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A Cytochrome *c* from a Lupanine-Transforming *Pseudomonas putida* Strain Is Expressed in *Escherichia coli* during Aerobic Cultivation and Efficiently Exported and Assembled in the Periplasm

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We have cloned, sequenced, and heterologously expressed a periplasmic cytochrome *c* from a lupanine-utilizing *Pseudomonas putida* strain. Aerobic batch cultivation of *Escherichia coli* TB1 harboring the cytochrome *c* gene placed downstream of the *lac* promoter in pUC9 vector resulted in significant production of the holo-cytochrome *c* in the periplasm (~4 mg of hemoprotein/liter of culture). The recombinant cytochrome *c* was purified to homogeneity and was found to be functional in accepting electrons from lupanine hydroxylase while catalyzing hydroxylation of lupanine. Comparison of the N-terminal amino acid sequence of the isolated cytochrome *c* with that deduced from the DNA sequence indicated that the signal sequence was processed at the bond position predicted by the SigPep program. The molecular size of the cytochrome *c* determined by electrospray mass spectrometry (9,595) was in precise agreement with that predicted from the nucleotide sequence.

Lupanine hydroxylase of a *Pseudomonas* sp. catalyzes conversion of lupanine, a quinolizidine alkaloid produced by plants of the genus *Lupinus*, to 17-hydroxylupanine (9). The periplasmic enzyme is induced by growth of the organism on lupanine. It is a monomeric protein of M_r 72,000, containing one molecule of pyrroloquinoline quinone and one covalently bound heme as cofactors. The enzyme catalyzes dehydrogenation of lupanine, a reaction in which the cofactor pyrroloquinoline quinone acts as the initial electron acceptor. The electrons are then shunted through the C-terminal cytochrome *c*-type domain to an external electron acceptor that has not yet been identified. However, the enzyme will rapidly reduce horse heart cytochrome *c*, which is used as electron acceptor in the enzyme assay (11). We have been interested in identifying the endogenous electron acceptor in the lupanine hydroxylase-catalyzed reaction.

In a previous study employing a strategy to isolate the *Pseudomonas* lupanine hydroxylase gene (10) which contains a cytochrome *c*-related domain, a shotgun cloning approach using a PINK reporter system that facilitates detection of overexpressed recombinant hemoproteins was employed (13). By using this system in place of cytochrome b_5, a gene coding for a cytochrome c_{552} was isolated, and the cytochrome *c* gene was then cloned into a pUC derivative. The presence of a secretory signal in the cytochrome *c* suggested that the hemoprotein could be targeted to the periplasmic space of the lupanine-utilizing organism, where it would be expected to play a role in accepting electrons from lupanine hydroxylase.

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Periplasmic *c*-type cytochromes are initially synthesized as preapocytochrome forms carrying an N-terminal signal sequence that facilitates precursor translocation via the *s*-dependent preprotein translocase (15). The covalent coupling of the imported heme to the signal-processed apocytochrome is generally thought to occur posttranslocationally in the more oxidizing periplasmic environment of facultative microorganisms. Many enteric bacteria, such as *Escherichia coli*, whose dependency on *c*-type cytochromes arises under anaerobic conditions, have evolved a complex pathway for the maturation of the hemoproteins. At least eight *ccm* genes of the so-called A-H cluster located downstream of the *napF* operon are thought to be essential for cytochrome *c* maturation (24). Related genes have also been found in *Bradyrhizobium japonicum* and *Pseudomonas denitrificans* (15, 21). Most of these components are localized in the periplasm or are membrane bound with their functional domains facing the periplasm. Although the detailed functioning of these proteins in the maturation process is incomplete, three broad groups of activities can be categorized. The first group translocates heme from the cytosol to the periplasm. The second group is thought to participate in the reduction of a disulfide bridge of the vicinal Cys residues in the apocytochrome for linkage with the vinyl groups of the incoming heme. The final group is thought to constitute the heme lyase activity (19).

In the present study, we report on the isolation and sequence of the lupanine-transforming *Pseudomondas putida* cytochrome c_{552} gene. Heterologous expression of the cytochrome gene resulted in abundant production of a functional heme-assembled cytochrome *c* in the periplasm of *E. coli* cultured under standard aerobic batch conditions without the need for *ccm* complement. The isolated cytochrome *c* was functional in accepting electrons from lupanine hydroxylase during metabolism of lupanine.

MATERIALS AND METHODS

Strains, culturing, and subcellular fractionation. *E. coli* strains used were TB1 genotype [F *ara*Δ(*lac-pro*AB)*rps* ϕ 80*d lacZ*Δ*M15hsdR17* (r_k^+ m_k^+)] and N4830-1 genotype {F^- *suo thi-1 thr-1 leuB6 lacY1 fhuA21 supE44 rfbD1 mcrA1 his ilv galK8* Δ(*hemF-esp*) Δ(*bio uvrB*) [λ Δ*Bam* N^+ *c*I857 Δ(CroattR)]}. N4830-1 is particularly suitable for high-level expression of recombinant proteins. It is a p_L transductant of strain N4830 (7) and carries the temperature-sensitive *c*I857 λ repressor. Cells harboring appropriate plasmid were batch cultured in Luria-Bertani broth (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, and 1% [wt/vol] sodium chloride) containing 75 μg of ampicillin/ml with orbital agitation at 30°C for the durations stated elsewhere in the text. Subcellular fractionations of *E. coli* (500 ml) cultivated for 15 h were prepared as described previously (12).

DNA manipulations. Plasmid and genomic DNA was isolated with the Wizard DNA purification system (Promega). The restriction and DNA modifying enzymes (New England Biologicals) were used as recommended. The standard procedures for DNA manipulations involving their restrictions, modifications, isolation, introduction into *E. coli* cells, and sequencing were performed as described previously (4). DNA sequencing was performed by employing a Long ReadIR 4200 Li-Cor automated fluorescent sequencer (MWG).

Protein characterization. The N-terminal analysis of the purified cytochrome *c* was performed by automated Edman degradation on an Applied Biosystems sequencer (model 473A) at the sequencing service of the University of Nottingham. The protein content in bacterial fractions was estimated by using the Bio-Rad Coomassie blue assay kit (Bio-Rad, Hemel Hampstead, United Kingdom) with bovine serum albumin as the standard. Polypeptide patterns were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) employing the discontinuous buffer system of Laemmli (14). Lupanine hydroxylase activity was measured by the spectrophotometric assay with the isolated *Pseudomonas* cytochrome *c* as the electron acceptor (10). Cytochrome *c* content was estimated from the absorption difference of the α peaks at 552 nm between the sodium dithionite-reduced and oxidized forms of the hemoprotein, using an absorption coefficient of 27.7 mM^{-1} cm^{-1} (26). The molecular mass of the desalted cytochrome *c* was measured by using liquid chromatography orthogonal acceleration time-of-flight mass spectrometry (Micromass). The capillary and cone voltages were 3.2 kV and 50 V, respectively. The air-dried protein dissolved in 0.2% (vol/vol) formic acid (200 ng of protein/μl) was mixed with an equal volume of acetonitrile and injected at a rate of 5μl/min with nebulizer gas at 100 liters/h. The instrument was precalibrated with myoglobin.

Purification of cytochrome *c*. Periplasmic fractions were prepared by an osmotic shock method as follows. The cells were plasmolysed by suspension in 20 ml of 20% (wt/vol) sucrose–0.3 M Tris-HCl (pH 8)–1 mM EDTA (STE buffer) and incubation at 22°C for 10 min. Cells were harvested and resuspended in residual STE and osmotically shocked by rapid immersion in 2 ml of ice-chilled 0.5 mM $MgCl_2$. After incubation on ice for 10 min, the periplasmic fraction was recovered by centrifugation at 15,000 × *g* for 10 min. The periplasmic extract was passed through a DEAE-cellulose column (1 by 2.5 cm) equilibrated with 42 mM Na/K phosphate buffer, pH 7.0. The cytochrome passed straight through the column. The solution was concentrated by pressure filtration to 2.5 ml and loaded onto a Superdex 75 (Hiload 16/60) column that was eluted by fast-protein liquid chromatography with phosphate buffer containing 0.15 M NaCl. The flow rate was 1 ml min^{-1}, and fractions of 2 ml were collected. The purity of the reduced cytochrome was estimated from the ratio of A_{418} to A_{280} (26).

Nucleotide sequence accession number. The nucleotide sequence of the *Pseudomonas* cytochrome *c* gene has been deposited in the GenBank/EMBL Nucleotide Sequence Database under the accession number AY279525.

RESULTS

Cloning and expression of the *Pseudomonas* cytochrome *c*. In a strategy to isolate the *Pseudomonas* lupanine hydroxylase gene (10), which contains a cytochrome *c*-related domain, we employed a shotgun cloning approach using the PINK reporter system that facilitates detection of overexpressed recombinant hemoproteins (13). *Hin*dIII-fragmented *Pseudomonas* chromosomal DNA (approximately 2 kbp) was cloned into a derivative of pEX-PINK vector. Several *E. coli* N4830 transformant colonies displaying a deep red color were isolated. The insert DNA sequence from one such colony was subsequently recloned in pUC9 vector downstream of the *lac* promoter, and recombinant plasmids were introduced into *E. coli* TB1 cells.

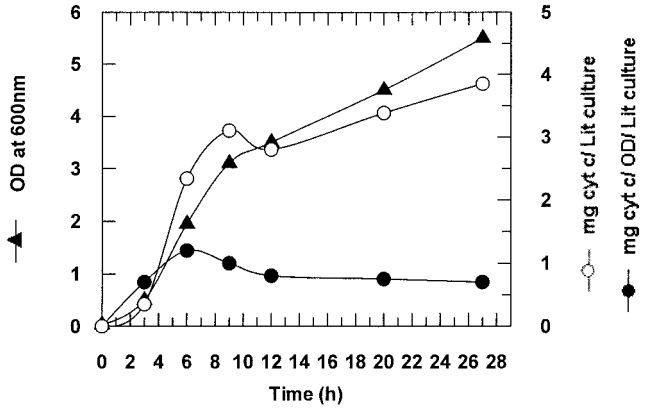

FIG. 1. Periplasmic production of cytochrome *c* in *E. coli* TB1 pLQ318 as a function of bacterial growth.

Spectral analysis of the cell lysate of a cell line that was batch cultured under standard aerobic conditions indicated expression of a recombinant hemoprotein that exhibited a characteristic absorption of spectrum of a *c*-type cytochrome 552 (see below). The abundant production of a potential holo-cytochrome *c* in a reduced state when cells were batch cultured under standard aerobic conditions indicated that the cloned gene was constitutively expressed in *E. coli*. In order to investigate the subcellular localization of the recombinant protein, *E. coli* TB1 cells cultured for 20 h were fractionated into periplasmic, cytoplasmic, and membrane fractions. Effective subcellular fractionation was confirmed from the greater-than-90% enrichment of the marker enzyme activity associated with each of the subcellular fractions, namely, alkaline phosphatase (periplasm), malate dehydrogenase (membranes), and fumarase (cytosol). More than 95% of the total cellular recombinant cytochrome *c* content was recovered with the periplasmic extract.

Periplasmic buildup of cytochrome *c* monitored as a function of growth time showed that this occurred throughout the aerobic growth phase but reached a peak of about 4 mg of the hemoprotein per liter of culture at the end of the exponential growth phase (Fig. 1). Under anaerobic growth conditions, no significant production of the hemoprotein was observed (data not shown).

Purification and characterization of the *Pseudomonas* cytochrome c_{552}. The cytochrome *c* was purified in two steps by using a periplasmic extract isolated from *E. coli* harboring plasmid pLQ318. The details are summarized in Table 1. The 17-fold purification yielded a highly purified cytochrome *c*,

TABLE 1. Purification of cytochrome *c*

Purification step	Volume (ml)	A_{418}/A_{280}	Cytochrome *c* (mg)	Yield (%)
Shock extract[a]	50	0.330	2.85	100
DEAE chromatography	60	0.416	2.42	84.9
Gel filtration	6	5.71	1.21	42.5

[a] Prepared from 500 ml of *E. coli* pLQ318 cultured for 18 h in Luria-Bertani medium containing 75 μg of ampicillin/ml.

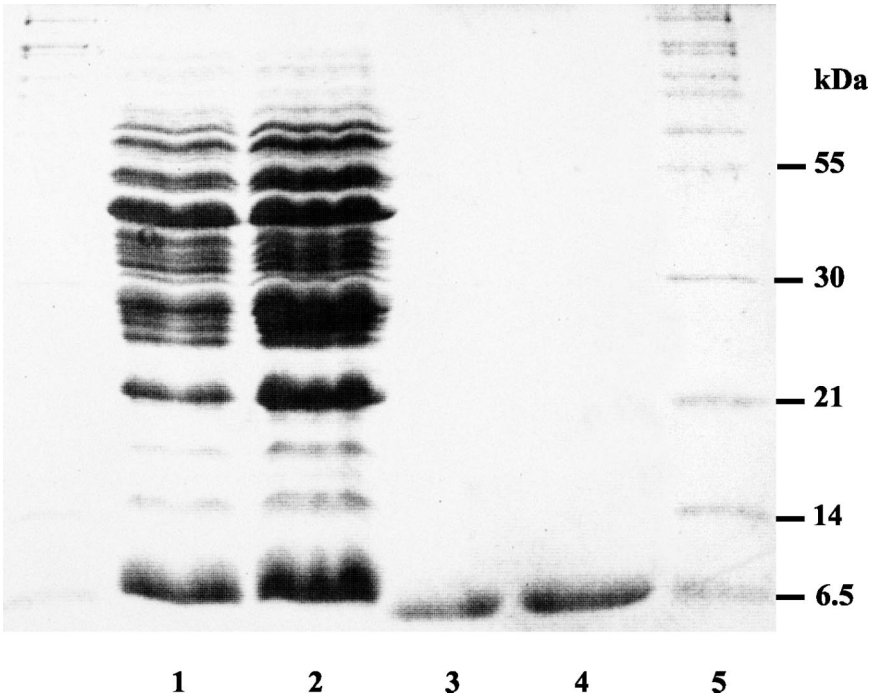

FIG. 2. SDS-PAGE analysis of the purified cytochrome *c*. Lanes 1 and 2, periplasmic fractions from the hosts harboring the progenitor plasmid pLQ (control) and the recombinant pLQ318, respectively; lanes 3 and 4, purified cytochrome *c* with approximate loadings of 1.5 and 3 μg of protein, respectively; lane 5, protein molecular markers.

with a recovery of 43%. The purity was estimated from a ratio of A_{418} to A_{280} for reduced cytochrome of 5.71. The A_{418}/A_{280} for pure horse heart cytochrome *c* has been reported to be 5.61 (26). It should to be pointed out that although the expressed cytochrome *c* in the periplasmic fraction is not visible by Coomassie blue staining following electrophoretic separation, possibly due to interference in this region by endogenous *E. coli* protein, the presence of the hemoprotein was clearly detected by heme staining. Furthermore, the high purity of the purified cytochrome *c* was also evident by detection of a single major band when examined by SDS-PAGE (Fig. 2). Spectrally, the recombinant hemoprotein had an oxidized protein Soret band at 416 nm, a reduced protein Soret peak of increased absorbance at 418 nm, and visible peaks at 522 and 552 nm, identifying it as a *c*-type cytochrome 552 (Fig. 3). The lesser absorbance in the UV region was indicative of a lower content of aromatic residues in the protein. The isolated cytochrome was functional (1.5 A_{551} units/min/mM cytochrome *c*) in accepting electrons from lupanine following its dehydrogenation by lupanine hydroxylase (10). The hemoprotein was resistant to denaturation following precipitation in 80% (vol/vol) acetone or to heat inactivation when boiled for 5 min. Upon recooling, the cytochrome was fully functional in its ability to give a typical reduced spectrum.

The first 20 amino acids from the N terminus of the purified cytochrome *c* were sequenced by automated Edman degradation. This gave the following sequence (starting with N terminus at the left): Q E G E E I F K A K P C V A C H A V E T. The molecular masses of the purified hemoprotein determined by SDS-PAGE and electrospray mass spectrometry were about 6,500 and 9,595.

Sequence of the cytochrome *c* gene. The sequence of the cloned DNA segment containing the cytochrome *c* gene is shown in Fig. 4. The region coding for the cytochrome *c* was identified by using the Blast search (1), which revealed the presence of the hemoprotein whose sequence potentially encoded a polypeptide of 82 residues that was strongly related to hemoproteins from *Pseudomonas mendonica* (58% identity)

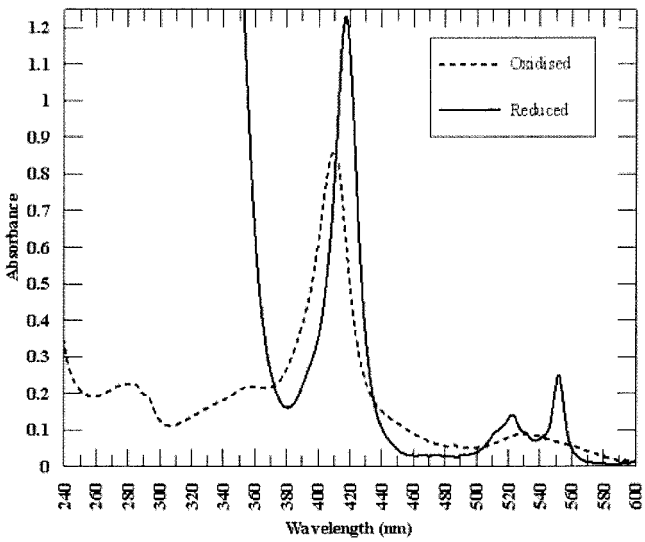

FIG. 3. Spectral characteristics of the isolated cytochrome *c*. The reduced spectrum was derived by reduction of the oxidized protein (approximately 100 μg of cytochrome *c*/ml in 10 mM Tris-HCl [pH 8]–1 mM EDTA) with sodium dithionite.

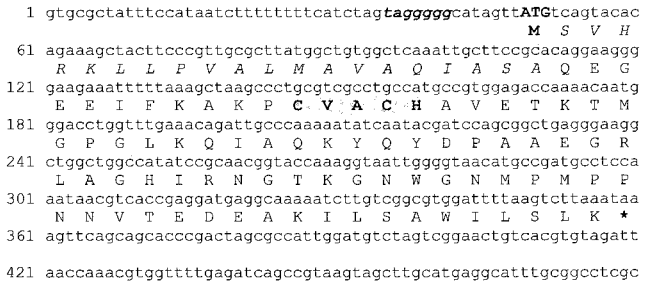

FIG. 4. DNA sequence coding for cytochrome *c*. Potential ribosomal binding sites are shown in bold italicized letters, initiation start codon is shown by bold uppercase letters, and the heme-binding domain in apocytochrome is shown in gray shadings. The asterisk denotes the termination codon.

and *Hydrogenobacter thermophilus* (48% identity) (Fig. 5). The derived sequence confirms a low content of aromatic residues in the protein shown by the low absorbance in the UV region of the isolated cytochrome *c* (Fig. 3). A potential reading frame starting with Met as the initiator residue, located at nucleotide 49 (Fig. 4), encoded a well-characterized cleavable signal sequence of 21 amino acid residues that precedes the hemoprotein sequence as determined by Edman degradation of the purified protein. These findings thus strongly indicated that hemoprotein was coded for by the cloned DNA sequence and that the precytochrome was processed by signal peptidase I at the predicted position (ASA↓QEGE) following its periplasmic localization. The molecular mass of 9,595 of the cytochrome *c* as determined by the electron spray method was in precise agreement with that calculated from the processed precursor polypeptide sequence determined from the DNA sequence. The lower estimate by SDS-PAGE (Fig. 2) may be due to a more compact globular fold preserved by the thioether link between the heme and the protein backbone.

DISCUSSION

In the present study, we have isolated the gene coding for a cytochrome c_{552} from a lupanine-transforming *P. putida*. On the basis of sequence similarity, the cytochrome *c* is of class I type and can be subgrouped among other bacterial proteins such as *Pseudomonas* sp. c_{551}, *H. thermophilus* c_{552}, and *Rhodospirillum tenue* c_{553}. Sequence characteristics include several Pro residues around the potential sixth heme ligand, a Met (18), and also a conserved Trp residue near the C terminus (2). In these proteins, the heme group is covalently attached by thioether bonds to two conserved Cys residues in the apoprotein. The consensus sequence for this site is Cys-X-X-Cys-His, and the histidine residue is one of the two axial ligands of the heme iron (16). In common with other bacterial *c*-type cytochromes, the *P. putida* cytochrome *c* resides in the periplasmic space, and so their mode of biogenesis has attracted significant interest, and a model for the assembly of *c*-type cytochromes has been proposed (23).

In facultative anaerobic organisms such as *E. coli*, *c*-type cytochromes are not essential for aerobic growth. However, under anaerobic growth, several *c*-type related cytochromes are expressed in response to availability of trimethylamine-*N*-oxide. In a study on the heterologous production of *Desulfovibrio vulgaris* cytochrome c_3, it was shown that *E. coli* was capable of synthesizing and exporting the heterologous cytochrome polypeptide but failed to insert the heme (17). Production of other heterologous monoheme cytochrome *c* proteins in a number of *E. coli* strains has also proved difficult under a range of growth conditions. A deletion mutant of *E. coli* which lacked the entire *ccm* cluster annulled maturation of indigenous *c*-type cytochromes synthesized under anaerobic respiratory conditions, with nitrite or trimethylamine-*N*-oxide as the electron acceptors (25). However, significant production of various *c*-type cytochromes, including *Desulfovibrio* tetraheme cytochrome *c* (5), under aerobic conditions in *E. coli* has been made possible by complementing strains with plasmid encoding the full complement of *ccm* gene cluster (3, 20). Clearly, dependable expression of *c*-type cytochromes in *E. coli* has relied on the presence of the entire A-H cluster harbored in plasmids and culturing under anaerobic conditions (6).

In contrast to these findings, in the present study, *Pseudomonas* cytochrome *c* was efficiently expressed in *E. coli* under both aerobic conditions and in the absence of the full complement of *ccm* elements. Sinha and Ferguson (22) reported that an *E. coli* strain carrying a *ccm* deletion substantially expressed a signal sequence-less *H. thermophilus* cytochrome c_{552} in the cytoplasm. This mode of maturation of *H. thermophilus* cytochrome c_{552} in the cytoplasm of *E. coli* was indeed unique among bacterial *c*-type cytochromes. These workers suggested that the thermostability of the apocytochrome c_{552} may have a sufficiently stable tertiary structure to allow the heme to slot into its binding pocket, which in turn triggers the spontaneous covalent attachment between apocytochrome c_{552} and heme. The *Pseudomonas* cytochrome *c* is closely related in structure to that of *H. thermophilus*, and both show significantly high thermostability. The greater thermostability may be due to unique aromatic amino acid interactions between Asp 37 and Tyr 34 and the possible hydrophobic contacts with the side chains of Tyr 34, Ala 41, and Leu 46 (8). Thus, it is possible that the assembly of *Pseudomonas* cytochrome *c* in *E. coli*

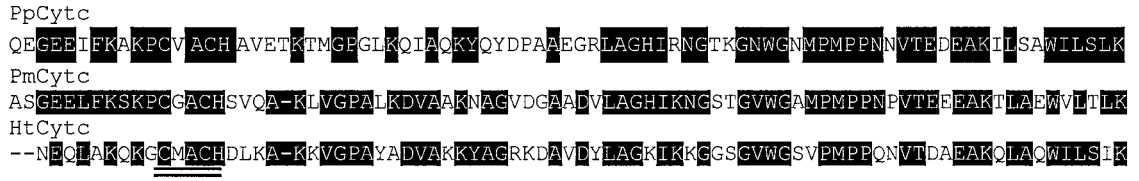

FIG. 5. Alignment of the amino acid sequences of cytochrome *c* from *P. putida* (PpCytc) with periplasmic forms of *P. mendonica* (PmCytc) species and *H. thermophilus* (HtCytc). The comparisons were performed without the signal sequences. Residues that are common to all sources are shaded in black. The double underlined regions are putative heme-binding regions of the cytochromes.

under aerobic conditions without the need for induced *ccm* cluster may be possible in light of the finding that the processed apoprotein in the periplasm may have a sufficiently stable structure to allow spontaneous heme association. The presence of a secretory signal that is functional in exporting the *Pseudomonas* cytochrome *c* in *E. coli* is indicative of its periplasmic location in the lupanine-utilizing organism, where it may play a role in accepting electrons from lupanine hydroxylase.

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