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Plasmid-Mediated Formaldehyde Resistance in *Escherichia coli*: Characterization of Resistance Gene

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The formaldehyde resistance mechanisms in the formaldehyde-resistant strain *Escherichia coli* VU3695 were investigated. A large (4.6-kb) plasmid DNA fragment encompassing the formaldehyde resistance gene was sequenced. A single 1,107-bp open reading frame encoding a glutathione- and NAD-dependent formaldehyde dehydrogenase was identified and sequenced, and the enzyme was expressed in an in vitro assay and purified. Amino acid sequence homology studies showed 62.4 to 63.2% identity with class III alcohol dehydrogenases isolated from horse, human, and rat livers. We demonstrated that the resistance mechanism in the formaldehyde-resistant strain *E. coli* VU3695 and in other formaldehyde-resistant members of the family *Enterobacteriaceae* is based on the enzymatic degradation of formaldehyde by a formaldehyde dehydrogenase.

Bacterial strains resistant to the disinfectant formaldehyde have been identified among several members of the family *Enterobacteriaceae* and *Pseudomonas* species (5, 7–10, 15, 22, 25). Such resistant strains were isolated from hospitalized patients with infections and from contaminated disinfection solutions. In our studies on the resistance mechanisms it was shown that formaldehyde resistance in strains of the family *Enterobacteriaceae* is plasmid mediated and self-transmissible (15, 25). In *Pseudomonas* strains, however, only chromosomally located formaldehyde resistance has been found up to now (15, 17, 25). We cloned a 4.6-kb DNA fragment from the clinical isolate *Escherichia coli* VU3695 encompassing formaldehyde resistance (17). It was shown by hybridization studies with the cloned fragment with the resistance gene(s) as a probe that DNAs of different formaldehyde-resistant *Serratia marcescens*, *Enterobacter cloacae*, *Citrobacter freundii*, and *Klebsiella pneumoniae* strains hybridize with this DNA probe of *E. coli* (25). This indicates that the resistance genes in *E. coli* and the other formaldehyde-resistant strains of the family *Enterobacteriaceae* have a high degree of homology.

Moreover, high-level glutathione- and NAD-dependent formaldehyde dehydrogenase activity in cell extracts from various formaldehyde-resistant strains of the family *Enterobacteriaceae* has been demonstrated previously (16). In contrast, in cell extracts from formaldehyde-resistant *Pseudomonas* strains, only a little enzyme activity was detected (16). The enzymatic degradation of formaldehyde has been thought to play a key role in resistance to formaldehyde in the *Enterobacteriaceae*. Here we report the characterization of a formaldehyde resistance gene in *E. coli* and other members of the family *Enterobacteriaceae* and the mechanisms responsible for formaldehyde resistance.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The *E. coli* strains and plasmids used in the study were *E. coli* HB101 (20), *E. coli* XL-Blue (Stratagene), *E. coli* DH5α (Gibco BRL), and pCl40 (16). pCl40 was revealed by cloning a large (4.6-kb) *Bam*HI fragment from wild-type *E. coli* plasmid pVU3695 on which the formaldehyde resistance gene(s) was located in pBR322. This recombinant plasmid was transformed in *E. coli* HB101 and was selected for formaldehyde resistance. The formaldehyde-resistant transformants were designated *E. coli* Cl40. The vectors M13mp19 (Stratagene), pBluescript II SK$^+$ (Stratagene), and pMal-C2 (New England Biolabs) were used for the cloning experiments.

E. coli B was obtained from Gutheil (Boston, Mass.) (6). The bacteria were grown at 36°C in Luria-Bertani (LB) broth or on LB agar. When necessary, the medium was supplemented with ampicillin (100 μg/ml). For formaldehyde resistance testing, Trypticase soy broth or agar was supplemented with 0.02% formaldehyde (Merck, Darmstadt, Germany).

DNA isolation and cloning. Plasmid DNA was isolated as described by Birnboim and Doly (2). For large-scale preparation we used a modified method that we have described previously (25). Cloning was performed as described by Maniatis et al. (20). The *E. coli* strains were transformed by the CaCl procedure (18).

Nucleotide sequence analysis. Deletion subclones for sequencing were constructed by DNase I treatment (19). Single-stranded M13 DNA was sequenced by the dideoxy chain termination method with the *Taq* Dye Primer Cycle Sequencing Kit and the 373 A DNA Sequencer (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Nucleotide and derived amino acid sequence analysis as well as sequence homology studies were carried out by using the DNA Star program (DNA STAR Inc., Madison, Wis.).

DNA amplification. DNA amplification was performed by PCR as described previously (3). For amplification of the sequenced 1,107-bp DNA fragment, a sense primer with an *Eco*RI restriction site (CTCGAATTC ATGAAATCA) and an antisense primer with an *Hin*dIII restriction site (CTCAAGCTTTCA GTA GTGGAT) were used (underscoring indicates the restriction sites).

Expression of recombinant proteins and measurement of enzyme activity. The amplified DNA fragment of 1,107 bp was cloned into pMal. After transformation into *E. coli* DH5α, expression was induced by the addition of isopropyl-β-D-galactopyranoside to a final concentration of 0.3 mM; this was followed by incubation for 2 h at 37°C. The cells were chilled on ice and were centrifuged at 8,000 × *g* for 10 min. Preparation of cell extracts and measurement of formaldehyde dehydrogenase activity were performed as described previously (17). Fusion proteins were separated by affinity chromatography with maltose-binding protein–agarose (New England Biolabs), as recommended by the manufacturer.

Nucleotide sequence accession number. The described sequence was assigned accession number X73835 (EMBL Data Library Heidelberg, Germany).

RESULTS

Sequencing of the resistance gene. For detailed characterization of formaldehyde resistance, a 4.6-kb *Bam*HI fragment encompassing the resistance gene(s) from the formaldehyde-resistant *E. coli* Cl40 was analyzed. Since attempts to subclone this fragment were not successful, the whole 4.6-kb *Bam*HI fragment was sequenced (data not shown). Analysis of the sequence of this large fragment revealed only one complete open reading frame (ORF). This ORF consists of 1,107 bp and codes for 369 amino acids (Fig. 1).

Expression of gene products. For expression of the gene products, the sequenced 1,107-bp fragment of the formalde-

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ATG AAA TCA CGT GCA GCT GTA GCA TTT GCT CCT GGT AAG CCC CTC
 M   K   S   R   A   A   V   A   F   A   P   G   K   P   L

GAG ATC GTT GAA TTG GAT GTG GAG CCG CCT CGT AAG GGT GAA GTA
 E   I   V   E   I   D   V   E   P   P   R   K   G   E   V

CTG GTA AAA ATC ACC CAT ACC GGC GTC TGC CAC ACT GAT GCA TTT
 L   V   K   I   T   H   T   G   V   C   H   T   D   A   F

ACC TTG TCC GGT GAT GAT CCG GAA GGT GTG TTC CCG GCA GTA CTG
 T   L   S   G   G   D   D   P   E   G   V   F   P   A   V

GGT CAT GAA GGT GCG GGT GTT GTT GTG GAA GTC GGC GAA GGG GTC
 L   G   H   E   G   A   G   V   V   V   E   V   G   E   G

ACC AGT GTG AAA CCT GGC GAT CAT GTT ATT CCG CTT TAC ACG GCA
 V   T   S   V   K   P   G   D   H   V   I   P   L   Y   T

GAA TGC GGC GAG TGT CGT TTC TGT AAA TCC GGA AAA ACT AAC CTG
 A   E   C   G   E   C   L   F   C   K   S   G   K   T   N

TGT GCT TCT GTT CGC GCC ACC CAG GGT AAA GGA CTT ATG CCT GAT
 L   C   V   S   V   R   A   T   Q   G   K   G   L   M   P

GGC ACG ACC CGT TTC TCT TAC AAG GGC CAG CCT CTT TTC CAC TAT
 D   G   T   T   R   F   S   Y   K   G   Q   P   L   F   H

ATG GGC TGC TCT ACA TTC AGT GAG TAT ACC GTC GTC GCA GAA GTG
 Y   M   G   C   S   T   F   S   E   Y   T   V   V   A   E

TCT TTA GCC AAA ATT AAT CCA CAG GCG AAT CAT GAA CAT GTC TGT
 V   S   L   A   K   I   N   P   Q   A   N   H   E   H   V

CTG CTG GGT TGT GGC GTA ACG ACA GGT ATC GGT GCG GTT CAC AAC
 C   L   L   G   C   G   V   T   T   G   I   G   A   V   H

ACT GCG AAA GTA CAG CCA GGT GAC ACG GTT GCT ATT TTT GGC CTG
 N   T   A   K   V   Q   P   G   D   T   V   A   I   F   G

GGT GGT ATT GGC CTT GCA GCG GTA CAG GGC GCG CGT CAG GCA AAA
 L   G   G   I   G   L   A   A   V   Q   G   A   R   Q   A

GCG GGT CGT ATC TTT GCC ATC GAT ACC AAT CCG GAG AAA TTT GAC
 K   A   G   R   I   F   A   I   D   T   N   P   E   K   F

CTG GCC CGT TCA TTT GGT GCA ACT GAC TGC ATT AAC CCG AAA GAC
 D   L   A   R   S   F   G   A   T   D   C   I   N   P   K

TAC GAC AAA CCT ATC CAG GAC GTT CTC ATT GAA CTG ACA GGC TGG
 D   Y   D   K   P   I   Q   D   V   L   I   E   L   T   G

GGT GTC GAC CAT ACA TTT GAA TGT ATC GGT AAC GTA AAC GTA ATG
 W   G   V   D   H   T   F   E   C   I   G   N   V   N   V

CGT TCT GCA CTT GAA GCG GCA CAC CGT GGC TGG GGT CAG TCC GTC
 M   R   S   A   L   E   A   A   H   R   G   W   G   Q   S

GTT ATC GGT GTT GCT GGC GCA GGT AAA GAA ATC TCC ATC CGT CCA
 V   V   I   G   V   A   G   A   G   K   E   I   S   T   R

TTC CAG CTG GTC ACC GGC CGT GTA TGG AAG GGT TCT GCC TTT GGT
 P   F   Q   L   V   T   G   R   V   W   K   G   S   A   F

GGT GTT AAA GGT CGT ACC CAG CTG CCG GGC ATG GTT GAA GAT GCG
 G   G   V   K   G   R   T   Q   L   P   G   M   V   E   D

ATG AAA GGC GAA ATC GAT CTC GCA CCA TTC GTG ACT CAT ACC ATG
 A   M   K   G   E   I   D   L   A   P   F   V   T   H   T

GGC CTT GAA GAG ATT AAT GAC GCC TTC GAC CTG ATG CAT GAA GGC
 M   G   L   E   E   I   N   D   A   F   D   L   M   H   E

AAA TCT ATT CGT ACA GTG ATC CAC TAC TGA
 G   K   S   I   R   T   V   I   H   Y
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FIG. 1. Enzyme sequence in 5′-3′ direction (1,110 bases, 369 amino acids).

hyde-resistant strain *E. coli* Cl40 was amplified, cloned into the expression vector pMal, and transformed into *E. coli* DH5α. The transformants were designated *E. coli* pMal1107. Resistance testing of the transformants showed that all were susceptible to formaldehyde. No growth was observed on Trypticase soy agar plates supplemented with 0.01% formaldehyde. However, after lysis of the cells it was possible to demonstrate in all cell extracts of the transformants glutathione-dependent formaldehyde dehydrogenase with an average specific enzyme activity of 8.7 U/mg of protein. In contrast, in cell extracts of *E. coli* DH5α, which harbored only the vector plasmid pMal, no enzyme activity was found.

Analysis of the expressed cell proteins of *E. coli* pMal is presented in Fig. 2. Lane 1 shows the maltose-binding protein with a band of 42 kDa. For the cloned strain pMal1107, one main band of 87 kDa is shown in the sodium dodecyl sulfate (SDS)-polyacrylamide gels (lane 2). This band represents a fusion protein (maltose-binding protein plus the protein corresponding to the 1.1-kb fragment).

The fusion protein was purified by affinity chromatography. SDS-polyacrylamide gel electrophoresis (PAGE) after separation showed one band of 87 kDa (Fig. 2). This purified fusion protein was tested in the enzyme assay and revealed a specific formaldehyde dehydrogenase activity of 30.9 U/mg of protein.

Homology studies. Since it was demonstrated that the resistance gene(s) codes for an enzyme with formaldehyde dehydrogenase activity, the sequence of the ORF and the corresponding amino acid sequence were analyzed for their identities with published sequences from other formaldehyde dehydrogenases. High degrees of identity were found only with four class III alcohol dehydrogenases, from livers of rats

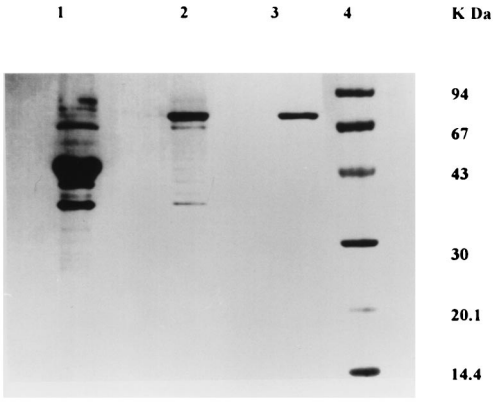

FIG. 2. SDS-PAGE of expressed cell proteins of *E. coli* pMal strains. Lane 1, expressed proteins of the pMal vector strain; lane 2, expressed fusion protein of cloned strain pMal1107; lane 3, purified fusion protein of strain pMal1107; lane 4, molecular mass markers (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin).

(63.2%) (12), humans (62.7%) (4, 13, 23), and horses (62.4%) (14).

DISCUSSION

Resistance against the disinfectant formaldehyde seems to be a phenomenom that is found most often in gram-negative bacteria like members of the family *Enterobacteriaceae* and *Pseudomonas* species (5, 7–10, 15, 22, 25). We could demonstrate plasmid-mediated formaldehyde resistance in a clinical *E. coli* isolate designated strain VU3695 (15). Restriction analysis studies and cloning experiments with the resistance plasmid pVU3695 revealed that the formaldehyde resistance gene(s) is located on a 4.6-kb *Bam*HI fragment (17). To characterize the formaldehyde resistance in more detail, the resistance gene(s) should be sequenced. Since attempts to subclone this 4.6-kb fragment were at first not successful, the whole 4.6-kb fragment was sequenced. Then the sequence was analyzed for ORFs. Only one complete frame of 1,107-bp length encoding 369 amino acids was found. Moreover, a second unfinished ORF consisting of 1,001 bp in the 5′ to 3′ direction was found; it ends at a *Bam*HI restriction site and therefore it is not relevant for formaldehyde resistance.

Since we have demonstrated formaldehyde dehydrogenase activities in cell extracts of formaldehyde-resistant *E. coli* VU3695 and cell extracts of other formaldehyde-resistant strains, *S. marcescens*, *C. freundii*, *E. cloacae*, and *K. pneumoniae* (16), we supposed that the resistance gene in VU3695 encodes a formaldehyde dehydrogenase. For that reason the 1,107-bp fragment that was sequenced was amplified by PCR, cloned into the expression vector pMal, and transformed into *E. coli* DH5α (*E. coli* pMal1107). All transformants were formaldehyde susceptible but glutathione-dependent formaldehyde dehydrogenase activity could be demonstrated in all cell extracts. In contrast, transformants which harbored only the vector pMal without the 1,107-bp insert showed no enzyme activity. As shown in Fig. 2, SDS-PAGE of the cell extract of *E. coli* pMal1107 revealed a main band of 87 kDa resembling the fusion protein. After purification the fusion protein showed highly specific formaldehyde dehydrogenase activity. This indicates that this gene encodes a glutathione-dependent formaldehyde dehydrogenase.

Since the maltose-binding protein has a size of 42 kDa, one can calculate the size of the enzyme (45 kDa). This is in agreement with the size of a typical protein band detected in all formaldehyde-resistant *Enterobacteriaceae* strains tested and in our cloning experiments with *E. coli* VU3695 (17). By using an average molecular mass of 126.7 Da per amino acid, the sequenced 369 amino acids revealed a protein of nearly 46 kDa.

The NAD-dependent enzyme formaldehyde dehydrogenase (EC 1.2.1.1) which oxidizes formaldehyde appears to be universally present in animal cells, in some plant cells, in methanol-utilizing yeasts, and in some methanol-utilizing methylotrophic bacteria like *Pseudomonas methanica*, *Pseudomonas extorquens*, *Protaminobacter* species, and some other formaldehyde-utilizing bacteria like *Pseudomonas aeruginosa*, *Pseudomonas putida*, and one strain of *E. coli* (for a review, see reference 24). Formaldehyde dehydrogenase belongs to a group of enzymes that specifically require glutathione for function. This glutathione-dependent formaldehyde dehydrogenase must be distinguished from a different glutathione-independent enzyme also termed formaldehyde dehydrogenase (EC 1.2.1.46), but this enzyme appears to have limited distribution. It has only been described for some bacteria and has been purified from *P. putida* (1, 11). The glutathione-dependent formaldehyde dehydrogenase catalyzes the reversible formation of *S*-formylglutathione and NADH from formaldehyde, glutathione, and NAD. The enzyme has been purified from several sources, including human and rat liver, yeasts, and bacteria (6, 24). All investigated enzymes have a molecular mass of about 80,000 Da and are dimers consisting of two similar units. All belong to the so-called class III alcohol dehydrogenases and are quite similar (6, 24). Therefore, we compared the sequences of the 369 amino acids of *E. coli* VU3695 sequenced in the present study with the amino acid sequences of other published formaldehyde dehydrogenases. The studies revealed a high degree of identity (62.4 to 63.2%) to class III alcohol dehydrogenases isolated from a horse, a human, and rat liver (4, 12–14, 23), but not with bacterial enzymes published up to now.

Gutheil et al. (6) also demonstrated a glutathione-dependent formaldehyde dehydrogenase in an *E. coli* strain (*E. coli* B), but the specific enzyme activity in the crude cell extract was 6,500-fold lower than the specific enzyme activity from *E. coli* VU3695 (16). Purification and characterization of the enzyme extracted from *E. coli* B revealed a class III alcohol dehydrogenase. The complete sequence is not available. The published N-terminal sequence of this enzyme (47 amino acids) showed a high degree of similarity to the N-terminal sequence of our strain (6); both differ by only six amino acids. The following are the N-terminal amino acid sequences of the formaldehyde dehydrogenases from *E. coli* VU3695 and the formaldehyde-resistant strain *E. coli* B, respectively (the underscored amino acids are those that are different): MKSRAAVAFAPG KPLEIVEIDVEPPRKGEVLV KITHTGVCHTDAFTL and MKSRAAVAFAPGKPLEIVEIDV<u>A</u>PX<u>K</u>KGEVLI K<u>V</u>THT GVC<u>E</u>TDAF<u>G</u>L. Interestingly, *E. coli* B was formaldehyde susceptible, whereas *E. coli* VU3695 and the cloned variant *E. coli* Cl40 were highly resistant to formaldehyde.

These data indicate that resistance to formaldehyde in *E. coli* VU3695 depends on the function of a highly active glutathione-dependent formaldehyde dehydrogenase which inactivates formaldehyde. To protect the resistant bacteria against formaldehyde, it is necessary that the enzyme work outside the cytoplasm. This requires transport of the enzyme through the cytoplasmic membrane. In the case of the formaldehyde-susceptible strain *E. coli* Mal1107, in cell extracts of which high levels of formaldehyde dehydrogenase activity could be demonstrated, transport of the fusion protein through the membrane is blocked, and therefore, the resistance phenotype is not completely developed.

Colony blot hybridization studies with other formaldehyde-resistant members of the family *Enterobacteriaceae* by using the 4.6-kb fragment with the resistance gene from *E. coli* VU3695 as a DNA probe showed hybridization with all other formaldehyde-resistant *Enterobacteriaceae* strains tested (25). All these resistant strains showed glutathione-dependent formaldehyde dehydrogenase activity. This indicates that the resistance mechanisms in resistant *Enterobacteriaceae* strains are the same and that the genes are probably identical.

In contrast, hybridization studies with the same DNA probe with formaldehyde-resistant *P. aeruginosa* strains indicates that the resistance genes and resistance mechanisms in *P. aeruginosa* are probably quite different from those in the *Enterobacteriaceae* (25).

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