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Construction and Screening of Metagenomic Libraries Derived from Enrichment Cultures: Generation of a Gene Bank for Genes Conferring Alcohol Oxidoreductase Activity on *Escherichia coli*

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Enrichment of microorganisms with special traits and the construction of metagenomic libraries by direct cloning of environmental DNA have great potential for identifying genes and gene products for biotechnological purposes. We have combined these techniques to isolate novel genes conferring oxidation of short-chain (C_2 to C_4) polyols or reduction of the corresponding carbonyls. In order to favor the growth of microorganisms containing the targeted genes, samples collected from four different environments were incubated in the presence of glycerol and 1,2-propanediol. Subsequently, the DNA was extracted from the four samples and used to construct complex plasmid libraries. Approximately 100,000 *Escherichia coli* strains of each library per test substrate were screened for the production of carbonyls from polyols on indicator agar. Twenty-four positive *E. coli* clones were obtained during the initial screen. Sixteen of them contained a plasmid (pAK101 to pAK116) which conferred a stable carbonyl-forming phenotype. Eight of the positive clones exhibited NAD(H)-dependent alcohol oxidoreductase activity with polyols or carbonyls as the substrates in crude extracts. Sequencing revealed that the inserts of pAK101 to pAK116 encoded 36 complete and 17 incomplete presumptive protein-encoding genes. Fifty of these genes showed similarity to sequenced genes from a broad collection of different microorganisms. The genes responsible for the carbonyl formation of *E. coli* were identified for nine of the plasmids (pAK101, pAK102, pAK105, pAK107 to pAK110, pAK115, and pAK116). Analyses of the amino acid sequences deduced from these genes revealed that three (*orf12*, *orf14*, and *orf22*) encoded novel alcohol dehydrogenases of different types, four (*orf5*, *sucB*, *fdhD*, and *yabF*) encoded novel putative oxidoreductases belonging to groups distinct from alcohol dehydrogenases, one (*glpK*) encoded a putative glycerol kinase, and one (*orf1*) encoded a protein which showed no similarity to any other known gene product.

Interconversion of alcohols, aldehydes, and ketones is involved in an astonishingly wide range of essential metabolic reactions in microorganisms. These redox reactions are catalyzed by oxidoreductases, including dehydrogenases. Most of these enzymes require cofactors such as flavin adenine dinucleotide, pyrroquinoline quinone, and $NAD(P)^+$ (25, 35). The nicotinamide-dependent alcohol dehydrogenases especially are of considerable industrial importance. These enzymes catalyze the reaction alcohol + $NAD(P)^+ \rightleftarrows$ aldehyde + $NAD(P)H + H^+$.

Enzymes of this type are useful catalysts, with high enantioselectivity for the synthesis of carbonyl compounds, hydroxy acids, amino acids, and chiral alcohols, which are chemically difficult to prepare (19, 25, 26). Alcohol dehydrogenases are employed for the preparation of deuterium- or tritium-labeled compounds, production of dihydroxyacetone, and as tools for enzymatic analysis of serum lipids (19, 47). For large-scale applications, regeneration of the nicotinamide cofactors by effective regeneration reactions such as the glucose-6-phosphate dehydrogenase or the formate dehydrogenase system is necessary (9). These systems reduce costs and provide an elegant way of driving thermodynamically unfavorable reactions towards the desired product (9, 19, 26).

The isolation of new or improved catalysts from environmental sources can be facilitated by two different approaches. The classical approach to isolate new enzymes by enrichment and screening of a wide variety of microorganisms for the desired activity. The enzymes and the corresponding genes are then recovered from the identified organisms. In this way, a large fraction (>99%) of the microbial diversity in an environment is lost due to difficulties in enriching and isolating microorganisms in pure culture (2). The alternative way is the assessment and exploitation of the entirety of the microbial genomes found in nature (4, 7, 11, 23, 31), which is termed the metagenome by some authors (21, 37). This approach comprises the construction of metagenomic libraries by direct extraction and cloning of DNA from environmental samples. Subsequently, the resulting libraries are screened for the targeted genes (12, 37). The number of positive clones in a screen can be increased by with environmental samples which exhibit an enrichment of microorganisms containing the desired activity for library production (12).

Previous studies have shown that the metagenomic approach offers an almost unlimited pool for encountering novel genes encoding biotechnologically relevant gene products such as lipases (23), cellulases (22), amylases (36, 37), chitinases (11), esterases (23), and enzymes involved in biotin synthesis (17).

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Nevertheless, the list of enzyme activities encountered in this way is still rather small in comparison to the classical discovery of new enzymes by enrichment and isolation of individual microorganisms.

The main goal of this study was to combine enrichment technology and metagenomics for the identification of a large number of diverse genes which confer the ability to oxidize short-chain polyols or reduce the corresponding carbonyl compounds. The recovered genes can serve as a resource (gene bank) for the rapid identification of suitable genes and gene products during development or improvement of industrial processes. The DNA used for preparation of the libraries was isolated directly from different environmental samples after a short enrichment for polyol-fermenting microorganisms. The resulting DNA libraries were screened for the presence of genes conferring a carbonyl compound-producing phenotype on *Escherichia coli* ECL707 (41) during incubation in the presence of short-chain (C_2 to C_4) polyols with adjacent hydroxyl groups. Crude extracts of the positive *E. coli* clones obtained were analyzed for nicotinamide-dependent dehydrogenase activity. Subsequently, the plasmids encoding the targeted activity were recovered from the positive *E. coli* strains and sequenced.

MATERIALS AND METHODS

Sample sites, bacterial strains, and plasmids. For the construction of environmental DNA libraries after enrichment for polyol-consuming microorganisms, soil from a sugar beet field near Göttingen (Germany), sediment from the river Grone (Germany), sediment from Solar Lake (Egypt), and sediment from the Gulf of Eilat (Israel) were collected. *E. coli* strains DH5α (3) and ECL707 (41) were used as hosts for the cloning experiments and the activity-based screening procedures, respectively. The plasmid pBluescript SK^+ (pSK^+) (Stratagene, San Diego, Calif.) was employed as the vector for the cloning experiments. The recombinant vectors pRD1 (13, 14) and pFL1 (30) were applied as positive controls during activity-based screening.

Media and growth conditions. *E. coli* was routinely grown in Luria-Bertani (LB) medium at 30°C (3). For activity-based screening of environmental libraries, recombinant *E. coli* strains were grown on carbonyl indicator plates. These plates were prepared according to the method of Conway et al. (10), but ethanol was replaced with 100 mM 1,2-ethanediol, 1,2-propanediol, glycerol, 2,3-butanediol, or combinations of these substrates. The enrichment for polyol-fermenting microorganisms was performed under anaerobic conditions for 24 h in a medium containing (per liter) K_2HPO_4, 14.0 g; KH_2PO_4, 6.0 g; $(NH_4)_2SO_4$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CoCl_2 \cdot 6H_2O$, 0.012 g; yeast extract, 0.2 g; cysteine-HCl, 0.2 g; and trace element solution SL4 (33), 1 ml (pH 7.5). The medium was supplemented with 100 mM glycerol and 1,2-propanediol. The enrichment was initiated by adding 50 g (wet weight) of environmental sample to flasks (1 liter) containing enrichment medium (500 ml). For the preparation of crude extracts, recombinant *E. coli* strains were grown in LB medium at 37°C. All growth media for *E. coli* strains harboring plasmids contained 100 μg of ampicillin per ml to maintain the plasmids.

Preparation of cell extracts. Cells in the stationary growth phase from 500-ml cultures were harvested by centrifugation at 6,000 × *g* for 20 min, washed once with 100 mM potassium phosphate buffer (pH 8.0), and resuspended in 2 to 3 ml of the same buffer. The cells were disrupted by French pressing (1.38×10^8 Pa), and the extract was cleared by centrifugation at 32,000 × *g* and 4°C for 30 min.

Enzyme assays. Alcohol dehydrogenase activity was determined by measuring the NAD(P)H-dependent reduction of carbonyls or by measuring the $NAD(P)^+$-dependent oxidation of alcohols according to Daniel et al. (14) and Ruch et al. (38), respectively. For the oxidation reaction, the substrates were 1,2,4-butanetriol, 2,3-butanediol, 1-butanol, 2-butanol, glycerol, 1,2-propanediol, 1-propanol, 2-propanol, 1,2-ethanediol, ethanol, and methanol at a final concentration of 100 mM; for the reduction reaction, the substrates were diacetyl, methylglyoxal, glyceraldehyde, dihydroxyacetone, hydroxyacetone, acetone, glycolaldehyde, and acetaldehyde at a final concentration of 10 mM. The specific background activities of the *E. coli* host with the substrates were measured in each case and subtracted from those of the recovered carbonyl-producing *E. coli* strains.

Protein concentrations were determined by the method of Bradford (6) with bovine serum albumin as the standard.

Molecular procedures. The isolation and purification of DNA from environmental samples were performed as described previously (12). The purified environmental DNA was partially digested with *Bsp*143I and, in order to avoid cloning of very small DNA fragments, size fractionated by sucrose density centrifugation (10 to 40% [wt/vol]). Fractions containing DNA fragments of >2 kb were ligated into the *Bam*HI-digested high-copy vector pSK^+ (500 to 700 copies per cell). The ligation products were then transformed into *E. coli* DH5α. To amplify the libraries, the resulting clones were grown on LB agar plates containing ampicillin for 18 h. Subsequently, the colonies were collected, and the plasmids were prepared by employing the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. To initiate the screening, the recovered plasmids were used to transform *E. coli* ECL707.

All other manipulations of DNA, PCR, and transformation of plasmids into *E. coli* were done according to routine procedures (3). The Göttingen Genomics Laboratory (Göttingen, Germany) determined the DNA sequences. Sequence analysis was performed with the Genetics Computer Group program package (16).

Nucleotide sequence accession numbers. The nucleotide sequences of the inserts of pAK101 to pAK116 have been deposited in the GenBank database. The accession numbers are given in Table 3.

RESULTS AND DISCUSSION

Enrichment for polyol-fermenting microbial consortia and construction of metagenomic DNA libraries. The enrichments for polyol-utilizing microorganisms were performed with samples derived from four different environments (soil from a sugar beet field and sediments from the Grone River, Solar Lake, and the Gulf of Eilat). Generally, the use of enrichment steps is associated with the loss of major portions of the microbial populations present in environmental samples (8). In order to maintain a high microbial diversity in the samples during the enrichment for microorganisms harboring the desired genes, we applied mild selective pressure for a short period. In addition, the subsequent DNA isolation was performed by the direct lysis approach. This method is less biased than the fractionation approach, in which the microbial cells are separated from the environmental matrix substances prior to DNA isolation (for a review, see reference 12).

The enrichment medium contained glycerol and 1,2-propanediol as selective agents. These substrates were chosen because several different nicotinamide-dependent dehydrogenases, such as propanol dehydrogenases, propionaldehyde dehydrogenases, 1,2-propanediol dehydrogenases, 1,3-propanediol oxidoreductases, and glycerol dehydrogenases, are known to be involved in the degradation of these substrates (15, 34, 38, 42, 45). In addition, the latter three types of enzymes exhibit a rather broad substrate specificity and are most active with short-chain polyols and the correlating carbonyl compounds (13, 14, 28, 29, 42, 43, 45). After inoculation of the enrichment cultures and growth for 1 day at 30°C, microorganisms and environmental matrix substances were pelleted, used to inoculate fresh medium, and incubated for another day. The enrichment of polyol-utilizing microorganisms was indicated by recording significant substrate consumption in the four different cultures (data not shown). A high degree of diversity within the four enrichment cultures was microscopically verified. Different forms of microbial cells, including cocci, rods, endospore-forming microbes, and chains of short rods, were observed.

Subsequently, the cells and the remaining environmental matrix substances were harvested by centrifugation, and the

TABLE 1. Activity-based screening of four environmental librarics for genes conferring the ability to form carbonyl compounds from polyols on *E. coli*[a]

Test substrate	Library (avg insert size)	Sample site	No. of carbonyl-forming *E. coli* clones after initial screen	No. of carbonyl-forming *E. coli* clones with stable phenotype (plasmid[s])
Glycerol/1,2-propanediol	I (3.3 kb)	River Grone	5	3 (pAK101–pAK103)
	II (5.4 kb)	Sugar beet field	6	6 (pAK111–pAK116)
	III (3.0 kb)	Solar Lake	1	0
	IV (5.6 kb)	Gulf of Eilat	1	1 (pAK108)
1,2-Ethanediol	I (3.3 kb)	River Grone	3	2 (pAK106–pAK107)
	II (5.4 kb)	Sugar beet field	0	0
	III (3.0 kb)	Solar Lake	0	0
	IV (5.6 kb)	Gulf of Eilat	0	0
2,3-Butanediol	I (3.3 kb)	River Grone	3	2 (pAK104–pAK105)
	II (5.4 kb)	Sugar beet field	1	0
	III (3.0 kb)	Solar Lake	4	2 (pAK109–pAK110)
	IV (5.6 kb)	Gulf of Eilat	0	0

[a] Approximately 100,000 *E. coli* clones from each library per test substrate were screened for carbonyl formation.

resulting pellets were used as starting material for the direct isolation of genomic DNA, which was performed as described previously (12, 24). Approximately 1 to 2 mg of DNA was recovered in each case. The DNA obtained was partially digested and size fractionated. DNA fragments of >2 kb were ligated in pSK^+ and then transformed into *E. coli*. The vector pSK^+ was selected for library construction because of its high copy number. This allows the detection of even weakly expressed genes in the subsequently performed activity-based screening of the four libraries constructed, because heterologous expression of environmental genes in *E. coli* is often dependent upon the native promoters (12). The quality of the four different libraries produced was controlled by determination of the average insert size and the percentage of recombinant plasmids containing inserts. The four libraries revealed average insert sizes of 3.0 to 5.6 kb (Table 1). The percentage of plasmids containing inserts was approximately 70 to 80%.

Screening for genes conferring alcohol oxidoreductase activity. In order to access the alcohol oxidoreductase-encoding genes of the four different environmentally derived libraries, the recombinant *E. coli* strains of each library were screened for production of carbonyl compounds from short-chain (C_2 to C_4) polyols with adjacent hydroxyl groups. The screening was performed on indicator plates (10) that contained 1,2-ethanediol, 2,3-butanediol, or a mixture of 1,2-propanediol and glycerol as test substrates and a mixture of pararosaniline and bisulfite for the detection of carbonyl compounds formed by the *E. coli* clones. Upon production of carbonyls from the test substrates by recombinant *E. coli* strains, an intensely red Schiff base is formed. Thus, *E. coli* colonies capable of carbonyl formation appeared red on indicator medium and were surrounded by a red zone, whereas colonies failing to produce carbonyl compounds remained uncolored. To avoid a high background coloring of the indicator plates by glycerol dehydrogenase activity of *E. coli*, the glycerol-minus mutant *E. coli* ECL707 (41) was used as the host for the libraries during the screening procedure.

Positive colonies were best visualized after overnight incubation at 37°C. Such colonies completely colored the indicator plates after prolonged incubation. The reliability of the entire activity-based screening procedure was controlled by transferring pRD1 (13, 14) or pFL1 (30), harboring the genes encoding NAD^+-dependent alcohol dehydrogenases from *Citrobacter freundii* and *Clostridium pasteurianum*, respectively, into *E. coli* ECL707. Both resulting recombinant *E. coli* strains showed a strong reaction on the indicator plates, in contrast to *E. coli* ECL707. Subsequently, the screening of the four metagenomic libraries was initiated by transformation of the recombinant plasmids of each constructed library in *E. coli* ECL707, followed by transfer of the resulting *E. coli* clones on indicator plates containing the different types of test substrates (see above). Approximately 100,000 *E. coli* clones of each library per test substrate were screened for formation of carbonyls.

Twenty-four *E. coli* clones were positive during the initial screen of the four libraries constructed (Table 1). Most of the clones were obtained from libraries I and II and with the glycerol/1,3-propanediol mixture as the test substrate (Table 1). In order to confirm that the carbonyl-forming phenotype of the positive clones was plasmid encoded and stable, the recombinant plasmids were isolated and retransformed into *E. coli*, and the resulting *E. coli* strains were screened again on appropriate indicator plates. Sixteen different recombinant plasmids, designated pAK101 to pAK116, conferred a stable carbonyl-forming phenotype on the resulting recombinant *E. coli* strains. Seven of these plasmids were derived from library I, six from library II, two from library III, and one from library IV (Table 1). The insert sizes of pAK101 to pAK116 were in the range of 651 to 8,249 bp (Fig. 1). The corresponding *E. coli* strains (*E. coli*/pAK101 to *E. coli*/pAK116) were studied further.

Biochemical characterization of carbonyl-forming *E. coli* clones. Crude extracts of the 16 positive *E. coli* clones obtained during the screening procedure were prepared and tested for $NAD(P)^+$-dependent oxidation and NAD(P)H-dependent reduction of various short-chain alcohols and carbonyl compounds, respectively. $NADP^+$- or NADPH-dependent reactions with the tested substrates were not detectable in the crude extracts of all *E. coli* clones. *E. coli*/pAK103, *E. coli*/pAK104, *E. coli*/pAK108, *E. coli*/pAK109, *E. coli*/pAK110, *E.*

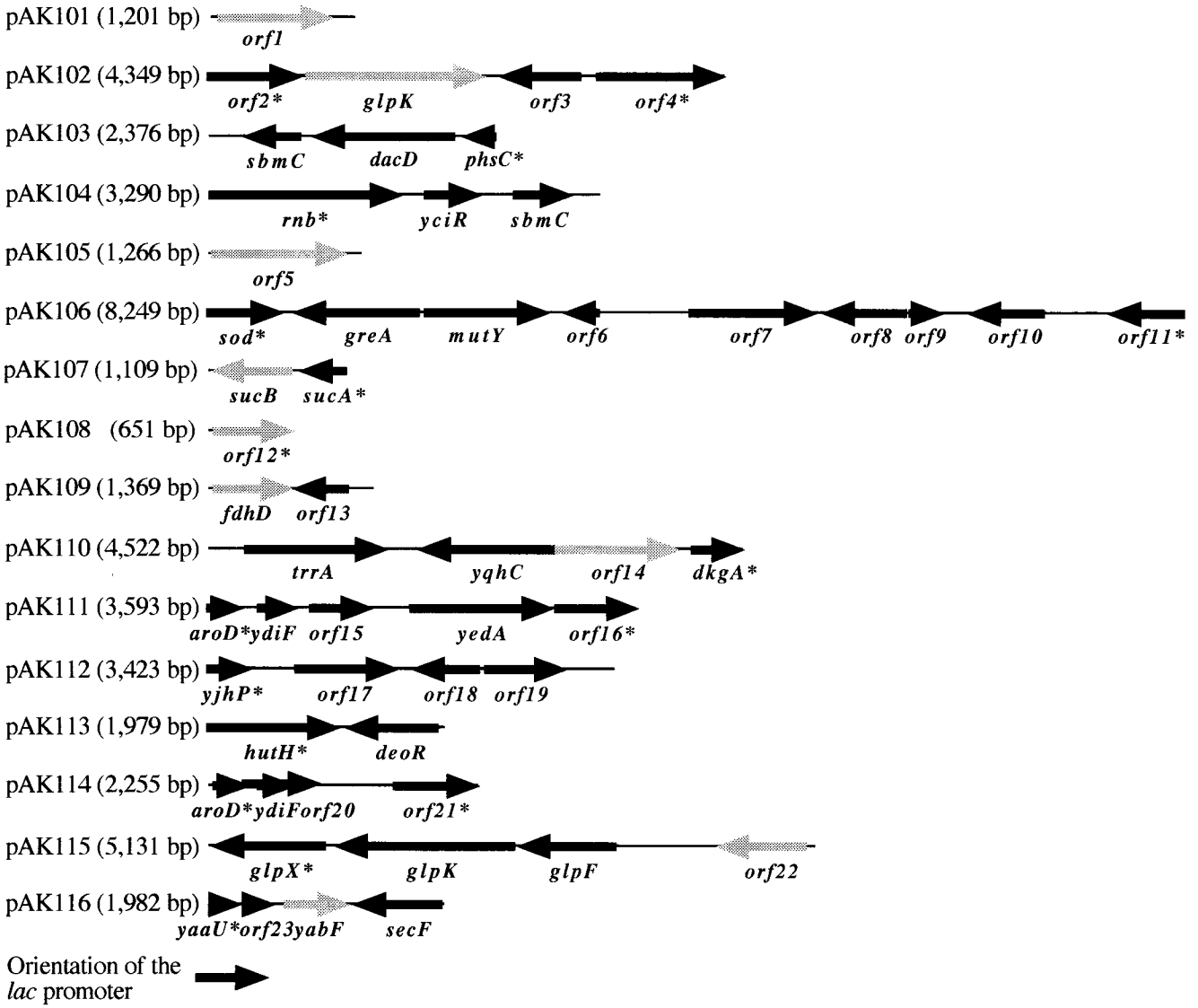

FIG. 1. Genetic organization and length of the inserts of pAK101 to pAK116. The insert sizes of the plasmids are given in brackets. Arrows and arrowheads represent the lengths, locations, and orientations of the potential genes within the inserts. The arrows of potential genes which were identified as being responsible for the carbonyl-forming phenotype of the *E. coli* clones are shaded gray. The gene designations are written below the arrows. Incomplete open reading frames are marked with asterisks. Observed similarities of the deduced amino acid sequences to sequences available in the NCBI databases are listed in Table 3.

coli/pAK112, *E. coli*/pAK113, and *E. coli*/pAK116 showed NAD^+-dependent oxidation or NADH-dependent reduction of one or more substrates (Table 2). Substrate specificity studies revealed that the enzymes produced by these clones were capable of catalyzing a number of oxidation and reduction reactions except the enzyme encoded by pAK116, which was specific for 1,2-ethanediol under the assay conditions employed (Table 2).

The enzymes were most active with polyols containing two adjacent alcohol groups. The preferred substrates were glycerol (pAK103), 1,2-propanediol (pAK104, pAK108, pAK109, pAK110, pAK112, and pAK113), and 1,2-ethanediol (pAK116). A significant oxidation of primary or secondary alcohols containing single hydroxyl groups such as ethanol, 1-propanol, 2-propanol, 1-butanol, and 2-butanol was not detected (data not shown). The reduction reaction was more specific (Table 2). The enzymes produced were most active with dihydroxyacetone (pAK104, pAK110, pAK112, and pAK113) or glycolaldehyde (pAK103 and pAK109). No significant reduction of glyceraldehyde, diacetyl, acetaldehyde, or acetone was observed. Although *E. coli*/pAK108 and *E. coli*/pAK116 exhibited significant activity in oxidation reactions, NADH-dependent reductions of the tested carbonyl compounds were not detected under these assay conditions.

In contrast to the above-mentioned carbonyl-forming *E. coli* clones, the other eight positive clones (*E. coli*/pAK101, *E. coli*/pAK102, *E. coli*/pAK105, *E. coli*/pAK106, *E. coli*/pAK107, *E. coli*/pAK111, *E. coli*/pAK114, and *E. coli*/pAK115) exhibited nicotinamide-dependent activities with the tested substrates that did not significantly exceed the background activities of

TABLE 2. Substrate specificity of nicotinamide-dependent dehydrogenase reactions in crude extracts of *E. coli* carrying various plasmids[a]

Reaction and substrate	Relative sp act in crude extracts of corresponding clones (%)							
	pAK103	pAK104	pAK108	pAK109	pAK110	pAK112	pAK113	pAK116
Oxidation								
1,2-Ethanediol	—	—	—	—	—	5	—	100
1,2-Propanediol	81	100	100	100	100	100	100	—
2,3-Butanediol	—	22	50	31	44	32	43	—
Glycerol	100	35	91	36	40	44	68	—
1,2,4-Butanetriol	46	14	27	26	27	16	36	—
Reduction								
Dihydroxyacetone	76	100	—	78	100	100	100	—
Hydroxyacetone	9	39	—	41	70	—	—	—
Glycolaldehyde	100	19	—	100	93	—	53	—
Methylglyoxal	33	—	—	—	—	—	—	—

[a] The specific alcohol dehydrogenase activities were determined with various short-chain (C_2 to C_4) alcohols or carbonyls as substrates and NAD^+ or NADH, respectively, as the cofactor. The oxidation of the alcohols and the reduction of the carbonyl compounds were assayed as described in Materials and Methods. The specific background activities of the *E. coli* host containing the cloning vector were measured in each case and subtracted. The specific activities in oxidation reactions were expressed relative to that obtained with the substrate yielding the highest value in each case (pAK103, 0.024 U/mg; pAK104, 0.033 U/mg; pAK108, 0.022 U/mg; pAK109, 0.039 U/mg; pAK110, 0.048; pAK112, 0.057 U/mg; pAK113, 0.028 U/mg; and pAK116, 0.040). —, no detectable activity. The specific activities in reduction reactions were expressed relative to that obtained with the substrate yielding the highest value in each case (pAK103, 0.054 U/mg; pAK104, 0.080 U/mg; pAK109, 0.044 U/mg; pAK110, 0.137 U/mg; pAK112, 0.032 U/mg; and pAK113, 0.068 U/mg).

the *E. coli* control harboring the cloning vector (data not shown). This result indicated that these strains did not contain genes encoding an NAD(H)-dependent dehydrogenase activity. The carbonyl-forming phenotype of these *E. coli* clones on indicator plates was probably caused by the activities of other classes of enzymes acting on alcohols, such as metallooxidases, dehydratases, and flavin adenine dinucleotide-dependent or pyrroloquinoline quinone-dependent dehydrogenases (15, 19, 25, 40). In addition, the assay conditions employed may not be suitable for the activity of the gene products produced by these clones and may also account for the lack of activity of the dehydrogenase activity-exhibiting clones with certain substrates.

Molecular analyses. The inserts of pAK101 to pAK116 were sequenced and compared to the sequences available in the National Center for Biotechnology Information (NCBI) databases. The identified genes and the observed similarities of the deduced amino acid sequences to known gene products are summarized in Table 3 and Fig. 1. Thirty-six complete and 17 incomplete protein-encoding genes were predicted within the sequences of all inserts, with 50 showing significant similarity to sequenced genes from other organisms. All were most similar to bacterial homologues derived from a wide variety of different genera, i.e., *Salmonella*, *Yersinia*, *Thermoanaerobacter*, *Pseudomonas*, *Sinorhizobium*, *Agrobacterium*, and *Ralstonia*.

Although the observed similarities of some of the identified genes to known genes, especially to genes from enteric bacteria, were surprisingly high, the sequences of almost all predicted genes showed significant differences from sequenced genes from individual microorganisms. Thus, we have no evidence indicating from which species the inserts of the recovered plasmids were derived. The similarity to genes from enteric bacteria could also be a result of the activity screen, which was based on the ability of *E. coli* clones to form carbonyl compounds from polyols. This allowed only the detection of cloned genes whose promoters were recognized by the *E. coli* host strain or of genes expressed via the plasmid-encoded *lac* promoter, which is induced in the presence of isopropyl-1-thio-β-D-galactopyranoside (IPTG). The activity of the *lac* promoter was probably very low under the growth conditions employed, since IPTG was not added to the growth medium. In addition, the latter mode of expression was only possible for genes oriented in the same direction as the vector-encoded *lac* promoter (Fig. 1).

The use of other hosts, such as *Bacillus* or *Streptomyces*, and mobilizable expression vectors might have increased the diversity and number of resulting clones. However, in all published reports, *E. coli* has been used as the host for metagenomic libraries during activity-based screening programs. Sequencing of more than one recombinant plasmid recovered from the positive *E. coli* clones has been performed in only a few studies (23, 24, 31, 46). The results obtained have shown that activity-based screens of metagenomic libraries are suitable for the detection of genes which are similar to genes derived from microorganisms weakly related to the *E. coli* host. For example, our screening of metagenomic libraries derived from soil samples led to the identification of genes encoding 4-hydroxybutyrate dehydrogenases which were most similar to the genes from *Ralstonia eutropha*, *Clostridium acetobutylicum*, and *Synechocystis* sp. strain PCC6803 (24).

The known and sequenced alcohol oxidoreductases can be divided into three major categories (35). (i) The $NAD(P)^+$-dependent dehydrogenases are the best characterized of the three groups. These can in turn be separated into type I long-chain zinc-containing enzymes, such as alcohol dehydrogenase I of *Saccharomyces cerevisiae* and *Zymomonas mobilis* (44); type II short-chain zinc-independent enzymes, such as ribitol dehydrogenase of *Enterobacter aerogenes* and glucose dehydrogenase of *Bacillus megaterium* (32); and type III iron-dependent enzymes, such as alcohol dehydrogenase II of *Z. mobilis* (10) and glycerol dehydrogenase of *Citrobacter freundii* (13). Most of the characterized alcohol oxidoreductases, which are involved in the degradation of glycerol and 1,2-propanediol by individual microorganisms such as *Citrobacter freundii* (13, 14), *Clostridium pasteurianum* (30), *Erwinia aroidea* (43), *Desulfo-*

vibrio sp. strain HDv (34), *Cellulomonas* sp. (47), *Lactobacillus reuteri* (45), and *Klebsiella pneumoniae* (28), belong to the last family of NAD(H)-dependent alcohol dehydrogenases (13, 14, 28, 30). (ii) $NAD(P)^+$-independent enzymes use other cofactors, such as pyrroloquinoline quinone or F_{420}, exemplified by the ethanol dehydrogenase of *Acetobacter aceti* (27) and the alcohol dehydrogenase of *Methanobacterium palustre* (5). (iii) Oxidases that catalyze an essentially irreversible oxidation of alcohols, such as the flavin adenine dinucleotide-dependent alcohol oxidase 1 of *Candida boidinii* (39).

DNA sequence analyses of the inserts of pAK101 (1,201 bp), pAK105 (1,266 bp), and pAK108 (651 bp) revealed in each case one large potential gene of 969, 1,137, and 534 bp, respectively, within the sequence (Fig. 1). The amino acid sequence deduced from *orf1* located on pAK101 exhibited no significant similarities to the above-mentioned alcohol oxidoreductases or to sequences and signatures of proteins available in the NCBI and PROSITE databases, whereas the proteins deduced from *orf5* (pAK105) and *orf12* (pAK108) showed similarities to oxidoreductases from other organisms. The *orf5* gene product showed 62% identity to a putative monooxygenase/oxidoreductase of *Sinorhizobium meliloti*, and the partial *orf12* gene product showed 31% identity to a putative NAD^+-dependent zinc-containing (type I) alcohol dehydrogenase of *Mesorhizobium loti* (Table 3). These results corresponded well with those obtained during the determination of the alcohol dehydrogenase activity in crude extracts of the correlating *E. coli* clones, since solely *E. coli*/pAK108 harboring *orf12* exhibited NAD^+-dependent dehydrogenase activity with polyols as the substrates (Table 2).

Sequencing of the other 13 plasmids revealed in each case more than one potential gene within the sequence of the insert (Fig. 1). In order to identify the genes on the plasmids that were responsible for the formation of carbonyl compounds by the corresponding recombinant *E. coli* strains, the inserts were partially subcloned by restriction digestion with various enzymes or PCR with primers derived from sequencing and subsequent ligation into pSK^+. The resulting constructs were transformed into *E. coli*, and the recombinant *E. coli* strains were screened again on indicator plates containing 1,2-ethanediol, glycerol/1,2-propanediol, or 2,3-butanediol as the substrate. This method was successful for the inserts of pAK102, pAK107, pAK109, pAK110, pAK115, and pAK116. The corresponding subclones were indistinguishable from the original clones with respect to NAD(H)-dependent alcohol dehydrogenase activities in crude extracts. In all six cases, single genes were responsible for the carbonyl-forming phenotype (Fig. 1). All attempts to subclone a DNA region conferring the carbonyl-forming phenotype of pAK103, pAK104, pAK106, pAK111, pAK112, pAK113, and pAK114 failed. For most of these plasmids, we were unable to recover all of the possible subclones. Since subcloning can lead to changes in expression levels of genes, this may have resulted from toxic effects caused by increased levels of the foreign gene products in *E. coli*.

All six identified genes conferring the carbonyl-forming phenotype were preceded by a potential ribosome-binding site appropriately spaced from the start codon. The deduced gene products of all genes except the one located on pAK102 revealed significant similarities to known oxidoreductases (Table 3). The protein deduced from the *glpK* gene of pAK2 (502 amino acids) was 46% identical to the glycerol kinase of *Yersinia pestis*. This indicated that production of the carbonyl compounds from glycerol on indicator agar by *E. coli* ECL707/pAK102 was initiated by phosphorylation of glycerol catalyzed by the heterologously produced kinase. Subsequently, the resulting glycerol 3-phosphate is oxidized by a dehydrogenase, i.e., glycerol 3-phosphate dehydrogenase, provided by the *E. coli* host. This mode of glycerol consumption can also account for the lack of NAD(H)-dependent enzyme activity with the tested substrates in crude extracts of the *E. coli* clone. We cannot exclude that indirect reactions like this also account for the carbonyl-forming phenotype of some other positive clones, since the introduction of unknown environmental DNA into the *E. coli* host can yield hybrid products whose synthesis is directed in part by the host genome and in part by the introduced DNA.

Interestingly, one of the proteins deduced from the potential genes located on pAK115 was also highly similar to glycerol kinases of enteric bacteria, but the *orf22* gene product (247 amino acids) identified as being responsible for the carbonyl-forming phenotype of *E. coli* ECL707 was related to a type II short-chain alcohol dehydrogenase of *Agrobacterium tumefaciens* (55% identity). The short-chain dehydrogenase/reductase family is very large, and most members are proteins of approximately 250 to 300 amino acids. The signature pattern for this protein family (LIVSPADNK-x_{12}-Y-PSTAGNCV-STAGNQ-CIVM-STAGC-K-PC-SAGFYR-LIVMSTAGD-x_2-LIVM-FYW-x_3-LIVMFYWGAPTHQ-GSACQRHM) includes two perfectly conserved residues, a tyrosine and a lysine. The tyrosine residue participates in the catalytic mechanism (18). This motif was retained in the deduced amino acid sequence of *orf22* (amino acids 143 to 171) except that the first amino acid residue (LIVSPADNK) was replaced by a cytosine residue (see GenBank accession).

In addition to this gene product, the one responsible for the carbonyl-forming phenotype encoded by pAK110 (Orf14; 387 amino acids) was also similar to one of the above-mentioned categories of alcohol oxidoreductases. The deduced Orf14 protein sequence showed 90% identity to the putative iron-containing type III alcohol dehydrogenase of *Salmonella enterica*. For the detection of type III alcohol dehydrogenases, two specific fingerprint patterns are available in the PROSITE database (18); the first one is STALIV-LIVF-x-DE-$x_{6,\ 7}$-P-x_4-ALIV-x-GST-x_2-D-TAIVM-LIVMF-x_4-E and the second one is GSW-x-LIVTSACD-GH-x_2-GSAE-GSHYQ-x-LIVTP-GAST-GAS-x_3-LIVMT-x-HNS-GA-x-GTAC. The *orf14* gene product showed both fingerprint patterns (amino acids 173 to 201 and 264 to 284). In addition, the correlating *E. coli* clone (Table 2) and the purified gene product (data not shown) exhibited NAD^+-dependent activity with a variety of polyols, which is typical of some type III alcohol dehydrogenases, such as glycerol dehydrogenases and 1,3-propanediol dehydrogenases (13, 14, 28, 30).

The deduced amino acid sequences of the other three presumptive oxidoreductase-encoding genes *sucB*, *fdhD*, and *yabF* identified during subcloning of pAK107, pAK109, and pAK116, respectively, were similar to those of members of the groups of oxidoreductases, which were different from the above-described alcohol oxidoreductases. The amino acid sequences deduced for the *sucB*, *yabF*, and *fdhD* products (214,

TABLE 3. Identified genes, GenBank accession numbers, and observed similarities[a]

Plasmid	Gene identified (accession no.)	Similar protein	Organism (accession no. of similar protein)	% Identity	No. of similar amino acids	Score
pAK101	***orf1*** (AF543467)	No similar protein				
pAK102	*orf2*∗ (AF543469)	Hypothetical protein	*Yersinia pestis* (NP_406777)	77	268	424
	glpK (AF543469)	Glycerol kinase	*Y. pestis* (NP_406778)	46	498	427
	orf3 (AF543469)	Putative acetyltransferase	*Y. pestis* (NP_405514)	44	222	201
	orf4∗ (AF543469)	Conserved hypothetical protein	*Salmonella enterica* (NP_455160)	83	356	626
pAK103	*sbmC* (AF543472)	SbmC protein, unknown function	*Escherichia coli* (NP_288514)	74	155	254
	dacD (AF543472)	Carboxypeptidase	*Salmonella enterica* serovar Typhimurium (NP_461007)	90	390	723
	phsC∗ (AF543472)	Thiosulfate reductase, cytochrome *b* subunit	*S. enterica* (NP_456611)	85	71	128
pAK104	*rnb*∗ (AF543475)	RNase II	*S. enterica* serovar Typhimurium (NP_460661)	88	536	957
	yciR (AF543475)	Hypothetical protein	*E. coli* (NP_415802)	66	106	152
	sbmC (AF543475)	SbmC protein, unknown function	*E. coli* (NP_288514)	74	155	254
pAK105	***orf5*** (AF543478)	Probable monooxygenase/oxidoreductase	*Sinorhizobium meliloti* (T46818)	62	380	499
pAK106	*sodA*∗ (AF543480)	Superoxide dismutase	*Thermoanaerobacter tengcongensis* (NP_622509)	34	184	91
	greA (AF543480)	Transcription elongation factor	*Caulobacter crescentus* (NP_421644)	31	151	65
	mutY (AF543480)	Hypothetical adenine glycosylase	*Ralstonia solanacearum* (NP_518522)	45	365	286
	orf6 (AF543480)	Hypothetical protein	*Pseudomonas aeruginosa* (NP_254046)	51	111	112
	orf7 (AF543480)	No similar protein				
	orf8 (AF543480)	Putative aminopeptidase	*Agrobacterium tumefaciens* (NP_355456)	39	241	144
	orf9 (AF543480)	Probable response regulator	*Rhizobium solanacearum* (NP_518671)	52	91	100
	orf10 (AF543480)	Hypothetical protein	*Mesorhizobium loti* (NP_104373)	33	106	58
	orf11∗ (AF543480)	No similar protein				
pAK107	***sucB*** (AF543482)	2-Oxoglutarate dehydrogenase, E2 subunit	*Vibrio cholerae* (NP_231718)	60	225	251
	sucA∗ (AF543482)	2-Oxoglutarate dehydrogenase, E1 subunit	*Vibrio cholerae* (NP_231719)	74	123	198
pAK108	***orf12***∗ (AF543483)	Putative zinc-type alcohol dehydrogenase	*Mesorhizobium loti* (NP_104464)	31	217	118
pAK109	***fdhD*** (AF543481)	Putative FdhD protein	*S. enterica* (NP_458012)	88	218	402
	orf13 (AF543481)	Putative membrane protein	*Y. pestis* (NP_404224)	24	148	57
pAK110	*trrA* (AF543479)	Putative transcriptional regulator	*Bacillus cereus* (CAB69794)	25	415	121
	yqhC (AF543479)	AraC-type regulatory protein	*E. coli* (NP_417483)	89	299	531
	orf14 (AF543479)	Iron-containing alcohol dehydrogenase	*S. enterica* (NP_457557)	90	387	704
	dkgA∗ (AF543479)	2,5-Diketo-D-gluconic acid reductase	*E. coli* (Q56857)	86	140	254
pAK111	*aroD*∗ (AF543477)	3-Dehydroquinate dehydratase	*Salmonella enterica* serovar *Enteritidis* (Q9RN77)	88	90	157
	ydiF (AF543477)	Probable membrane protein	*E. coli* (NP_416209)	85	103	182
	orf15 (AF543479)	Sensor histidine kinase, PleD homologue	*Synechocystis* sp. (NP_441080)	38	221	150
	yedA (AF543477)	IS*10*, transposase	Plasmid R100 (NP_052934)	99	401	818
	orf16∗ (AF543477)	Hypothetical protein	*Pseudomonas aeruginosa* (NP_251460)	46	228	189
pAK112	*yjhP*∗ (AF543476)	Putative methyltransferase	*E. coli* (NP_418726)	93	114	225
	orf17 (AF543476)	Hypothetical protein	*Y. pestis* (NP_405552)	84	277	498
	orf18 (AF543476)	Putative transmembrane efflux protein	*Streptomyces coelicolor* (NP_628101)	46	183	160
	orf19 (AF543476)	Probable integral membrane protein	*Clostridium perfringens* (NP_560977)	95	221	403
pAK113	*hutH*∗ (AF543474)	Histidine ammonia-lyase	*S. enterica* serovar *Typhimurium* (NP_459769)	83	361	606
	deoR (AF543474)	Transcriptional regulator	*S. enterica* serovar *Typhimurium* (NP_459841)	88	252	454
pAK114	*aroD*∗ (AF543473)	3-Dehydroquinate dehydratase	*S. enterica* (Q9RN77)	88	90	157
	ydiF (AF543473)	Probable membrane protein	*E. coli* (NP_416209)	85	103	182
	orf20 (AF543473)	Sensor histidine kinase	*Synechocystis* sp.(NP_441080)	38	221	150
	orf21∗ (AF543473)	Probable oxidoreductase	*Xanthomonas axonopodis* (NP_641091)	41	244	164
pAK115	*glpX*∗ (AF543471)	Glycerol metabolism, unknown function	*S. enterica* serovar Typhimurium (NP_462966)	94	308	561
	glpK (AF543471)	Glycerol kinase	*E. coli* (NP_290555)	92	502	952
	glpF (AF543471)	Glycerol uptake facilitator protein	*S. enterica* (NP_457965)	86	281	490
	orf22 (AF543471)	Putative short-chain alcohol dehydrogenase	*Agrobacterium tumefaciens* (NP_396110)	55	241	243
pAK116	*yaaU*∗ (AF543470)	Putative transport protein	*E. coli* (NP_414587)	78	66	109
	orf23 (AF543470)	Putative lipoprotein	*S. enterica* (NP_454690)	83	77	129
	yabF (AF543470)	Probable NAD(P)H oxidoreductase	*E. coli* (NP_414588)	89	176	355
	secF (AF543470)	Protein export protein	*E. coli* (AAB40165)	88	171	311

[a] The identification of proteins similar to the presumptive gene products encoded by the inserts of pAK101 to pAK116 and the determination of the values for the percent identity, region of similar amino acids, and score were performed by using the Blast programs (1). Incomplete genes are marked by asterisks. The names of genes identified as being responsible for carbonyl formation are shown in bold. The apparent gene organizations are given in Fig. 1.

176, and 212 amino acids, respectively) were most similar to a part of the 2-oxoglutarate dehydrogenase enzyme complex of *Vibrio cholerae*, a probable NAD(P)H oxidoreductase from *E. coli*, and a putative protein required for formate dehydrogenase activity from *Salmonella enterica*. The last putative gene product is found in other organisms, such as *Xanthomonas campestris* (NP_637709) and *Mesorhizobium loti* (NP_106066) as part of the formate dehydrogenase. The *yabF* and *fdhD* gene products conferred NAD(H)-dependent activity with short-chain polyols or carbonyl compounds as substrates on the *E.*

coli clones (Table 2). To our knowledge, the reactions with these substrates have not been reported for the related proteins of other organisms.

The sequence analyses of the seven plasmids for which the identification of a single gene responsible for the carbonyl-forming phenotype failed (see above) revealed for open reading frames (*phsC* and *orf21*, respectively) encoding putative enzymes similar to known oxidoreductases/reductases in pAK103, and pAK114, respectively, but both putative genes were incomplete (Table 3). In addition to these two deduced gene products, the inserts of the correlating plasmids and those of the remaining five plasmids (pAK104, pAK106, pAK111, pAK112, and pAK113) encoded putative gene products similar to proteins with unknown functions or to gene products involved in a broad range of different metabolic reactions (Table 3). Although pAK103, pAK104, pAK112, and pAK113 conferred significant NAD(H)-dependent dehydrogenase activities on *E. coli*, none of the plasmids encoded complete genes similar to those encoding oxidoreductases. These may therefore be entirely new genes encoding products mediating these reactions. Another possibility is that the gene products facilitate the dehydrogenase activity of the recombinant *E. coli* strains and the carbonyl-forming phenotype in a not yet understood way. The function of these genes will be unraveled by characterization of the purified gene products.

In summary, nine novel genes were identified as being responsible for the formation of carbonyls from short-chain polyols by recombinant *E. coli* strains during subcloning and sequence analyses. Three (*orf12*, *orf14*, and *orf22*) encoded presumptive alcohol dehydrogenases, four (*orf5*, *sucB*, *fdhD*, and *yabF*) encoded putative oxidoreductases belonging to groups different from alcohol dehydrogenases, one (*glpK*) encoded a putative glycerol kinase, and one (*orf1*) encoded a protein unrelated to known gene products. Thus, the majority of the gene products encountered were similar to groups of enzymes for which the formation of carbonyls from the polyols employed in this study have not been reported. In addition, type III alcohol dehydrogenases are involved in the consumption of glycerol and 1,2-propanediol by the characterized microorganisms (see above), but only one of the identified gene products (Orf14) was related to this enzyme type. These results are typical for activity-based screening programs of libraries derived from complex microbial assemblages.

Most of the genes and the corresponding gene products discovered are entirely novel, similar to genes with unknown functions, and weakly related or unrelated to known genes encoding the targeted functions (20, 23, 24, 37, 46). The novelty of the biocatalysts encountered in this line of work arises from the widely unexplored enormous genetic and metabolic diversity of uncultured microorganisms (12, 20, 37). Nevertheless, the involvement of enrichment steps yields metagenomic libraries that are more biased than those constructed without these steps, but the results presented here and by other authors (17, 22) showed that a diverse set of genes conferring the targeted reaction were still recovered by this approach. Thus, the coupling of classical enrichment and modern metagenomic technologies in combination with simple activity-based screening systems is a good way to isolate a large number of diverse genes conferring redox reactions with polyols and the corresponding carbonyls. The number of genes and gene products performing this reaction can be extended by increasing the number of clones screened. In this way, gene banks consisting of several hundred genes conferring certain types of industrially important reactions can be prepared rapidly. These gene banks or the corresponding clones can serve as starting material for the development of novel processes and products. Future studies targeting other biocatalysts will define the true scope and general usefulness of this approach.

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