Role of d-Cysteine Desulhydrase in the Adaptation of Escherichia coli to d-Cysteine*

Julie Soutourina, Sylvain Blanquet, and Pierre Plateau‡

From the Laboratoire de Biochimie, Unité Mixte de Recherche 7654, CNRS-Ecole Polytechnique, 91128 Palaiseau Cedex, France

D-Cysteine, a powerful inhibitor of Escherichia coli growth, is decomposed in vitro into pyruvate, H₂S, and NH₃ by d-cysteine desulhydrase. To assess the role of this reaction in the adaptation of the bacterium to growth on d-cysteine, the gene of the desulhydrase was cloned. It corresponds to the open reading frame yedO at 43.03 min on the genetic map of E. coli. The amino acid sequence deduced from this gene is homologous to those of several L-aminoacyclopropane-carboxylate deaminases. However, the E. coli desulhydrase does not use L-aminoacyclopropane-1-carboxylate as substrate. Various mutants in which the yedO gene was inactivated or overexpressed were constructed. They exhibited hypersensitivity or resistance, respectively, to the presence of d-cysteine in the culture medium. Growth protection against d-cysteine in minimal medium was conferred by the simultaneous addition of isoleucine, leucine, and valine. In agreement with this behavior, d-cysteine inhibited the activity of threonine deaminase, a key enzyme of the isoleucine, leucine, and valine pathway. Finally, in the presence of the intact yedO gene, E. coli growth was improved by addition of d-cysteine as the sole sulfur source. In agreement with a role of the desulhydrase in sulfur metabolism, yedO expression was induced under conditions of sulfate limitation.

Escherichia coli contains an enzyme activity capable of catalyzing the transformation of d-cysteine into pyruvate, H₂S, and NH₃ (1). This enzyme was named d-cysteine desulhydrase. A similar activity could be detected in Citrobacter freundii, Klebsiella pneumoniae, Enterobacter cloacae, Chlorella fusca, Spinacia oleracea, and various Fusobacterium species (1–4). On the other hand, no activity was found in the bacteria of genera Brevibacterium, Corynebacterium, Erwinia, Flavobacterium, Micrococcus, Proteus, Pseudomonas, Salmonella, Sarcina, Seratia, and Xanthomonas (1).

d-Cysteine desulhydrase from E. coli is a pyridoxal phosphate-containing enzyme. It catalyzes the α, β-elimination reaction of d-cysteine and of several d-cysteine derivatives, such as S-alkyl-, S-aryl-, and S-aralkyl-d-cysteine, d-cystine, DL-selenocysteine, DL-selenocysteine, β-chloro-d-alanine, O-methyl-th-serine, or O-acetyl-d-serine (1, 5). d-Cysteine desulhydrase also catalyzes the formation of d-cysteine or d-cysteine-related amino acids from β-chloro-d-alanine in the presence of various thiols or from O-acetyl-d-serine plus sulfide (1, 5).

The physiological role of bacterial d-cysteine desulhydrase is unknown. However, recent studies indicated that E. coli growth is impaired in the presence of micromolar amounts of d-cysteine (6), suggesting that d-cysteine desulhydrase has a detoxification function.

In the present work, we report the cloning of the E. coli gene (yedO) encoding d-cysteine desulhydrase activity. Involvement of the product of this gene in the adaptation of the cell to d-cysteine is established. Overexpression of the desulhydrase gene protects E. coli against d-cysteine, whereas its inactivation renders the bacterium hypersensitive to the d-amino acid. Moreover, our data suggest that, similarly to l-cysteine (7), d-cysteine exerts its toxicity through an inhibition of threonine deaminase. Decomposition of d-cysteine by the desulhydrase produces H₂S, which the bacterium can utilize as a sulfur source. Accordingly, we establish that the presence of the yedO gene stimulates cell growth in the presence of d-cysteine as sole sulfur source. In agreement with an involvement of the desulhydrase in sulfur metabolism, sulfate starvation induces expression of yedO.

MATERIALS AND METHODS

L- and D-Amino acids, β-chloro-d-alanine, N,N′-dimethyl-p-phenylenediamine, 1-aminoacyclopropane-1-carboxylate, and 2,4-dinitrophenylhydrazine were from Sigma. Pyridoxal phosphate and 2-oxobutyrate (sodium salt) were from Fluka. Rabbit muscle L-lactate dehydrogenase and NADH were from Roche Molecular Biochemicals. DEAE-Sephael and Superdex 75 were from Amersham Pharmacia Biotech.

Purification of d-Cysteine Desulhydrase—Cells from E. coli strain K37 (Table I) were grown at 37 °C in 8 liters of 2X TY medium and harvested by centrifugation for 35 min at 5,000 × g. All buffers used for the purification of d-cysteine desulhydrase contained 10 μM pyridoxal phosphate, 0.1 mM dithiothreitol, and 0.1 mM EDTA. The cell pellet was suspended in 0.1 M potassium phosphate (pH 7.0) at an optical density of 140 at 650 nm. Crude extract preparation, nucleic acid precipitation with streptomycin sulfate, and ammonium sulfate fractionation of proteins were performed as described previously (8), except that ammonium sulfate precipitations were at 35 and 55% saturation, instead of 50 and 80%, respectively. Then the protein pellet was dissolved in 50 ml of 0.1 M potassium phosphate (pH 7.0) and dialyzed overnight against 5 liters of the same buffer. The resulting solution was applied to a 250-ml column of DEAE-Sephael (4.4 × 16.5 cm) equilibrated in 0.1 M potassium phosphate (pH 7.0). Elution was carried out at a flow rate of 2.4 ml/min with a 6-liter linear gradient of 0–0.5 M KCl. d-Cysteine desulhydrase activity was recovered at 0.31 M KCl. After concentration by ammonium sulfate precipitation (80% saturation) and dialysis against 2 liters of 20 mM potassium phosphate (pH 7.0) containing 150 mM KCl, the sample was applied to a Superdex 75 column (120 ml, 1.6 × 60 cm) equilibrated in the same buffer and eluted at a flow rate of 0.5 ml/min.

N-terminal Sequencing of d-Cysteine Desulhydrase—Aliquots of the fractions recovered from the Superdex 75 column were analyzed by SDS-polyacrylamide gel electrophoresis. After migration, proteins were electroeluted from a ProBlot membrane (from Applied Biosystems) and stained with Amido Black. One protein band corresponding to a Mr of 35,000 was selected and submitted to 8 cycles of Edman degradation on an Applied Biosystems 473A Sequencer.

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‡ To whom correspondence should be addressed. Tel.: 33 1 69 33 41 81; Fax: 33 1 69 33 30 13; E-mail: plateau@botrytis.polytechnique.fr.

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Cloning of the yedO Gene—The yedO gene was amplified by PCR1 using *E. coli* K37 genomic DNA as template and oligonucleotides yedODEB (CCGATACCAATTTCCACCTGCATATATTAA CCDDTTTCCAGG- GGCAGCAAG) and yedOFIN (CCGCGCAACCTTTGAGCTCAGAACCTG- CTGCCGATATG) as primers. The amplified DNA fragment of 1010 bp was purified using the Qiagen PCR Purification Kit 50, digested by both EcoRI and HindIII, and inserted into the corresponding sites of plasmid pKK223-3 to give plasmid pKKyedO. The cloned DNA was verified by RI and was purified using the Qiagen PCR Purification Kit 50, digested by both EcoRI and HindIII, and inserted into the corresponding sites of plasmid pKK223-3 to give plasmid pKKyedO. The cloned DNA was verified by PCR amplification of chromosomal DNA using

Disruption of the yedO Gene—Disruption of the yedO gene was performed according to the method of Hamilton et al. (9), by using the plasmid pMAK705 carrying a thermosensitive replicon and a chloramphenicol resistance gene. The XbaI and KpnI sites of this plasmid were used to insert a DNA fragment carrying, in successive order as follows: (i) the ribosome-binding site of plasmid pKK223-3 (CCGATACCAATTTCCACCTGCATATATTAA CCDDTTTCCAGG-GGCAGCAAG) and yedOFIN (CCGCGCAACCTTTGAGCTCAGAACCTGCTGCCGATATG) as primers. The amplified DNA fragment of 1010 bp was purified using the Qiagen PCR Purification Kit 50, digested by both EcoRI and HindIII, and inserted into the corresponding sites of plasmid pKK223-3 to give plasmid pKKyedO. The cloned DNA was verified by PCR amplification of chromosomal DNA using

Purification of D-Cysteine Desulfhydrase from the Overproducing *E. coli* Strain XL1-Blue—D-Cysteine desulfhydrase activity was assayed at 37 °C for 30 min in 1-ml assays containing 0.1 mM potassium phosphate (pH 8.0), 2.5 mM diethiothreitol, and 0.2 mM freshly prepared D-cysteine (assay I). One unit corresponds to enzyme activity capable of producing 1 μmol of H2S per min.

The optical density of the culture reached 0.15 at 650 nm, 1 mM isopro- 

Assays of D-Cysteine Desulfhydrase Activity—D-Cysteine desulfhydrase activity could be followed by use of three different assays. In each case, the enzyme dilution buffer contained 0.1 mM potassium phosphate (pH 8.0), 50 μM bovine serum albumin, and 10 μM pyridoxal phosphate.

Measurement of H2S Formation (Assay I)—In this assay, H2S formation from D-cysteine was measured (3). Incubation was performed at 37 °C for 30 min in 1-ml assays containing 0.1 mM potassium phosphate (pH 8.0). 2.5 mM diethiothreitol, and 0.2 mM freshly prepared D-cysteine. The reaction was initiated by the addition of 0.1 mM of enzyme and quenched by the successive addition of 0.1 ml of solution I (20 mM N,N'-dimethyl-p-phenylenediamine dihydrochloride in 7.2 mM HCl) and 0.1 ml of solution II (30 mM FeCl3 in 1.2 mM HCl). After 30 min at room temperature, methylene blue formation was measured at 670 nm using a molar extinction coefficient of 28.5 × 103 M−1 cm−1. One unit of D-cysteine desulfhydrase activity corresponds to the activity capable of producing 1 μmol of methylene blue per min, i.e., 1 μmol of H2S per min.

Measurement of Pyruvate Formation (Assay II)—The activity of the purified enzyme was also monitored through measurement of the amount of pyruvate formed from D-cysteine by using a spectropho-
metric method with lactate dehydrogenase and NADH (1). The reaction was performed at 37 °C in cuvettes (250 μl) containing 50 mM potassium phosphate (pH 8.0), 0.13 mM NADH, 5 units of rabbit muscle l-lactate dehydrogenase, various concentrations (0.02–5 mM) of the substrate under study (D-cysteine, β-chloro-D-alanine, or L-cysteine), and catalytic amounts of enzyme in 10 μl. The reaction was started by adding 10 μl of the substrate solution in H₂O. Consumption of NADH and the resulting decrease in absorption at 340 nm were followed. A molar absorption coefficient of 6220 M⁻¹ cm⁻¹ was used for NADH.

Assay II was also used to study the conversion of 1-aminocyclopropane-1-carboxylate into 2-oxobutyrate (11). In this case, the final concentration of lactate dehydrogenase in the reaction mixture was 25 units per 250 μl. We verified that, under these conditions, the transformation of 2-oxobutyrate into the final product was not rate-limiting.

Measurement of 1-Aminocyclopropane-1-carboxylate Deamination (Assay III)—Colorimetric quantitation of 2-oxobutyrate, based on the reaction of this compound with 2,4-dinitrophenylhydrazine (12), was used to obtain evidence for the possible conversion of 1-aminocyclopropane-1-carboxylate into 2-oxobutyrate. Incubation was performed at 37 °C for 30 min in 0.5-ml assays containing 0.1 M potassium phosphate (pH 8.0), 10 μM pyridoxal phosphate, and 20 mM 1-aminocyclopropane-1-carboxylate. The reaction was started by addition of 20 μl of the enzyme solution and stopped by the addition of 0.1 ml of a freshly prepared solution containing 15% (w/v) trichloroacetic acid and 0.1% (w/v) phenyl hydrazine hydrochloride. After 10 min at 37 °C, 0.5 ml of 2.5 M NaOH was added, and absorption at 540 nm of the 2,4-dinitrophenylhydrazine derivative of 2-oxobutyrate was immediately measured. A molar absorption coefficient of 4000 M⁻¹ cm⁻¹ was used for this derivative.

Assay of Threonine Deaminase Activity—Threonine deaminase activity was measured in crude extracts through the production of 2-oxobutyrate, according to the procedure of Harris (7). The colorimetric determination of 2-oxobutyrate was the same as that used in assay III. One unit of threonine deaminase activity produces 1 μmol of 2-oxobutyrate per min.

Genomic and Phylogenetic Analyses—Amino acid sequences were obtained from the SwissProt data base (www.expasy.ch) or the GenBank® data base (www.ncbi.nlm.nih.gov/GenBank). In the cases of Pseudomonas syringae pv. tomato, Caulobacter crescentus, Bacillus anthracis, Desulfovibrio vulgaris, Burkholderia mallei, Mycobacterium tuberculosis, Mycobacterium smegmatis, and Aspergillus fumigatus, preliminary sequence data were obtained from the Institute for Genome Research website at www.tigr.org; for Salmonella typhi, Yersinia pestis, Bordetella bronchiseptica, and parapertussis and Burkholderia pseudomallei, the Sanger Center website at www.sanger.ac.uk was used; Salmonella typhimurium data were from the Washington University Genome Sequencing Center website at genome.wustl.edu. The ClustalX program (13) was used to align the sequences and to construct the phylogenetic tree.

RESULTS

Cloning of the Gene Encoding E. coli D-Cysteine Desulfhydrase—E. coli D-cysteine desulfhydrase was partially purified from the strain K37 through successive chromatographies on a DEAE-Sephadex and a Superdex column. At each stage, a single peak of D-cysteine desulfhydrase activity was recovered. In addition, along the DEAE-Sephadex elution profile, the D-cysteine-dependent activity was clearly separated from the activities liberating H₂S from t-cysteine.

The purification procedure resulted in an 880-fold enrichment of the β-cysteine desulfhydrase activity (3 units/mg), when compared with the activity of the initial crude extract (0.0034 units/mg). According to SDS-polyacrylamide gel electrophoresis analysis, the recovered enzyme was not homogeneous. However, one protein band, the intensity of which varied proportionally to the β-cysteine desulfhydrase activity measured in the fractions obtained from the SEPHADEX Superdex column chromatography, could be clearly distinguished. The position of this band corresponded to an apparent mass of 35 kDa, a value close to that already reported for the t-cysteine desulfhydrase polyphenyl chain under denaturing conditions (1).

After transfer from the gel to a polyvinylidene difluoride membrane, the above protein band was cut and submitted to 8 cycles of Edman degradation. The resulting N-terminal sequence, PLHNLTRF, corresponded to the open reading frame (ORF) yedO (14), located at 43.03 min on the E. coli genetic map (Fig. 1). In agreement with the size of the selected protein, this ORF encodes a polypeptide composed of 328 amino acids with an associated Mᵣ of 35,153 (35,022 upon removal of the N-terminal methionine).

The yedO gene could be amplified by PCR and cloned into the expression vector pKK223-3. After transformation of the strain XL1-Blue by the resulting plasmid (pKKyedO), overexpression of a protein with the expected mass of ~35 kDa was obtained. In addition, D-cysteine desulfhydrase activity in a crude extract of strain XL1-Blue (pKKyedO) was increased 500-fold as compared with the activity from a strain transformed by the control plasmid pKK223-3 (Table II).

The chromosomal yedO gene was inactivated by insertion of a kanamycin resistance cassette, as described under “Materials and Methods.” The resulting strain (K37ΔyedO) was viable. α-Cysteine desulfhydrase activity in a crude extract of this strain was 10–30-fold smaller than that measured in an extract of the parental strain K37 (Table II). One possible explanation for the residual production of H₂S from D-cysteine by the extract of the K37ΔyedO cells is the occurrence in E. coli of a D-amino acid dehydrogenase activity capable of converting D-cysteine into the corresponding α-keto acid. Subsequent transamination could thereby generate t-cysteine, another source of H₂S.

All these results indicate, first, that the D-cysteine desulfhydrase occurring in E. coli is actually encoded by the yedO gene and, second, that this protein is not essential to the growth of E. coli.

Specificity of D-Cysteine Desulfhydrase—Searches on the sequence data bases using the BLAST program revealed the presence of genes homologous to yedO in the genomes of several proteobacteria from the α-, β-, γ-, and δ-subdivisions, as well as in the genomes of the Gram-positive bacteria B. anthracis and M. smegmatis, of Thermotoga maritima, of archaeabacteria of the genus Pyrococcus, of some fungi (Penicillium citrinum, Schizosaccharomyces pombe, Hansenula saturnus, and A. fermentatius), and of the plant Arabidopsis thaliana (Fig. 2).

In several of the above organisms (Pseudomonas sp., Pseudomonas fluorescens, E. cloacae, and F. citrinum), the yedO orthologs are known to encode 1-aminocyclopropane-1-carboxylate (ACC) deaminases (15–18). ACC is a natural compound produced by plants, in which it serves as the direct precursor of the plant hormone, ethylene. The E. coli yedO gene product has from 34 to 36% identity with these ACC deaminases.

Because of above sequence similarities between E. coli D-cysteine desulfhydrase and proteins showing activity toward ACC, we investigated whether E. coli D-cysteine desulfhydrase could also use ACC as a substrate. For this purpose, the desulfhydrase was purified until homogeneity from the overproducing strain XL1-Blue (pKKyedO). The optical spectrum of purified D-cysteine desulfhydrase exhibited absorption maxima at 280 and 418 nm with an A₂₈₀/A₄₁₈ ratio of 3. The absorption maximum at 418 nm reflects the presence of a pyridoxal phosphate cofactor (1).
In LB medium, addition of 10 mM D- or L-cysteine had no effect on the growth of the two compared strains, K37 and K37ΔyedO. In contrast, in M9-glucose minimal medium, the addition of D-cysteine markedly slowed down the growth of the two strains. However, the inhibitory effect was more pronounced in the case of ΔyedO::kan strain. To quantify this observation, we determined the concentration of D-cysteine, further designated as toxicity index, causing a 2-fold reduction in the optical density of the culture after an 8 h-incubation as compared with the optical density of a control culture without D-cysteine. This concentration was found equal to 3.5 μM with strain K37ΔyedO and to 9 μM with strain K37 (Table III).

As reported previously (7), addition of L-cysteine also inhibited bacterial growth in minimal medium. Nevertheless, the growth inhibition by L-cysteine did not respond to the inactivation of yedO. Indeed, the indices of L-cysteine toxicity were 7 μM with strain K37 and 8 μM with strain K37ΔyedO (Table III).

During the first 8 h of growth of the yedO+ strain in minimal medium, D- and L-cysteine showed the same inhibitory effects. However, a difference between the two amino acid isomers became apparent after 24 h of growth. At that time, all cultures containing L-cysteine at concentrations up to 0.5 mM had been able to reach the stationary phase. In contrast, the D-amino acid had to be less than 25 μM to allow the cultures to reach the stationary phase, in the case of K37, and less than 5 μM in the case of K37ΔyedO. These observations suggest the following: (i) the yedO+ bacterium adapts more easily to the presence of L-cysteine than to that of D-cysteine, and (ii) specific adaptation to D-cysteine is partly impaired in a ΔyedO context.

As emphasized above, D- or L-cysteine addition remained without effect on cell growth in LB-rich medium. Since cysteine was reported to induce transient amino acid starvation (19), we hypothesized that the amino acids brought to the cell by the rich medium could compensate for the inhibitory properties of cysteine. To assess this idea, we studied the growth inhibition by D- or L-cysteine in M9-glucose medium supplemented with a mixture of 18 amino acids (all L-amino acids except sulfur-containing methionine and cysteine). Upon simultaneous addition of the 18 amino acids, the growth rate of the strain K37 increased by a factor of ~1.6-fold, as compared with standard M9-glucose growth conditions. Moreover, in the presence of the mixture of amino acids, the toxic effect of cysteine became significantly lower. Indeed, addition of up to either 100 μM D-cysteine or 150 μM L-cysteine was required to reach a 2-fold reduction in the optical density of the culture after 6 h of growth (Table III). Therefore, we could conclude at this stage that D-cysteine as well as L-cysteine interferes with the amino acid metabolism of E. coli. The mechanisms underlying these properties will be discussed further below.

Effect of D-Cysteine on the Growth of a yedO-overexpressing Strain—Strains XL1-Blue(pKKyedO) and XL1-Blue (pKK232-3) grew normally on LB-rich solid medium containing ampicillin. However, on M9-glucose minimal medium containing ampicillin, bacteria grew slowly and formed colonies of variable size. Consequently, to characterize the effect of yedO overexpression on D-cysteine resistance in minimal medium, we transformed strain JM101TR with plasmid pMAKyedO. The resulting strain grew in a normal manner in M9-glucose minimal medium containing chloramphenicol in the presence or absence of IPTG.

In liquid minimal medium containing IPTG, D-cysteine desulphydrase activity measured in the strain JM101TR carrying pMAKyedO was increased 6-fold if compared with the strain JM101TR containing the control plasmid pMAK705 (Table II). In the same culture conditions plus L-cysteine, the presence of the plasmid pMAKyedO conferred significant protection.
against the toxic effect of the D-amino acid. Indeed, the D-cysteine toxicity index was found to be 7 times greater in the presence of the pMAK-yedO plasmid than in the presence of the control plasmid (24 versus 3.5 μM). On the other hand, as in the above section, the index of L-cysteine toxicity did not depend on the harbored plasmid, i.e., did not depend on the concentration of desulfhydrase. These experiments reinforced our conclusion that the yedO gene product specifically protects E. coli against the toxic effect of D-cysteine.

In the course of our study, we searched for possible induction of the yedO gene by D-cysteine. For this purpose, strain JM101TR(pMAK705) was cultivated for 24 h in M9-glucose minimal medium in the presence of 25 μM D-cysteine, and D-cysteine desulfhydrase activity was measured in crude extracts. The values in Table II show that, in agreement with previous results (1), expression of the yedO protein product does not respond to the addition of D-cysteine.

**Mechanism of D-Cysteine Toxicity**—We showed above that the lack of growth inhibition by L- or D-cysteine could be related to the presence of amino acids in the culture medium. This observation is in agreement with a series of reports indicating that inhibition of E. coli growth by various sulfur compounds including L-cysteine, homocysteine, mercaptoethanolamine, and to a lesser extent cystine could be overcome by the simultaneous presence of L-isoleucine, L-leucine, and L-valine in the growth medium (7, 19, 20). This behavior was explained by an inhibition of threonine deaminase by the sulfur-containing compounds (21).

To explore whether the effect of D-cysteine could be explained in the same manner, we studied the effect of this D-amino acid on the growth of strains K37 and K37ΔyedO with the simultaneous presence in the growth medium of L-isoleucine (0.4 mM), L-leucine (0.8 mM), and L-valine (0.6 mM). Upon addition of these three L-amino acids to M9-glucose minimal medium, the inhibitory effect of D-cysteine significantly decreased. However, the inhibition remained more pronounced with the ΔyedO::kan strain (K37ΔyedO) than with the yedO+ strain (K37). The measured indices of D-cysteine toxicity increased up to 35 and 22 μM, respectively (Table III).

These results suggested that if the effect of L-cysteine was due to inhibition of threonine deaminase, D-cysteine would also inhibit the activity of threonine deaminase. To assess this idea, we directly measured the specific activity of this enzyme in a crude extract of the strain K37 cultivated in M9-glucose minimal medium. In the absence of added cysteine, the threonine deaminase activity in the crude extract was equal to 0.084 units per mg of total protein. Upon addition of L-cysteine, the activity decreased. Half-inhibition was reached at 5.1 mM L-cysteine. This value is in good agreement with that already reported in the literature (7). Addition of D-cysteine also inhibited the threonine deaminase activity. Half-inhibition was obtained with 5.8 mM of the D-amino acid. Therefore, D-cysteine had a behavior very similar to that of L-cysteine. Moreover, as already observed in the case of L-cysteine (7), inhibition by the D-amino acid was not modified by the addition to the assay of an excess concentration of pyridoxal phosphate (1 mM instead of 0.1 mM as in the standard assay).

The above results strongly suggest that threonine deaminase is one target of D-cysteine. Nevertheless, D-cysteine might also exert its toxicity by reacting with D-succinylhomoserine to give D-allolcystathionine, instead of L-cystathionine (19), in the methionine pathway. As a consequence, D-cysteine would consume homoserine and induce starvation for threonine. Indeed, in Bacillus subtilis, D-cysteine was shown to be a substrate of cystathionine γ-synthase (21). To assess whether, in E. coli, addition of D-cysteine induced threonine starvation, we grew strains K37 and K37ΔyedO in the presence or absence of 0.5 mM L-threonine and in the presence of various concentrations of D-cysteine. Since the growth of the two strains did not respond to the addition of threonine (Table III), we concluded that, under the used culture conditions, D-cysteine does not interfere with the E. coli cystathionine γ-synthase activity.
In E. coli, serine transacetylase converts serine into O-acetylserine. Because a slow β-replacement reaction from O-acetyl-α-serine to D-cysteine was reported to be catalyzed by the desulfhydrase in vitro (5), we wondered whether the yedO gene product could be involved in the in vivo conversion of D-serine into D-cysteine. Such a reaction might account for a part of the toxicity of D-serine (22). For this purpose, we compared the effect of D-serine addition (from 0.1 to 25 mM) on the growth of the yedO mutants and ΔyedO strains in liquid M9-glucose minimal medium. No significant difference on the growth of these strains could be observed, however. An in vivo role of D-cysteine desulfhydrase in D-serine metabolism is therefore unlikely. Indeed, the possibility that O-acetyl-α-serine can ever be produced in vivo seems unlikely in view of the results of studies on the stereospecificity of serine transacetylase from various sources (23, 24).

Finally, we attempted to understand why the toxicity index of D-cysteine measured with K37 was 3-fold greater in the presence of a mixture of 18 amino acids in the growth medium (100 μM) than in the presence of the Ile, Leu, and Val combination (35 μM) (Table III). To identify which amino acid(s) acted in synergy with Ile, Leu, and Val, we systematically added to minimal growth medium containing these three amino acids each of the 17 amino acids except Cys and Met. Out of the 15 amino acids, only L-aspartate (0.4 mM) conferred a significant protective effect (Table III). Addition of L-Asp either to the minimal medium without Ile, Leu, and Val or to medium with 14 amino acids (all but Asp, Cys, Met, Ile, Leu, and Val) confirmed the capacity of this L-amino acid to protect cell growth against D-cysteine (Table III). Interestingly, L-Asp also exerted protection against the toxicity of L-cysteine. Therefore, the conferred protection did not require the simultaneous presence of Ile, Leu, and Val. Such a behavior indicates that aspartate can protect the cell against both D- and L-cysteine at the level of the same enzyme target. As discussed further below, one possible candidate is homoserine dehydrogenase-aspartate kinase.

Utilization of D- and L-Cysteine as Sulfur Sources—D-Cysteine desulfhydrase converts D-cysteine into pyruvate, H₂S, and NH₃. Then hydrogen sulfide can react with O-acetylserine to give L-cysteine. Therefore, participation of the desulfhydrase in cellular sulfur metabolism through degradation of D-cysteine can be imagined.

To study D-cysteine utilization as a sulfur source, we compared the growth of strains K37 and K37ΔyedO at 37 °C in liquid M9-glucose minimal medium supplemented with either 0.1 or 0.3 mM D-cysteine. As a control, L-cysteine was also studied (Fig. 3). Cells were pre-grown to mid-exponential phase in M9-glucose minimal medium and were inoculated at an OD₅₀₀ of 0.001 in the same medium supplemented or not supplemented with different sulfur sources and containing 18 amino acids (all but sulfur-containing Met and Cys). The 18 L-amino acids were added to minimize the inhibitory effect of cysteine on cell growth. After that, cells were left to grow at 37 °C.

Under the above conditions, utilization of L-cysteine as sulfur source was observed. OD₅₀₀ values obtained after 16 h of growth were 2.6 and 3.7 in the presence of 0.1 or 0.3 mM L-cysteine, respectively (Fig. 3). In the presence of 0.5 mM MgSO₄ instead of L-cysteine, the OD₅₀₀ of the two cultures was 4. In the absence of any added sulfur source, the optical densities of the two strains reached a level of ~1, probably owing to sulfur traces in the chemicals used in the experiments.

D-Cysteine could also be utilized as sulfur source by both wild-type and ΔyedO strains (Fig. 3). In the presence of 0.1 mM D-cysteine, the optical density of the K37 culture reached a value of 2 after a 16-h incubation. At the same D-cysteine concentration, the growth of the ΔyedO culture leveled off at an OD₅₀₀ of 1.3, a value close to that measured with the culture without D-amino acid added (OD₅₀₀ = 1). The growth rate of the ΔyedO mutant was improved by increasing the D-cysteine concentration up to 0.3 mM. However, it remained slower than the growth rate of strain K37 (OD₅₀₀ = 1.7) for K37ΔyedO after 16 h of incubation versus 2.6 for K37. Eventually, after 64 h of growth, the culture of the K37ΔyedO strain leveled off at a maximal OD value of 3, therefore showing the utilization of the D-amino acid despite the yedO inactivation. In relation to these results, it should be recalled that a crude cell extract from a ΔyedO strain also contains a weak D-cysteine-dependent H₂S-producing activity, as noted above.

The results in this paragraph strongly suggest that, by degrading D-cysteine in vivo, D-cysteine desulfhydrase contributes to the utilization of this amino acid as sulfur source. Since the substrate specificity of E. coli D-cysteine desulfhydrase in α,β-elimination or β-replacement reactions is broad (1, 5), the enzyme might also participate in the catabolic degradation and metabolic utilization of naturally occurring D-cysteine derivatives.

E. coli Chromosomal Region Encompassing the yedO Gene—The environment of the yedO gene on the E. coli chromosome may also provide clues to the function of this gene. The yedO gene is located downstream from the flfZY motility operon (Fig. 1). The flfA gene encodes the alternative transcription factor rpf that controls the expression of many motility genes. The functions of the flfZ and flfY genes are not definitely established, although the product of the E. coli flfY gene was identified to be a periplasmic cystine-binding protein (25). These two genes are not essential for motility (26). Nevertheless, a study of the S. typhimurium flfA operon, which is
very similar to the *E. coli* operon, indicates that motility is somewhat impaired in the absence of *fliZ* (27).

The yecS and yecC genes, which lie downstream of *yedO*, code for proteins whose functions are still poorly known. Sequence analyses suggest that the yecC gene product corresponds to the ATP-binding subunit of an ABC (ATP-binding cassette) protein (28). The yecS gene codes for a putative transmembrane ABC protein subunit.

In addition, sequence analyses have revealed contiguity of the *fliY*, yecC, and yecS orthologs in the genome of *Helicobacter pylori*, the *fliY* and yecS orthologs are adjacent on the chromosome. It was, therefore, suggested that the *fliY*, yecC, and yecS gene products are associated within a same ABC protein (28). New sequence data add support to this hypothesis (Kyoto Encyclopedia of Genes and Genomes website: www.genome.ad.jp/kegg/ortholog/tab02010.html). Indeed, the *fliY*, yecC and yecS orthologs are also adjacent in the genomes of *Neisseria meningitidis*, *Bacillus subtilis*, *Vibrio cholerae*, and *Bacillus halodurans*. The *fliY* and yecS orthologs are associated in the genomes of *Pseudomonas aeruginosa* and *Deinococcus radiodurans*.

During studies on the transcription initiation of the *E. coli* *fliA* operon, two promoter sites could be identified upstream to the *fliA* gene (26, 27). One of these sequences is recognized by the *fliA*-encoded σ factor, suggesting a positive autoregulation of the gene. A third promoter sequence is located upstream to *fliY*. Therefore, *fliY* transcription might start either upstream to *fliA* or just upstream to *fliY*. Moreover, the RegulonDB (version 3.0) database of the *E. coli* operon organization (29) predicts that the chromosomal region encompassing the *yedO* gene consists of two transcription units, *fliAZY* and *fliY-yecO-yecS-yecC*. At present time, a transcription unit covering all six genes has not been experimentally demonstrated.

The *fliY* gene is known to be induced under sulfate starvation conditions (30). This behavior, together with the chromosomal organization of the *yedO* region on the *E. coli* chromosome, prompted us to search whether *yedO* expression also was sensitive to sulfate starvation. To this end, we measured d-cysteine desulfhydrase activity in a crude extract of strain K37 cultivated until the stationary phase, in M9-glucose minimal medium plus 0.5 mM ethanesulfonate or 0.5 mM MgSO_4_2 as sulfur sources. Measured d-cysteine desulfhydrase activities were 0.033 and 0.0078 units/mg in the presence of ethanesulfonate or MgSO_4, respectively. Such a marked induction of the desulfhydrase activity in response to sulfate starvation conditions suggests the occurrence of a genetic linkage between *fliY* and *yedO*.

**DISCUSSION**

**Comparison of *E. coli* D-Cysteine Desulfhydrase with Related Enzymes**

**Green Alga and Plant D-Cysteine Desulfhydrases—**D-Cysteine desulfhydrase activity was early identified in the green alga *C. fusca* and in the plant *S. oleracea* (2, 3). The enzymes of these two organisms react only with d-cysteine. D-Cysteine derivatives such as d-cystine, d-alloclastathionine, d-lantionine, S-alkyl-, S-aralkyl-, and S-aryl-d-cysteine, d-selenocysteine, and d-selenocystine are not substrates. Functions of these desulfhydrases in cellular metabolism were deduced from the observation that green algae can utilize d-cysteine as the sole sulfur source and that higher plants form hydrogen sulfide upon feeding with d-cysteine (3).

Cyanobacteria can also utilize d-cysteine as their sole sulfur source. From this property, it was suggested that these bacteria contain a d-cysteine desulfhydrase (3). However, no direct experimental evidence has yet confirmed this hypothesis. In addition, no gene related to *yedO* has been found in the *Synechocystis* sp. genome. In contrast, a gene homologous to *yedO* has been recognized in the *A. thaliana* genome.

**ACC Deaminase—**The sequence of *E. coli* d-cysteine desulfhydrase resembles those of the inducible ACC deaminases characterized in several bacteria and fungi isolated from the soil. An ACC deaminase is capable of converting ACC into ammonium and 2-oxobutyrate. Our results indicate that *E. coli* d-cysteine desulfhydrase does not catalyze this reaction. However, the marked sequence homology between the desulfhydrase and an ACC deaminase suggests similarities in the catalytic mechanisms of these enzymes.

**β-Chloro-d-alanine Dehydrochlorinase—**The catalytic properties of d-cysteine desulfhydrase resemble those of the β-chloro-d-alanine dehydrochlorinase (chloride lyase) identified in β-chloro-d-alanine-resistant strains of *Pseudomonas putida* (5, 31). The *P. putida* enzyme uses pyridoxal phosphate as cofactor and catalyzes the α,β-elimination of β-chloro-d-alanine to form pyruvate, NH_3, and HCl. *D*-Cysteine and d-cysteine can also be used as substrates but at slower rates than β-chloro-d-alanine.

A significant role of *E. coli* d-cysteine desulfhydrase in β-chloro-d-alanine detoxification is unlikely. Indeed, the desulfhydrase expression is not induced by β-chloro-d-alanine, and overproduction of the d-cysteine desulfhydrase does not help the bacterium to grow in the presence of β-chloro-d-alanine (1, 5).

**D-Selenocysteine α,β-Lyase—**The enzymatic properties of d-selenocysteine α,β-lyase produced by *Clostridium sticklandii* resemble those of *E. coli* d-cysteine desulfhydrase. It behaves as a pyridoxal phosphate-containing enzyme and, similarly to the *E. coli* d-cysteine desulfhydrase (1), catalyzes elimination reactions with d-selenocysteine as well as d-cysteine and d-cysteine as substrates (32). Because of these similarities, it could be suspected that d-cysteine desulfhydrase and d-selenocysteine α,β-lyase are the same enzyme. However, anti-d-selenocysteine α,β-lyase antibodies do not cross-react with purified *E. coli* d-cysteine desulfhydrase, and anti-desulfhydrase antibodies do not cross-react with purified *C. sticklandii* α,β-lyase (32).

**Inhibition of *E. coli* Growth by d-Cysteine and Role of d-Cysteine Desulfhydrase**

**d-Cysteine Toxicity—**Our results establish that d-cysteine desulfhydrase interferes with the bacterial metabolism through inhibitory mechanisms analogous to those accounting for the toxic effect of L-cysteine. Indeed, growth inhibition induced by L- or D-cysteine appears to be mediated by an inhibition of threonine deaminase, a key enzyme of the isoleucine biosynthesis pathway. Our observation that d-cysteine inhibits the threonine deaminase activity in crude extracts of *E. coli* is in agreement with the earlier report of an *in vitro* inhibition of rat threonine deaminase by d-cysteine (33).

In the present work, we observed a protective effect conferred by L-aspartate against the toxicity accompanying the presence of d- or L-cysteine. This behavior raises the possibility that other enzymes are also inhibited by cysteine. One possible candidate to explain the action of aspartate is homoserine dehydrogenase-aspartate kinase. This bifunctional enzyme catalyzes the conversion of L-aspartate to L-4-aspartyl phosphate and of L-aspartate 4-semialdehyde to homoserine. Homoserine is then used to synthesize methionine, threonine, and isoleucine. L-Aspartate 4-semialdehyde is a precursor in lysine biosynthesis. Both activities of this protein have already been reported to be inhibited by L-cysteine in *vitro* (34). Moreover, L- and D-cysteine are equally effective in the inhibition of *B. subtilis* homoserine dehydrogenase (35). Therefore, we propose that, by increasing the intracellular concentrations of L-aspar-
cysteine is found in malformin A, an antibiotic produced by *Aspergillus niger* (41). Epidermine, a peptide antibiotic produced by *Gram-positive bacteria*, contains D-cysteine resulting from a post-translational modification of C-cysteine yielding a C-sulfhydrated form. In general, little information is available on the occurrence and roles of D-amino acids. However, several enzymes like D-amino acid dehydrogenase, D-amino acid oxidase, D-serine deaminase, and racemases sustain D-amino acid utilization (44).

As shown in Fig. 2, yedO-like genes occur widely in the phylogenetic groups. The distribution is evocative of an expansion through horizontal transfer between species. Nevertheless, analysis of the data is complicated by the close similarity between the sequences of ACC deaminases and that of the *E. coli* desulfhydrase. For instance, in the group of γ-proteobacteria, yedO-like genes producing either a deaminase (*Pseudomonas* and *E. cloacae*) or a desulfhydrase (*E. coli*) coexist. Moreover, in *P. syringae* and *B. mallei*, two distinct yedO-like genes coexist in the same cell. At this time, it cannot be decided whether the respective distributions of the deaminase and desulfhydrase functions result from post-transfer adaptation of a same gene or from mixed transfers of two gene types. Whatever the case, one factor contributing to the selection or expansion of the desulfhydrase function should be or has been the availability of D-cysteine or one of its derivatives. This reconfirms the idea that the living world contains still undiscovered D-cysteine sources.

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Role of d-Cysteine Desulphydrase in the Adaptation of *Escherichia coli* to d-Cysteine
Julie Soutourina, Sylvain Blanquet and Pierre Plateau

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