identified were also used in protein domain similarity searches of the NCBI conserved domain database (21). SsuD1, SsuD2, and SeuA exhibited significant hits to the strictly $FMNH_2$-dependent al-

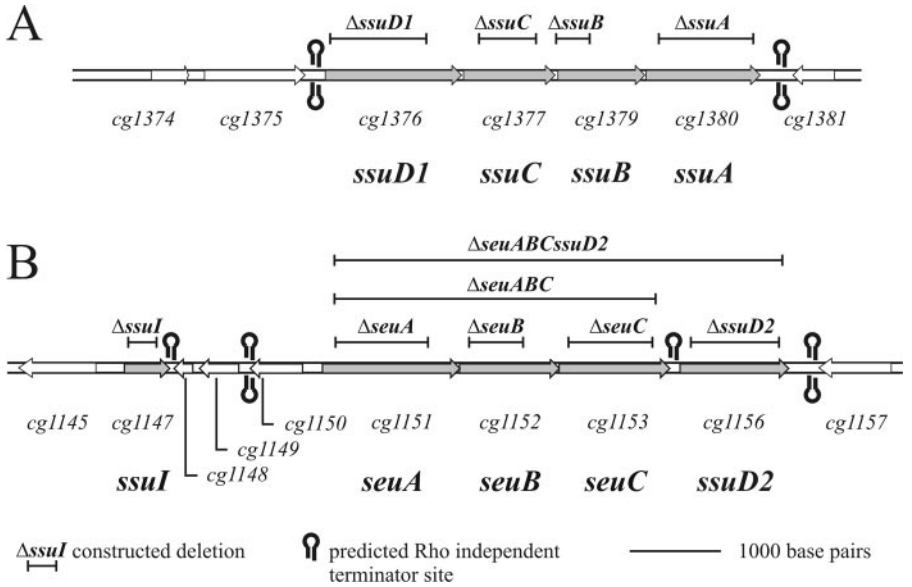

FIG. 2. Physical map of the DNA regions of the *C. glutamicum* ATCC 13032 genome carrying genes possibly involved in utilization of sulfonates and their esters. (A) Chromosomal region containing the genes *cg1374* to *cg1381*; (B) region containing the genes *cg1145* to *cg1157*. The extent and position of an introduced deletion for a given gene are indicated, as are sites for predicted Rho-independent terminators.

kanesulfonate monooxygenase protein family (6). Furthermore, the residues known for $FMNH_2$ binding and catalysis in this family were found to be conserved in the deduced SsuD1, SsuD2, and SeuA amino acid sequences (data not shown). A similar situation was found for SeuB and SeuC, which exhibited significant hits to the strictly $FMNH_2$-dependent dibenzothiophene desulfurization enzyme DszC, and the amino acid residues described as essential for $FMNH_2$ binding of DszC were identified as conserved residues in SeuB and SeuC (data not shown). These findings provided strong bioinformatic evidence that the enzymes SsuD1, SsuD2, SeuA, SeuB, and SeuC are $FMNH_2$-dependent monooxygenases. In addition, the predicted protein domain structure of the putative flavin mononucleotide (FMN) reductase SsuI exhibited significant similarity to the structure of FMN-binding proteins, with high levels of conservation in the residues important for FMN binding (data not shown).

The genomic locations of the genes described here and also of neighboring CDS are shown in Fig. 2. This figure also shows rho-independent transcription terminators found by analysis with the TransTerm software (9). The *ssuD1*, *ssuC*, *ssuB*, and *ssuA* genes form a dense cluster with proposed terminators up- and downstream, which supports the hypothesis that there is an operon structure. The *ssuI*, *seuA*, *seuB*, *seuC*, and *ssuD2* genes are interrupted by the CDS *cg1148*, *cg1149*, and *cg1150*, which are located downstream of *ssuI* and are oriented in the direction opposite the orientation of the *ssu* and *seu* genes. For these CDS, only the protein encoded by *cg1150* exhibited a significant hit in similarity searches, and it resembled a putative reductase. The genes in the cluster *seuABC* also seem to form an operon due to the lack of intergenic space between them and the presence of a predicted terminator downstream of the cluster. There are also terminators with the appropriate orientation located downstream of *ssuI*, *ssuD2*, and *cg1150*, indicating that these CDS are transcribed monocistronically, while no predicted terminator was found between *cg1148* and *cg1149*, indicating that there is polycystronic transcription.

Analysis of *ssuC*, *ssuB*, and *ssuA* deletion mutants. To analyze the importance of the CDS identified for sulfonate utilization, defined deletion mutants were constructed for each CDS (Fig. 2). To determine the relevance of the CDS in the *ssu* and *seu* gene clusters for sulfonate utilization, the growth of the *C. glutamicum* wild-type strain was compared with the growth of each constructed deletion mutant on various sulfur sources. The wild type and the mutants were grown on minimal agar plates containing one of the compounds shown in Fig. 1 as the sole sulfur source at a concentration of 2 mM. The results of the growth tests for all strains tested are summarized in Table 3 and given in detail in Table S3 in the supplemental material.

The growth tests with the deletion mutants showed that deletion of either *ssuC*, *ssuB*, or *ssuA* always resulted in the same phenotype: no growth on most aliphatic sulfonates. Thus, these genes are essential for utilization of nearly all aliphatic sulfonates except sulfonates with a chain length of eight carbon atoms or more. Based on the results of the growth tests and the similarity searches, these CDS were considered genes that encode an ABC-type sulfonate transporter and were designated *ssuC*, *ssuB*, and *ssuA*. As far as sulfonate esters are concerned, the phenotypes of the mutants were less homogeneous. The *ssuC*, *ssuB*, and *ssuA* deletion mutants could utilize all sulfonate esters tested with exception of (*S*)-1,2,4-butanetriol trimethanesulfonate and propanesultone, for which the SsuABC transporter was required (Table 3). The phenotypes obtained can be explained by the presence of an additional, as-yet-unidentified transporter for sulfonate esters which is not encoded in the *ssu* or *seu* gene cluster.

Analysis of *ssuD1* and *ssuD2* deletion mutants. The similarity searches revealed two candidate genes, *ssuD1* and *ssuD2*, that may encode sulfonatases. Deletion of *ssuD1* resulted in a

TABLE 3. Growth of *C. glutamicum* strains with deletions in *ssu* and *seu* genes on different classes of sulfonates and sulfonate esters[a]

Compound	Growth of strains								
	Wild type	Δ*ssuA* or Δ*ssuB* or Δ*ssuC* (transporter)[b,c]	Δ*ssuD1* (monooxygenase)[c]	Δ*ssuD2* (monooxygenase)[c]	Δ*ssuD1* Δ*ssuD2* (monooxygenases)[c,d]	Δ*seuA* or Δ*seuB* (monooxygenase)[b,c]	Δ*seuC* (monooxygenase)[c]	Δ*ssuD1* Δ*ssuD2* Δ*seuABC* (monooxygenases)[c,d]	Δ*ssuI* (reductase)[c]
Inorganic sulfur compounds									
Sulfate, sulfite	+	+	+	+	+	+	+	+	+
Sulfonates									
Simple aliphatic sulfonates (e.g., ethanesulfonate)	+	−	+	+	−	+	+	−	−
Long-chain simple aliphatic sulfonates (e.g., octanesulfonate)	+	+	+	+	−	+	+	−	−
Organic buffers (e.g., MOPS)	+	−	+	+	−	+	+	−	−
Aliphatic sulfonates with additional group (e.g., taurine)	+	−	—[e]	+	−	+	+	−	−
Sulfonate esters									
Busulfan	+	+	+	+	+	+	+	−	−
Butanesultone	+	+	+	+	+	−	+	−	−
(*S*)-1,2,4-Butanetriol-trimethane-sulfonate	+	−	+	+	−	+	+	−	−
Ethyl methanesulfonate	+	+	+	+	+	+	+	−	−
L-(−)-Methanesulfonylethyllactate	+	+	+	+	+	+	+	−	−
Methyl methanesulfonate	+	+	+	+	+	+	+	−	−
Propanesultone	+	−	+	+	−	+	+	−	−

[a] +, compound can be used as a sole sulfur source; −, growth was the same as the growth with no added sulfur after 72 h of incubation at 30°C. The test substances are shown in Fig. 1. Only compounds that could be utilized as sulfur sources by the wild type were used for the growth tests.
[b] Strains having a deletion in any of the genes had the same phenotype.
[c] The data in parentheses are the proposed functions of the deleted genes.
[d] A strain having multiple deletions was constructed and tested.
[e] Deletion of *ssuD1* resulted in a mutant able to utilize all sulfonates except L-cysteic acid, 1,2-ethanedisulfonate, and sulfoacetate.

mutant which was unable to utilize L-cysteic acid, 1,2-ethanedisulfonate, and sulfoacetate (Table 3). The results of the growth tests and similarity searches led to the conclusion that the *ssuD1* gene encodes a sulfonatase, but SsuD1 is not the only broad-range sulfonatase in *C. glutamicum*.

A mutant with a deletion only in the second possible sulfonatase gene, *ssuD2*, exhibited no growth defect on any of the substances tested. In contrast, deletion of both *ssuD1* and *ssuD2* resulted in a mutant that was not able to grow on any aliphatic sulfonate tested, including long-chain aliphatic sulfonates (Table 3). Thus, both of these genes can be considered genes that encode broad-range sulfonatases that together cover the complete spectrum of sulfonates with their enzymatic activities. However, a mutant having the *ssuD1 ssuD2* double deletion was still able to grow on most sulfonate esters, as were mutants having a deletion in either *ssuA*, *ssuB*, or *ssuC*. These results clearly indicate that there is a separate pathway for the degradation of sulfonate esters in *C. glutamicum*.

Analysis of *seuA*, *seuB*, and *seuC* deletion mutants. An *ssuD1 ssuD2* double-deletion mutant is still able to grow on most sulfonate esters, suggesting that other genes must be involved in the degradation of this class of compounds. Interestingly, mutants having a single deletion of either *seuA* or *seuB* (sulfonate ester utilization) were unable to grow on minimal agar plates containing butanesultone as the sole sulfur source (Table 3). In contrast, a mutant with a deletion in the *seuC* gene did not show this effect. However, the phenotypes observed occurred only if the medium containing butanesultone was fresh.

Sulfonate anions are weak bases and therefore groups that can be easily replaced (23). Therefore, sulfonate esters can generally be regarded as chemically unstable in the medium used, and the ester bond might be cleaved by spontaneous hydrolysis, leading to the release of a sulfonate and an alcohol. For example, it can be assumed that propanesultone is converted mainly to the appropriate 3-hydroxy-1-propanesulfonate after it is added to the medium, since this compound is known to be unstable in aqueous solution (10). Also intra- or extracellular *C. glutamicum* esterases might cleave sulfonate esters, leading to the same situation. To obtain information about the chemical stability of the sulfonate esters tested in aqueous solution, H^1-NMR studies were performed. The samples were analyzed after 24 h of incubation in D_2O, and the NMR data were compared to data for freshly dissolved samples (Table 4). Butanesultone and L-(−)-methanesulfonyl-ethyllactate were found to be completely stable for 24 h under these conditions, while for methyl methanesulfonate, propanesultone, and ethyl methanesulfonate, the remaining amounts of the original substances were 72%, 22%, and 90%, respectively. (*S*)-1,2,4-Butanetriol-trimethanesulfonate was completely converted to 1,2-butanediol-dimethanesulfonate, releasing an equimolar amount of methanesulfonic acid, while busulfan was completely converted to an unidentified substance. Although these experiments were conducted in cell-free aqueous solutions and not under exactly the same conditions as the biological growth tests, they showed that for most sulfonate esters tested, a significant amount of methanesulfonate was released by spontaneous hydrolysis, which eventually led to misinterpretation of growth test results.

To avoid such a misinterpretation, all growth tests with sulfonate esters were repeated for the mutants with deletions in the *seu* genes. The growth tests were conducted in liquid media with lower, growth-limiting concentrations so that possible sulfonate contamination alone could not result in complete growth of the *seu* deletion mutants. This led to significant reductions in the growth rate and final cell density for strains DK012 (Δ*seuA*), DK013 (Δ*seuB*), and DK016 (Δ*seuABC*) during growth with 25 μM ethyl methanesulfonate as the sulfur source, in contrast to the results obtained for DK014 (Δ*seuC*), which showed growth comparable to that of the wild type (Fig. 3). When growth-limiting concentrations were used, all sulfonate esters except propanesultone and butanetriol-trimethanesulfonate resulted in growth phenotypes for mutants with deletions in *seuA* or *seuB* that differed from the growth phenotype of the wild type (data not shown). However, the strains tested did not have different phenotypes when they were grown on limiting concentrations of sulfate or methanesulfonate, demonstrating that the mutants did not have a general growth deficiency in the minimal media used and that involvement of the *seuA* and *seuB* genes in the utilization of methanesulfonate is unlikely (data not shown).

The results of the growth assays can be simply explained by the stability of the sulfonate esters tested in aqueous environments. The growth phenotypes of the *seuA* and *seuB* mutant strains were negative in plate tests when the stable sulfonate ester butanesultone was used. In addition, growth-limiting concentrations of the partially stable sulfonate esters resulted in significantly reduced growth of these mutants, which was only due to sulfonates resulting from partial hydrolysis.

Mutants with the complete *seuABC* gene cluster deleted exhibited the same phenotype as mutants with a deletion in *seuA* or *seuB* in all growth tests. These findings can be explained by the assumption that SeuA and SeuB form an enzyme pair, which might work in an enzyme complex or degrade sulfonate esters sequentially as single enzymes. A specific role for SeuC in this degradation process could not be deduced.

Destruction of all degradation pathways for sulfonates and sulfonate esters through deletion of *ssuD1*, *ssuD2*, and *seuABC* resulted in a mutant that was not able to grow on any sulfonate or sulfonate ester in any liquid or solid medium growth test (Table 3), further strengthening the model for the action of the *seu* gene products in the degradation of sulfonate esters.

TABLE 4. Stability of sulfonate esters measured by H^1-NMR spectroscopy

Substance	Stability of compound (%)[a]	Released sulfonic acid
Busulfan	0	Unknown
Butanesultone	100	
(*S*)-1,2,4-Butanetriol-trimethanesulfonate	0	Methane sulfonic acid
Ethyl methanesulfonate	90	Methane sulfonic acid
L-(−)-Methanesulfonyl-ethyllactate	100	
Methyl methanesulfonate	72	Methane sulfonic acid
Propanesultone	22	3-Hydroxypropane sulfonic acid

[a] The values indicate the amount of original substance remaining after 24 h of incubation in D_2O.

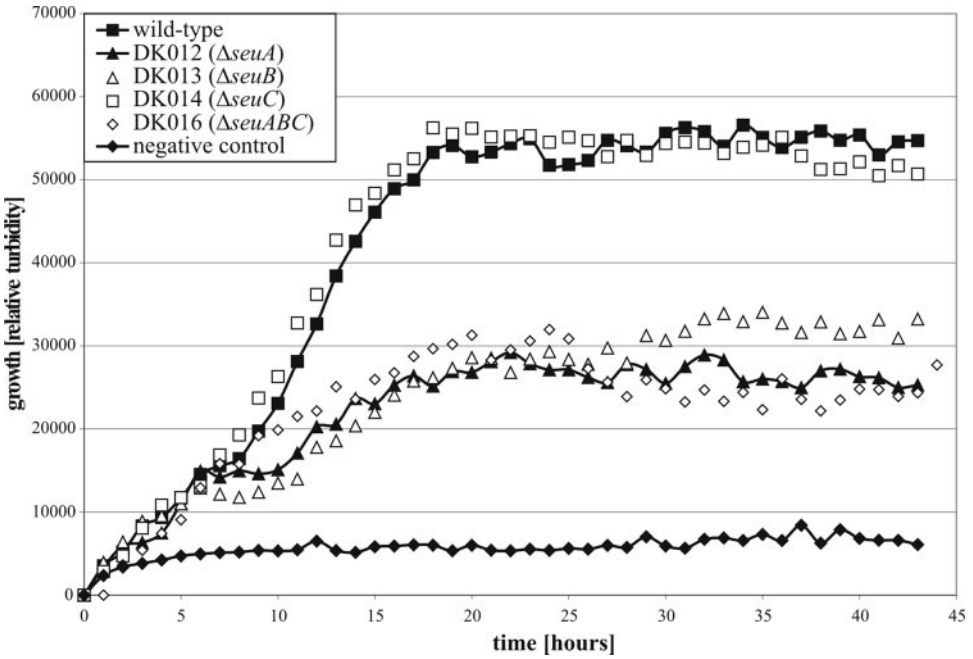

FIG. 3. Growth of the *C. glutamicum* wild-type and mutant strains. Growth in liquid minimal medium containing ethyl methanesulfonate at a concentration of 25 μM as the sole source of added sulfur was monitored with a nephelometer and is expressed as relative turbidity. Growth of the wild type without added sulfur was used as a negative control.

***ssuI* gene is essential for sulfonate and sulfonate ester utilization.** Additional deletion mutants were constructed and tested to analyze the importance of the CDS upstream of *seuABC ssuD2*. The growth of mutants with deletions in *cg1148*, *cg1149*, or *cg1150* did not differ from the growth of the wild type (data not shown). In contrast, deletion of *ssuI* (*cg1147*), which according to the similarity searches is the second CDS besides *cg1150* that potentially encodes a reductase, resulted in a mutant which exhibited growth on all sulfonates and sulfonate esters tested that was comparable to the growth of the negative control (Table 3). Thus, *ssuI* can be considered a gene that plays an essential role in the utilization of both classes of compounds. However, when the growth tests for the *ssuI* deletion mutant were prolonged beyond the normal observation time (72 h), cultures grew slowly to a final density comparable to that of the sulfate-grown positive control (data not shown). This phenotype could be confirmed when the *ssuI* deletion mutant was grown in liquid media. Evidently, the function of SsuI can be bypassed by prolonged cultivation.

Transcriptional studies of the *ssu* and *seu* genes. The growth tests with the deletion mutants showed that *ssuD1*, *ssuD2*, and *ssuI* are essential for the utilization of all aliphatic sulfonates. The *ssuCBA* genes can be considered genes that encode the transporter for nearly all aliphatic sulfonates, and the *seu* genes are involved in sulfonate ester utilization. It was of interest to analyze whether these genes are transcriptionally regulated for dependence on the sulfur source used. To answer this question, the mRNA levels of the genes studied were measured using real-time RT-PCR. Thus, *C. glutamicum* cultures were grown in liquid minimal medium containing different sulfur sources, including a short-chain aliphatic sulfonate (ethanesulfonate), a long-chain aliphatic sulfonate (decanesulfonate), a sulfonate ester (ethyl methanesulfonate), sulfate, or sulfite, at a concentration of 2 mM and were harvested at the logarithmic growth phase. Two independently grown cultures were used for each growth condition, and RNA was isolated, purified, and used in an automated real-time RT-PCR experiment with a LightCycler. All genes in the two gene clusters were analyzed, and the mRNA abundance ratios were determined by comparison to the values obtained from a culture grown on sulfate (reference culture).

The transcription ratios are shown in Fig. 4. Very high ratios (up to 10,000) for all genes in the *ssu* and *seu* clusters were observed for growth on a sulfonate or a sulfonate ester compared to growth on sulfate or sulfite. The CDS *cg1374* and *cg1375* were also tested, but they showed constant expression for all test conditions, confirming that there is no observable relevance of these genes for sulfur compound utilization (data not shown). An interesting result is the strong induction of the *seu* genes during growth on sulfonates, although these genes are considered genes that are not important for sulfonate utilization. This observation indicates that there is no differentiation in terms of regulation between sulfonates and sulfonate esters.

The CDS *cg1148* to *cg1150* were also expressed more highly during growth on a sulfonate or sulfonate ester than during growth on sulfate, albeit to a significantly lesser extent than the neighboring *ssu* and *seu* genes. Since mutants with a deletion in one of these CDS showed no growth phenotype, it can be speculated that these genes might have nonessential functions in the utilization of sulfonates and sulfonate esters.

To analyze the transcriptional organization of the *ssuD1CBA* and *seuABC ssuD2* gene regions, primer pairs amplifying sequences of two adjacent genes, including the com-

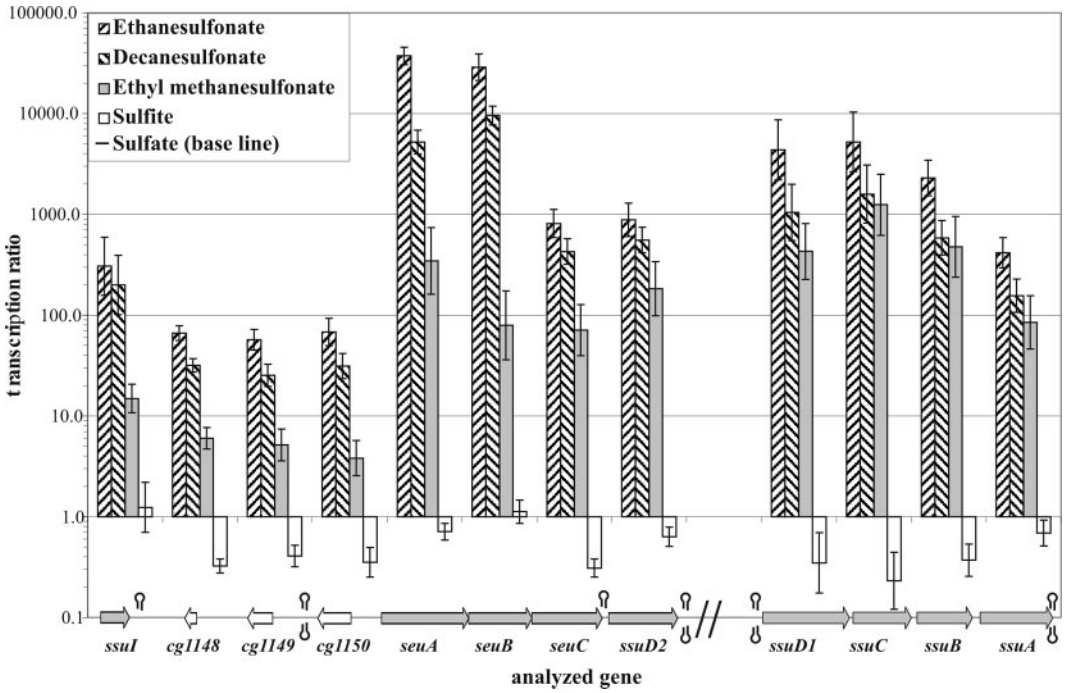

FIG. 4. Expression of the *C. glutamicum ssu* and *seu* genes depending on the available sulfur source. The transcription rates of the genes examined for growth of *C. glutamicum* ATCC 13032 on sulfonates, a sulfonate ester, or sulfite as the sole sulfur source were correlated with the transcription rates for growth on sulfate (baseline). The values are the means of at least two independent experiments with two technical replicates each.

plete intergenic regions, were tested for both clusters by real-time RT-PCR analysis (data not shown). By analyzing total RNA from cultures grown in the presence of ethanesulfonate, the transcription of the intergenic regions of *ssuD1CBA* and the *seuABC* gene cluster was found to be indistinguishable from that of the intragenic regions. Based on the presence of putative transcriptional terminators in front of as well as downstream of the gene clusters analyzed, operon structures for *ssuD1CBA* and *seuABC* seem likely. Interestingly, the intergenic region between *seuC* and *ssuD2* also showed the same expression pattern, indicating that there is a polycystronic *seuABC ssuD2* mRNA, despite the predicted rho-independent transcriptional terminator in front of *ssuD2*.

DISCUSSION

The purposes of this study were elucidation and characterization of the genes involved in the degradation of sulfonates and sulfonate esters in *C. glutamicum*. Therefore, bioinformatic analyses were combined with phenotypic analyses of targeted deletion mutants to infer the involvement of specific genes and to predict functions for their gene products.

Utilization of aliphatic sulfonates in *C. glutamicum*. The *ssu* gene class in *C. glutamicum* consists of the genes *ssuA*, *ssuB*, *ssuC*, *ssuD1*, *ssuD2*, and *ssuI*. According to their sequences, SsuA, SsuB, and SsuC are similar to the ABC transport system for sulfonates in *E. coli* or *B. subtilis* (35, 36), while SsuD1 exhibits similarities to known sulfonatases. As in all other organisms analyzed so far (18), the *C. glutamicum ssuD1CBA* genes also form a predicted operon.

Deletion of *ssuA*, *ssuB*, or *ssuC* resulted in mutants that were not able to grow on any short-chain aliphatic sulfonate. Therefore, we concluded that the putative ABC transporter consisting of SsuA (extracellular binding protein), SsuB (ATP-binding protein), and SsuC (transmembrane protein) is responsible for the import of this class of compounds. However, utilization of long-chain aliphatic sulfonates like decanesulfonate was found to be not dependent on the *ssuCBA* genes. Therefore, an additional uptake system in *C. glutamicum* has to be postulated (for example, uptake via a fatty acid transporter).

It is worthwhile to consider the situation in *B. subtilis*, in which, as in *C. glutamicum*, the *ssuA* and *ssuC* genes are necessary for utilization of short-chain aliphatic sulfonates (36). In *E. coli* the situation is different, since in addition to SsuABC a second sulfonate transporter, TauABC, is known (8). While SsuABC is thought to be responsible for the uptake of most sulfonates, including long-chain sulfonates, TauABC is essential only for the uptake of the short-chain sulfonate taurine. As concluded for *C. glutamicum*, taurine is also imported by the SsuABC transporter in *B. subtilis* (36).

In this study, *C. glutamicum* was found to have two genes encoding proteins with high levels of similarity to known SsuD sulfonatases, the *ssuD1* and *ssuD2* genes encoding broad-range sulfonatases with overlapping substrate spectra. In *E. coli* and all other bacteria described previously, only a single broad-spectrum sulfonatase, designated SsuD and encoded by the *ssuD* gene, was found (18). However, it should be mentioned that additional sulfonatases which are responsible for the degradation of specific sulfonate subgroups are known, like TauD in *E. coli*, which is mainly responsible for the degradation of taurine (33), and MsuD in *P. aeruginosa*, which is capable of

degrading methanesulfonate and other small sulfonates (18). This is in contrast to the situation in *C. glutamicum*, in which no specialized sulfonatases could be detected and SsuD1 as well as SsuD2 are apparently able to degrade a broad range of sulfonates, including taurine and methanesulfonate.

The *C. glutamicum ssuI* gene was shown to be essential for efficient degradation of all sulfonates or sulfonate esters tested. From sequence similarity analyses it could only be determined that *ssuI* encodes a putative reductase. Similar analyses predicted that the *ssuD1*, *ssuD2*, and *seuABC* genes encode $FMNH_2$-dependent monooxygenases with a high level of conservation of the residues important for $FMNH_2$ binding. This type of monooxygenase does not possess a prosthetic flavin group but essentially depends on a reductase which produces $FMNH_2$ by using NAD(P)H as a reducing agent (17). We therefore suggest that *ssuI* encodes a reductase which is essential and specific for the enzymatic action of all monooxygenases involved in sulfonate and sulfonate ester degradation in *C. glutamicum*. It is of interest that a BLAST search did not reveal any protein in other organisms with a high level of similarity to SsuI. It can be assumed that *ssuI* is replaced in other organisms by *ssuE* encoding the reductase for the sulfonate utilization system (17). The isolated enzymes SsuE and SsuD from *E. coli* were characterized biochemically, which showed that the action of the sulfonatase SsuD essentially depends on a reductase restoring $FMNH_2$ and that SsuE has this function (7). Interestingly, in all organisms analyzed so far, the *ssuE* gene was found to be not essential for growth on aliphatic sulfonates (17). It is supposed that SsuE can be replaced by other nonspecific reductases. Possible explanations for the finding that SsuI is essential for sulfonate and sulfonate ester degradation in *C. glutamicum* are (i) that the SsuD and SeuAB monooxygenases must form a complex with SsuI to be fully functional and (ii) that the monooxygenases cannot efficiently interact with the FMN reductases in *C. glutamicum* other than SsuI. This is in contrast to the situation found for *E. coli*, in which the appropriate reductase, SsuE, was shown to efficiently deliver the $FMNH_2$ needed for the sulfonate cleavage mediated by SsuD in vitro, although SsuE is not essential for this process in vivo (7, 35). It has been postulated that SsuD of *E. coli* and other organisms, like *P. aeruginosa* and *P. putida*, is able to interact with several FMN reductases, including SsuE (17).

Utilization of sulfonate esters in *C. glutamicum*. The *C. glutamicum* gene cluster *seuABC* encodes proteins resembling the $FMNH_2$-dependent Dsz monooxygenases from *Rhodococcus* sp. strain IGTS8, which are responsible for the utilization of dibenzothiophene as a sulfur source (5). Growth tests with deletion mutants showed that the *seuA* and *seuB* genes in *C. glutamicum* are involved in sulfonate ester utilization. The finding that sulfonate ester utilization in *C. glutamicum* depends on the *ssuI* gene encoding a putative reductase supports the assumption that *seuA* and *seuB* encode $FMNH_2$-dependent monooxygenases.

The ability of a *C. glutamicum ssuD1 ssuD2* double-deletion mutant to grow on most sulfonate esters showed that these substances can be degraded by a pathway different from that used for degradation of sulfonates. Not only the degradation but also the import of sulfonate esters differs from that of sulfonates. The *ssuA*, *ssuB*, and *ssuC* deletion mutants were still able to grow on most sulfonate esters, demonstrating the presence of an as-yet-unidentified transporter for this class of compounds in *C. glutamicum*. Therefore, sulfonate esters can be considered compounds that represent a distinct group of compounds, which in *C. glutamicum* is handled differently than the sulfonate group, but the possibility that the *seu* genes are also responsible for the utilization of another, as-yet-unidentified class of substances cannot be excluded. The results obtained for the utilization of sulfonate esters in *C. glutamicum* cannot be compared to previously described data since so far the metabolism of these compounds has not been studied in any other organism.

Transcriptional regulation of the *ssu* and *seu* genes. In this study, it was shown that the expression rates for all *ssu* and *seu* genes were very high during growth on sulfonates or a sulfonate ester compound compared to growth on sulfate or sulfite. Evidently, the *ssu* and *seu* genes exhibit a tight connection not only at the functional level but also at the regulatory level. This situation has also been described for other organisms, like *B. subtilis* (36) or *E. coli* (35), in which the *ssu* genes are highly expressed during growth on different sulfonates but not during growth on sulfate (17). In *E. coli* it was shown with a transcriptional *lacZ* reporter gene fusion that sulfate actually repressed *ssu* gene expression. However, the exact mechanism by which sulfate acts on the transcription of the *ssu* genes is still not known (34).

No CDS similar to one of the known *E. coli* regulators of sulfonate utilization, like *cysB* or *cbl* (35), was found in the *C. glutamicum* genome. On the contrary, *ssuD1* was shown to be regulated by the McbR repressor (28). This repressor, however, seems to represent a global regulator of *C. glutamicum* sulfur metabolism and is therefore not a likely candidate for selection between different sulfur sources. Therefore, the question of how gene regulation in *C. glutamicum* distinguishes between different sulfur sources remains. To identify the regulator(s) involved, additional experiments are necessary.

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