Construction of an Expression and Site-Directed Mutagenesis System of Haloalkane Dehalogenase in *Escherichia coli*

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Haloalkane dehalogenase from *Xanthobacter autotrophicus* was efficiently expressed in *Escherichia coli* BL21(DE3) and *E. coli* JM101. After introduction of restriction sites by PCR the haloalkane dehalogenase gene (*dhlA*) was translationally fused behind the T7 ($\phi 10$), *trc*, and *tac* promoters. This resulted in expression at 30°C up to 38 and 18% of the total soluble cellular protein with the T7 and *trc* promoters, respectively. Dehalogenase expression under control of the *tac* promoter was below 1% of the soluble cell protein, however. Aggregation of haloalkane dehalogenase into inclusion bodies was found during growth at 37°C but not at 30°C. Aggregates were also formed from intact enzyme upon incubation at 37°C of cells or crude extracts containing active mature dehalogenase. The high level of expression resulted in a short purification procedure in which 30–35 mg highly enriched haloalkane dehalogenase was obtained from an 0.5 l culture. For the production of single-stranded DNA an f1(+) origin was introduced in the T7 expression system. © 1993 Academic Press, Inc.

Haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10 catalyzes the hydrolytic conversion of chloroalkanes to alcohols (1). The gene, *dhlA*, was cloned and sequenced (2) and the X-ray structure of the enzyme is known (3). The 35-kDa protein consists of two domains: a cap domain on top of an α/β hydrolase fold domain (4). Between these, there is a hydrophobic cavity, which is the putative active site (3). The haloalkane dehalogenase belongs to a group of hydrolytic enzymes that have a similar α/β hydrolase fold and a con-

served arrangement of the catalytic triad (4). The enzyme is involved in the bacterial detoxification of 1,2-dichloroethane and other chlorinated aliphatic compounds. The enzyme has a rather high K_m (0.68 mM) and low k_{cat} (6 s⁻¹) and the substrate range is limited, however (1). This makes the haloalkane dehalogenase an interesting target for protein engineering toward enzymes with improved activity and broadened or altered substrate specificity.

Although the *dhlA* gene was isolated in *X. autotrophicus*, it can be well expressed in *Escherichia coli* (2). The original clone is a large (31 kb) pLAFR1 derivative (5) with a low copy number and is not suitable for protein engineering. Attempts to clone the *dhlA* gene with its own promoter sequence in more suitable high- and mid-copy number vectors such as pUC19 and pBR322 failed, probably due to lethal overexpression of the gene product (2).

In order to obtain inducible high-level expression of the *dhlA* gene, we decided to clone the gene behind three different promoters: the T7 ($\phi 10$) promoter (plasmid pET-3d, (6)); the *trc* promoter (plasmid pKK233-2, (10)); and the *tac* promoter (plasmid pTTQ18, (11)). The T7 expression system developed by Studier *et al.* (6) is widely used to express proteins (7–9) and uses the strong $\phi 10$ promoter of bacteriophage T7. Both the *tac*- and *trc* promoters are hybrid *trp-lac* promoters which are also used for efficient expression of various proteins in *E. coli* (10–13).

One of the major problems in the expression of foreign proteins in *E. coli* is the aggregation into an inactive form in insoluble inclusion bodies (14,15). Inclusion bodies are considered to be products of incorrect folding pathways of the expressed protein rather than being derived from mature or completely unfolded protein. Their formation seems to be strongly dependent on environmental conditions (14,15).

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FIG. 1. Construction of haloalkane dehalogenase expression vectors. (A) Construction of pGELAF+. The vector contains the origin of replication of pBR322, the *dhIA* gene translationally fused behind the T7 promoter of pET-3d, and the f1(+) origin of pGEM7+. (B) pKLA is pKK233-2 with the *dhIA* gene translationally fused behind the *trc* promoter. (C) pPJ113 is pTTQ18 with the *dhIA* gene translationally fused behind the *tac* promoter. Inactivation of the *lac*^a gene was done by deletion of an internal part of the gene which resulted in pPJ113Δ. Abbreviations used: A, *Apa*I; B, *Bgl*II; Ba, *Bam*HI; E, *Eco*RV; EI, *Eco*RI; H, *Hind*III; N, *Nco*I; S, *Sal*I; Sc, *Sca*I. The β-lactamase, the haloalkane dehalogenase, and *lac*I^a genes are indicated as Ap^r, *dhIA*, and *lac*I^a, respectively. The promoters are indicated as open triangles and by T7, *trc*, and *tac*. The transcription terminators are indicated by T, the origins of replication by ori, and the f1(+) origin by f1(+).

FIG. 1—Continued

We report here the construction of expression- and site-directed mutagenesis vectors for haloalkane dehalogenase in *E. coli* and present an optimized purification protocol for the enzyme. We also describe the formation of inclusion bodies at 37°C which occurs both during synthesis and from preformed native enzyme, suggesting an additional route for their formation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

E. coli strain BL21(DE3) (F^- *ompT* r_b^- m_b^- λ DE3), a strain expressing the T7 RNA polymerase of bacteriophage T7, was obtained from F. W. Studier (6) and strains JM101 (*thi*, Δ (*lac-proAB*), [F' , *traD36*, *proAB*, *lacI*^q Δ M15]) and HB101 (F^- , *hsdS20*(r_b^- , m_b^-), *supE44*, *recA13*, *ara14*, *proA2*, *rpsL20*, (*str*^r), *xyl-5*, *mlt-5*, *supE44*, λ^-) from Promega. Plasmid pET-3d was obtained from F. W. Studier (6), plasmid pGEM7 from Promega, plasmid pBR322 (16) from Boehringer-Mannheim, plasmid pKK233-2 (10) from Pharmacia, and plasmid pTTQ18 (11) from Amersham.

LB medium was used in all experiments (17) and supplemented when appropriate with 100 μ g/ml ampicillin. LBi medium, used for pH indicator plates, is solid LB medium (17) supplemented with 80 mg/liter bromothymolblue (Merck) and adjusted to pH 7.5. LBz medium used for qualitative dehalogenase activity determination was solid LB medium (17) without NaCl.

Construction of Expression and Mutagenesis Vectors

In order to obtain the *dhlA* gene under control of the T7, *trc* or *tac* promoters, restriction sites for translational fusion were introduced by the polymerase chain reaction (PCR), followed by ligation behind the different promoters (Fig. 1).

Construction of pGELAF+. Expression and site-directed mutagenesis vector pGELAF+ was constructed according to Fig. 1A. *NcoI* restriction sites in the ATG start codon and downstream of the dehalogenase gene were introduced with PCR (Hybaid thermoreactor), using the standard amplification protocol as described by Innis and Gelfand (18), except that primer annealing was done at 40°C instead of at 55°C. The primers used were pNcoI (5'-GAGGCTCCATGGTAAATGCAAT-3', start codon in bold) and p90B032 (5'-ATAGAATTCCATGGATCCTCAGTTTTCGTACCGGCACCGG-3', *NcoI* sites underlined). The PCR product was digested with *NcoI* and ligated into *NcoI* restricted T7 expression vector pET-3d, resulting in pELA. After testing the expression of pELA (see below), an *f1*(+) origin for the production of single-strand DNA was introduced by ligation of the *SalI*-*EcoRV* fragment of pELA into *XhoI*/*SmaI*-digested pGEM7+, resulting in pGELA+. This construct, however, was stable in *E. coli*

JM101 but not in *E. coli* BL21(DE3). After exchanging the origin of replication of pGELA+ with that of pBR322, the resulting construct (pGELALOW+) was stable in both *E. coli* JM101 and BL21(DE3). The exchange was done by ligating the *ScaI*-*HindIII* fragment (filled in with Klenow) of pGELA+ in *ScaI*-*PvuII*-digested pBR322. In order to prevent the presence of direct repeats, the extra T7 promoter originally present in pGEM7+ was removed by cutting pGELALOW+ with *PvuII* and *BglII*, filling in with Klenow, and self ligation. This resulted in pGELAF+.

Construction of pKLA. Expression vector pKLA was constructed as depicted in Fig. 1B. The same PCR product as used for the construction of pELA (Fig. 1A) was ligated into *NcoI*-digested *trc* expression vector pKK233-2, resulting in pKLA.

Construction of pPJ113. *dhlA* DNA was PCR amplified with primer pEcoRI (5'-ATGATGAATTCCA-TTCGCACCCCG-3', start codon in bold and *EcoRI* restriction site underlined) and primer p90B032 (5'-ATAGAATTCCATGGATCCTCAGTTTTCGTACCGGCACCGG-3', *BamHI* restriction site underlined). After restriction with *EcoRI* and *BamHI*, the product was ligated into *EcoRI*/*BamHI*-digested expression vector pTTQ18, which resulted in pPJ113 (Fig. 1C). Inactivation of the *lacI*^q gene of pPJ113 was done by deleting part of the gene. This was done by restriction of pPJ113 with *EcoRV* and *ApaI* followed by treatment with Mungbean Nuclease and self-ligation.

PCR-amplified parts of all constructs (pGELAF+, pKLA, and pPJ113) were sequenced (19) to ensure the absence of errors introduced by enzymatic amplification.

Protein Expression and Purification

Expression of pGELAF+. *E. coli* BL21(DE3) was transformed with enzyme expression vector pGELAF+, plated out on LB plates containing ampicillin (100 μ g/ml), and incubated overnight at 37°C. The next day, transformants were collected from the plates by resuspending in 1 ml LB medium, which was used to inoculate a culture of 10 ml LB containing 100 μ g/ml ampicillin at an OD₆₀₀ of 0.06–0.08. The culture was incubated at 30°C until it reached an OD₆₀₀ of 1 after which it was induced with 0.4 mM IPTG and cultivated for another 2.5 h at 30°C. Uninduced cultures were treated identically without the addition of IPTG.

Expression of pKLA and pPJ113. An overnight culture of *E. coli* JM101(pKLA) or JM101(pPJ113) was inoculated into 10 ml LB medium containing ampicillin (100 μ g/ml) to an OD₆₀₀ of 0.06–0.08 and cultivated at 30°C. The culture was induced with 0.4 mM of IPTG when it reached an OD₆₀₀ of approximately 1 and incubated further for 3 h at 30°C. Uninduced cultures were treated identically without the addition of IPTG.

Cells were harvested by centrifugation (10 min at 6500g), washed with 10 ml of TEM buffer (10 mM Tris-sulfate buffer, pH 7.5, 1 mM EDTA, and 1 mM β -mercaptoethanol) and resuspended in 1 ml TEM. The cell suspension was sonicated three times 5 s (Vibra-cell, using a microtip) followed by removal of the cell debris by centrifugation (20 min at 12,000g).

Purification of haloalkane dehalogenase. A preculture of 50 ml of *E. coli* BL21(DE3)(pGELAF+) (see above) was incubated at 30°C until it reached an OD₆₀₀ of approximately 1. The culture was diluted into 500 ml LB medium containing 100 μ g/ml ampicillin which resulted in a starting culture with an OD₆₀₀ of about 0.1. This culture was cultivated at 30°C until it reached an OD₆₀₀ of 1 and then induced by adding 0.4 mM IPTG. After 3 h the cells were harvested by centrifugation (10 min at 6500g), washed with 500 ml TEM, and resuspended in 20 ml of TEM. All further steps were carried out at 0–4°C to protect the enzyme against inactivation.

The crude extract was prepared by sonication of the cell suspension (Vibra-cell, standard tip, 20 times for 10 s), followed by centrifugation (20 min at 30,000g) to remove unbroken cells and debris. An extra centrifugation step (3.5 h at 50,000 rpm in a 70 Ti rotor (Beckman)) was added to remove all the cell debris. The supernatant was applied to a DEAE-cellulose column (DE52 from Whatman, 2.5 by 8 cm) which was equilibrated with TEM. The column was washed with 1 column volume of TEM and eluted with a linear gradient of 0 to 1 M ammonium sulfate in TEM (total volume, 400 ml; flow rate, 70 ml/h; fraction volume, 5 ml). Active dehalogenase eluted at an ammonium sulfate concentration of 0.28 to 0.36 M. These fractions were pooled and dialyzed overnight against PEM buffer (5 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA, and 1 mM of β -mercaptoethanol). The dialysate was loaded on a hydroxylapatite column (Bio-Gel HTP from Biorad, 2.5 by 8 cm) which was equilibrated with PEM. The enzyme was eluted with a linear gradient of 5 to 100 mM potassium phosphate in PEM (total volume, 300 ml; flow, 30 ml/h; fraction volume, 7 ml). Fractions with highly enriched haloalkane dehalogenase eluted from 31 to 44 mM potassium phosphate.

Dehalogenase activities and protein analysis. Quantitative haloalkane dehalogenase assays were routinely carried out by incubating appropriate amounts of enzyme solution with 5 mM of substrate (1,2-dichloroethane) in 50 mM Tris-sulfate buffer, pH 8.2, at 30°C. Halide liberation was followed colorimetrically (1). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μ mol of halide per minute (1).

The K_m of haloalkane dehalogenase for 1,2-dichloroethane was determined by incubation of 0.1 U enzyme with different concentrations of substrate in the range of 0.1 to 4.0 mM. The samples were incubated for 15 min

at 30°C in gas-tight tubes and extracted with 1/3 vol of diethylether containing 0.05 mM 1-bromohexane as the internal standard. The samples were analyzed for 2-chloroethanol production by injection on a Chrompack 438S gas chromatograph equipped with a CPWax 52 CB column, a flame ionization detector, and an integrator. The carrier gas was 60 kPa nitrogen and the temperature program was 3 min isothermal at 45°C followed by an increase to 200°C at 10°C/min. The data were fitted with the nonlinear regression data analysis program Enzfitter, version 1.05, from Elsevier-Biosoft (20).

For qualitative dehalogenase tests, cells were grown on solid LBz medium from which a small amount of cells was incubated in a 100- μ l solution of 5 mM of substrate in 50 mM Tris-sulfate (pH 8.2). After incubation for 60 min at 30°C, 0.1 ml of 0.25 M $\text{NH}_4\text{Fe}(\text{SO}_4)_2$ in 6 M HNO_3 was added, followed by a drop of saturated $\text{Hg}(\text{SCN})_2$ in ethanol. A red color indicated the presence of dehalogenase activity. Another qualitative dehalogenase test was performed on LBi plates. Cells grown on these plates (usually overnight) were incubated for 15–30 min with substrate (15 μ l on a small filter in the lid of the petri dish). Colonies which turned from blue/green to yellow had dehalogenase activity.

Protein determination was carried out with the method of Bradford (21) with bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (22) on gels containing 12.5% polyacrylamide. Samples contained 10 μ g of protein. Molecular weight markers (designated as m) were obtained from Pharmacia and contained the following proteins: phosphorylase b, M_r 94 kDa; bovine serum albumin, M_r 67 kDa; ovalbumin, M_r 43 kDa; supplemented in Figs. 2, 3, and 5 with haloalkane dehalogenase, M_r 35 kDa; carbonic anhydrase, M_r 30 kDa; soybean trypsin inhibitor, M_r 20 kDa and α -lactalbumin, M_r 14 kDa. Gels were stained with Coomassie brilliant blue.

For protein analysis of whole cells, cultures were centrifuged, resuspended in 1 $\times$ loading buffer (17), boiled for 5 min, and subjected to SDS-PAGE. The amount of cells to be applied was calculated from the OD₆₀₀. It was assumed that a culture with an OD₆₀₀ of 1 corresponds with approximately 0.150 mg cells/ml and that a cell consist of $\pm$ 50% of protein.

To determine the amount of dehalogenase in cell-free extracts, samples were subjected to SDS-PAGE. Dry stained gels were scanned with a Geniscan Grayscale scanner, using the iPhoto Deluxe program of U-lead Systems, Inc. The scanned gels were analyzed with the program Quantiscan, version 1.31 from Microsoft Systems, Biosoft.

RESULTS

Expression of haloalkane dehalogenase under control of the T7, trc, and tac promoters. The highest expression

FIG. 2. SDS-PAGE of cell-free extracts prepared from strains carrying the different expression vectors. Lane 1, pGELAF+ uninduced; lane 2, pGELAF+ induced; lane 3, pKLA uninduced; lane 4, pKLA induced; lane 5, pPJ113 uninduced; lane 6, pPJ113 induced.

of haloalkane dehalogenase was obtained with the T7 promoter in construct pGELAF+, using *E. coli* BL21(DE3) as the host (Fig. 2, Table 1). Haloalkane dehalogenase was produced up to 38% of the total soluble cell protein (Fig. 2, lane 2). pGELAF+ is a pET-3d derivative with the haloalkane dehalogenase under control of the T7 ($\phi 10$) promoter of bacteriophage T7 and an *f1*(+) origin for the production of single-stranded DNA.

Construct pGELA+ (see Fig. 1A), which also carries the *dhlA* gene and the T7 promoter, could be transformed to *E. coli* strain JM101 but not to strain BL21(DE3). This is probably caused by expression of haloalkane dehalogenase up to lethal levels in strain BL21(DE3) due to the elevated copy number in pGELA+ which was obtained by cloning the T7 expression cassette into pGEM7+. After reducing the copy number to that of pBR322 (mid-copy) the resulting construct (pGELALOW+) could be transformed to and was stable in both *E. coli* JM101 and BL21(DE3).

Even in uninduced cells there was significant expression of haloalkane dehalogenase with pGELAF+ in *E. coli* BL21(DE3) (Fig. 2, lane 1). This is due to the basal

TABLE 1

Specific Activities of Produced Haloalkane Dehalogenase in Cell-Free Extracts of *E. coli* Containing the Different Constructs

	Uninduced (U/mg protein)	Induced (U/mg protein)
pGELAF+	1.79	2.12
pKLA	0.37	0.81
pPJ113	0.08	0.11

TABLE 2

Summary of Haloalkane Dehalogenase Purification from a 0.5-Liter Culture of *E. coli* BL21(DE3)(pGELAF+)

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification factor
Crude extract	169	367	2.17	100	
DEAE-cellulose	72	295	4.10	80	2.0
Hydroxylapatite	34	174	5.12	47	2.4

level of T7 RNA polymerase present in *E. coli* strain BL21(DE3) (6). pGELAF+ produces good single-strand DNA for Kunkel mutagenesis. After a mutagenesis round, transformants plated out on LBI indicator plates can directly be screened for the presence of dehalogenase activity, thereby making it easy to screen large numbers of transformants in a short time.

The construct pKLA carrying the *dhlA* gene under control of the *trc* promoter gives good expression of dehalogenase in *E. coli* JM101 (Fig. 2, lane 4). pKLA is similar to pELA (compare Figs. 1A and 1B) since both are translational fusions constructed by introducing a *Nco*I restriction site for fusion into the ATG of *dhlA* gene. This resulted in a N-terminal sequence of MVNAIR instead of MINAIR (wild type). From the literature it is known that the T7 promoter is stronger than the *trc* promoter (6), which is also visible in Fig. 2 (compare lanes 2 and 4) and Table 1. Uninduced pKLA also gives some haloalkane dehalogenase expression.

Plasmid pPJ113, a vector with the haloalkane dehalogenase under control of the *tac* promoter, gave poor expression of haloalkane dehalogenase in *E. coli* JM101

FIG. 3. Aggregation of haloalkane dehalogenase. SDS-PAGE of whole-cell lysates and cell-free extracts of IPTG-induced *E. coli* BL21(DE3)(pGELAF+) cultivated at 30 and 37°C. Lane 1, whole-cell lysate at 30°C; lane 2, cell-free extract at 30°C; lane 3, whole-cell lysate at 37°C; and lane 4, cell-free extract at 37°C.

FIG. 4. Phase-contrast light micrographs of *E. coli* BL21(DE3)(pGELAF+) showing formation of inclusion bodies at 37°C. A 50-ml culture was induced for 2 h at 30°C and divided in two parts. One part was switched to 37°C and cultivated further for 2 h (panel A, see also Fig. 5) the other part of the culture was further incubated at 30°C for 2 h (panel B).

(Fig. 2, lane 6). Only a small difference in dehalogenase level is seen between induced and uninduced cells (Fig. 2, lanes 5 and 6, Table 1). The construct differs from pELA and pKLA in that the translational fusion was

done with an *Eco*RI restriction site which comprises only one base (the G) of the ATG start codon (Fig. 1C). Two amino acids are changed and one is deleted resulting in a N-terminal sequence of MNSIR. Expression of

FIG. 5. Aggregation of mature haloalkane dehalogenase into inclusion bodies. A 50-ml culture was induced for 2 h at 30°C and divided in two parts. One culture was switched at $t = 0$ to 37°C. Samples were taken at $t = 0, 15, 45, 75$, and 120 min and extract was prepared. The other culture was further incubated for 2 h at 30°C, and samples were taken at $t = 0$ and $t = 120$ min. (A) Curve a, activities from the culture that was held at 30°C; curve b, activities calculated assuming that haloalkane dehalogenase production stopped at $t = 0$ and that active protein was diluted by growth; curve c, activities in cell-free extracts from cultures incubated at 37°C. Activities are expressed as the percentage of activity found at $t = 0$. (B) SDS-PAGE of whole-cell lysates (C) and cell-free extracts (E) at different time points at 37°C and 30°C. The amount of protein applied was the same in each lane.

this construct in *E. coli* HB101 did not improve the expression of pPJ113 (not shown). Inactivation of the *lacI^a* gene, which was present in the construct to ensure maximal repression of the *tac* promoter, also did not result in an increased expression level.

Aggregation of haloalkane dehalogenase. High-level expression of haloalkane dehalogenase in *E. coli* at 37°C resulted in aggregation of the enzyme into inclusion bodies. The aggregation at 37°C led to large differences in

the haloalkane dehalogenase content of whole-cell lysates and cell-free extracts (Fig. 3, lanes 3 and 4), since the aggregates are spun down during cell-free extract preparation. Only a minor part of the haloalkane dehalogenase was found in inclusion bodies when the protein was expressed at 30°C (Fig. 3, lanes 1 and 2). Aggregation of dehalogenase was accompanied by inactivation of the protein, resulting in a fourfold higher activity in extracts prepared from cells grown at 30°C compared to cells grown at 37°C.

The formation of inclusion bodies from soluble mature haloalkane dehalogenase was investigated in a temperature shift experiment in which a culture of *E. coli* BL21(DE3)(pGELAF+) grown at 30°C and induced for

FIG. 6. Aggregation of haloalkane dehalogenase *in vitro*. Samples of a cell-free extract (induction temperature 30°C) of *E. coli* BL21(DE3)(pGELAF+) (7.5 mg of protein/ml) and of purified haloalkane dehalogenase (concentration 8 mg/ml) were incubated for 1.5 h at 37°C. The samples together with controls at 4°C were centrifuged for 20 min at 14,000g and analyzed with SDS-PAGE. Lane 1, 4°C cell-free extract; lane 2, 37°C cell-free extract; lane 3, pellet from 37°C cell-free extract (20 µl); lane 4, 4°C purified enzyme (8 mg/ml); lane 5, 37°C purified enzyme (8 mg/ml).

FIG. 7. Protein patterns during haloalkane dehalogenase purification. The samples were analyzed by SDS-PAGE. Lane 1, crude extract; lane 2, pooled DEAE fractions; and lane 3, pooled hydroxylapatite fractions.

2 h with 0.4 mM IPTG was shifted to 37°C (Fig. 5). Active haloalkane dehalogenase was rapidly converted into aggregates, leading to a 79% decrease of dehalogenase activity within 75 min. Only 15% of the activity was left after an additional 45 min (Fig. 5A). The reduction in soluble active haloalkane dehalogenase could not be explained by a stop in formation of new soluble haloalkane dehalogenase combined with dilution of the protein caused by growth (Fig. 5A). The control at 30°C showed no reduction in activity after 2 h (Fig. 5A). SDS-PAGE of whole cell lysates and cell free extracts taken at different time points (Fig. 5B) confirmed the activity data and clearly showed formation of dehalogenase aggregates which were spun down during the preparation of cell free extract. Formation of inclusion bodies at 37°C, present as large light scattering particles in the cell, was clearly visible by phase-contrast microscopy (Fig. 4A), whereas the control culture at 30°C only showed some small inclusion bodies (Fig. 4B).

To check whether aggregation of haloalkane dehalogenase was also possible *in vitro* we incubated extract (7.5 mg of protein/ml) of *E. coli* BL21(DE3)(pGELAF+) cultivated at 30°C and purified haloalkane dehalogenase (8 mg/ml) for 1.5 h at 37°C. The samples were centrifuged and supernatant and pellet (if present) were analyzed with SDS-PAGE (Fig. 6). Aggregation of haloalkane dehalogenase was observed with cell-free extract (Fig. 6, lanes 1, 2, and 3) but not with purified enzyme (Fig. 6, lanes 4 and 5). Additional experiments with other concentrations of purified enzyme (2, 4, 6, and 10 mg/ml) also showed no aggregation if incubated at 37°C (not shown).

Purification and properties of haloalkane dehalogenase expressed in *E. coli*. Starting with an 0.5-liter culture of *E. coli* BL21(DE3)(pGELAF+) with an OD₆₀₀ of 3.2, we obtained 34 mg of highly enriched haloalkane dehalogenase. The purification scheme is summarized in Table 2 and Fig. 7. The enzyme was purified 2.4-fold with an overall yield of 47%. This implies that in cell-free extract the dehalogenase represents 42% of the total cellular protein. The dehalogenase produced in *E. coli* BL21(DE3)(pGELAF+) had a $V_{\max}$ of 5.7 ± 0.1 U/mg protein and a K_m of 0.53 ± 0.11 mM for the substrate 1,2-dichloroethane.

DISCUSSION

The goal of this work was to obtain expression of haloalkane dehalogenase in *E. coli* under the control of a strong inducible promoter. Dehalogenase levels of up to 38% of the soluble cell protein were found when the expression was directed by the T7($\phi 10$) promoter. Expression was also good under the control of the *trc* promoter, but not under control of the *tac* promoter. Exam-

FIG. 8. Proposed scheme for inclusion body formation. The scheme is adapted and simplified from Kane and Hartley (14) and Mitraki and King (15) (solid arrows); 1, pathway to mature protein; 2, pathway to inclusion bodies. The pathway for inclusion body formation from mature haloalkane dehalogenase is represented with an open arrow (pathway 3).

ples of expression up to 20% of the soluble cell protein with the *tac* promoter in the pTTQ18 system are known, indicating that its *tac* promoter and ribosomal binding site are suitable for the expression of foreign proteins (11,12). Thus, the system functions well but is unable to express haloalkane dehalogenase up to high levels.

The main difference between the *tac* fusion and the T7 and *trc* fusions is the introduction of the fusion restriction site in the *dhlA* gene. In order to fuse the *dhlA* gene behind the *tac* promoter, the N-terminal sequence of the enzyme was changed from MINAIR to MNSIR. In contrast, the T7 and *trc* fusions had little change of the N-terminal sequence of the dehalogenase (from MINAIR to MVNAIR). It has been shown that the 5' region of mRNA can influence both translation initiation and mRNA stability (23,24). We propose that in construct pPJ113 the N-terminal sequence was changed in a way that either formation of a proper secondary mRNA structure for efficient translation initiation was prevented or mRNA stability was lowered.

While the haloalkane dehalogenase was expressed up to 38% of the total soluble cell protein at 30°C, most of the enzyme formed at 37°C was found in inclusion bodies. Inclusion bodies are considered to be formed from the aggregation of partially folded intermediates, rather than from mature protein or completely unfolded protein, by a process that seems to be strongly dependent on the environmental conditions (for reviews see 14,15). Kane and Hartley (14) and Mitraki and King (15) proposed a hypothetical scheme for inclusion body formation (Fig. 8, solid arrows) in which, starting from the transcription-translation complex, the partially folded intermediates can have two fates: (1) continuing the folding pathway resulting in mature protein; or (2) following an "off pathway" that results in an unstable conformation, which then aggregates to form inclusion bodies. This theory could also hold for inclusion body

formation of haloalkane dehalogenase. The off pathway is clearly induced by an increase in temperature (see Figs. 3, 5, and 6). This temperature effect has been found in a number of cases (15,25–27) and it was suggested that higher temperatures favor the formation of the inclusion bodies by reducing the stability of the thermolabile intermediates, which melt and form aggregates (15).

We have shown the existence of an additional pathway for inclusion body formation with haloalkane dehalogenase, namely from mature completely folded protein (Fig. 8, open arrow). This occurs both *in vivo* and in cell-free extract, but not with purified enzyme. Therefore, we suggest that an increase in temperature induces partial unfolding which results in aggregation of the protein. The presence of other components such as proteins, low-molecular-weight compounds, or membrane fragments could stimulate aggregation. Aggregation of native protein is thus not simply a matter of thermal instability. This was also found for ricin A chain (27).

The high level of expression of haloalkane dehalogenase facilitated the purification of the protein in large amounts. The ammonium sulfate precipitation step in the original purification protocol (1) could be omitted. The example of 34 mg of highly enriched haloalkane dehalogenase from a 0.5 liter (Fig. 7, Table 2) culture could easily be scaled up to 4 liters, which resulted in 300 mg highly enriched enzyme (not shown). The haloalkane dehalogenase produced by *E. coli* strain BL21(DE3)(pGELAF+) had properties comparable to enzyme isolated from *X. autotrophicus*. The $V_{\max}$ value of dehalogenase produced in *E. coli* with 1,2-dichloroethane was 5.7 U/mg of protein. This is somewhat lower than the $V_{\max}$ value of dehalogenase isolated from *X. autotrophicus* and suggests that the enzyme is slightly different or heterogenous. The K_m for 1,2-dichloroethane of 0.53 mM for the *E. coli*-produced dehalogenase is nearly the same as the one produced by *X. autotrophicus* (K_m of 0.68). The dehalogenase produced by the *E. coli*-pGELAF+ system formed good crystals which resulted in an electron density map identical to that of the one presented by Franken *et al.* (3) (K. Verschuere, personal communication).

In conclusion, we describe a system for expression, site-directed mutagenesis, and convenient purification of large amounts of haloalkane dehalogenase for further studies. The pGELAF+ system has now successfully been used in our laboratory to construct several active site mutants of haloalkane dehalogenase.

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REFERENCES

1. Keuning, S., Janssen, D. B., and Witholt, B. (1985) Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. *J. Bacteriol.* **163**, 635–639.
2. Janssen, D. B., Pries, F., van der Ploeg, J., Kazemier, B., Terpstra, P., and Witholt, B. (1989) Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. *J. Bacteriol.* **171**, 6791–6799.
3. Franken, S. M., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1991) Crystal structure of haloalkane dehalogenase: An enzyme to detoxify halogenated alkanes. *EMBO J.* **10**, 1297–1302.
4. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Haral, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschuere, K. H. G., and Goldman, A. (1992) The α/β hydrolase fold. *Prot. Eng.* **5**, 197–211.
5. Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J., and Ausubel, F. (1982) Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* **16**, 289–296.
6. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes, in "Methods in Enzymology" (Goeddel, D. V., Ed.), Vol. 185, pp. 60–89, Academic Press, San Diego.
7. Burton, N., Cavallini, B., Masamoto, K., Moncollin, V., and Egly, J.-M. (1991) Expression in *Escherichia coli*: Purification and properties of the yeast general transcription factor TFIID. *Protein Expression Purif.* **2**, 432–441.
8. Shuttlesworth, W. A., Hough, C. D., Bertrand, K. P., and Evans, J. N. S. (1992) Over-production of 5-enolpyruvylshikimate-3-phosphate synthase in *Escherichia coli*: Use of the T7 promoter. *Prot. Eng.* **5**, 461–466.
9. Li, B.-Y., Frankel, A. E., and Ramakrishnan, S. (1992) High-level expression and simplified purification of recombinant ricin A chain. *Protein Expression Purif.* **3**, 386–394.
10. Amann, E., and Brosius, J. (1985) "ATG vectors" for regulated high-level expression of cloned genes in *Escherichia coli*. *Gene* **40**, 183–190.
11. Stark, M. J. R. (1987) Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. *Gene* **51**, 255–267.
12. Harms, E., Wehner, A., Pennings, M. P., Pugh, K. J., Beacham, I. R., and Röhm, K. H. (1991) Construction of expression systems for *Escherichia coli* asparaginase II and two-step purification of the recombinant enzyme from periplasmic extracts. *Protein Expression Purif.* **2**, 144–150.
13. Beernink, P. T., and Tolan, D. R. (1992) Construction of a high-copy "ATG vector" for expression in *Escherichia coli*. *Protein Expression Purif.* **3**, 332–336.
14. Kane, J. F., and Hartley, D. L. (1988) Formation of recombinant inclusion bodies in *Escherichia coli*. *Tibtech.* **7**, 95–101.
15. Mittraki, A., and King, J. (1989) Protein folding intermediates and inclusion body formation. *Biotechnology* **7**, 690–697.
16. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynaker, H. L., and Boyer, H. W. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–113.
17. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
18. Innis, M. A., and Gelfand, D. H. (1990) Optimization of PCRs, in "PCR Protocols: A Guide to Methods and Applications" (Innis,

- M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., Eds.), pp. 3-11, Academic Press, San Diego.
19. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.
 20. Leatherbarrow, R. J. (1987) "Enzfitter: A Non-linear Regression Data Analysis Program for the IBM PC," Elsevier Science Publishers BV, Amsterdam, The Netherlands.
 21. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
 22. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
 23. Stanssens, P., Remaut, E., and Fiers, W. (1985) Alterations upstream from the Shine-Dalgarno region and their effect on bacterial gene expression. *Gene* **36**, 211-223.
 24. Balbas, P., and Bolivar, F. (1990) Design and construction of expression plasmid vectors in *Escherichia coli*, in "Methods in Enzymology" (Goeddel, D. V., Ed.), Vol. 185, pp. 14-37, Academic Press, San Diego.
 25. Liao, H. H. (1991) Effect of temperature on the expression of wild-type and thermostable mutants of kanamycin nucleotidyltransferase in *Escherichia coli*. *Protein Expression Purif.* **2**, 43-50.
 26. Lin, K., Kurland, I., Xu, L. Z., Lange, A. J., Pilakis, J., El-Maghrabi, M. R., and Pilakis, S. J. (1990) Expression of mammalian liver glycolytic/gluconeogenic enzymes in *Escherichia coli*: Recovery of active enzyme is strain and temperature dependent. *Protein Expression Purif.* **1**, 169-176.
 27. Piatak, M., Lane, J. A., Liard, W., Bjorn, M. J., Wang, A., and Williams, M. (1988) Expression of soluble and fully functional ricin A chain in *Escherichia coli* is temperature sensitive. *J. Biol. Chem.* **263**, 4837-4843.