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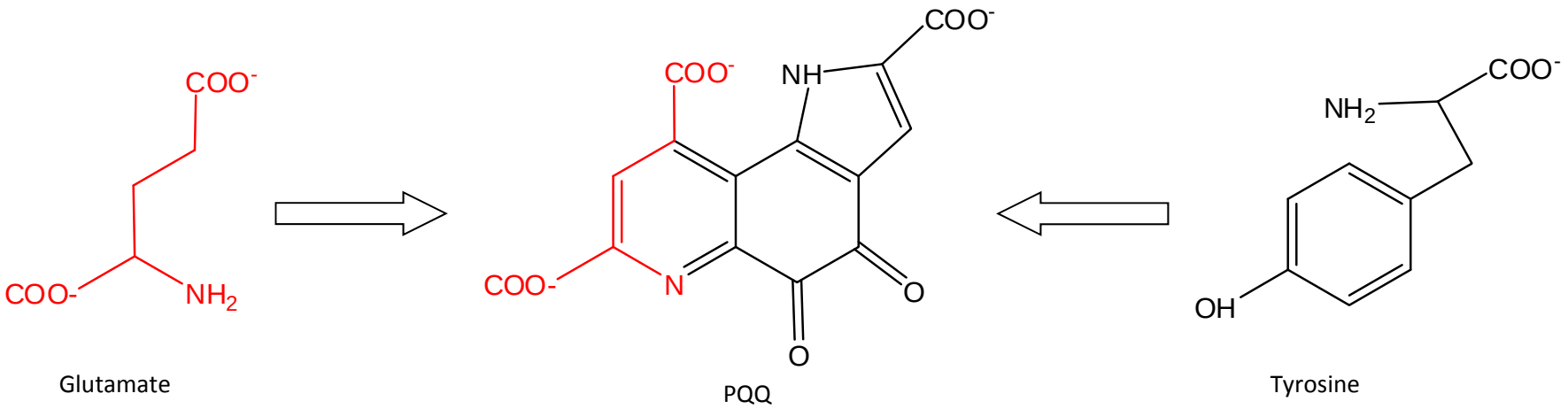
Keywords:	pyrroloquinoline quinone, Escherichia coli periplasmic space, lupanine hydroxylase

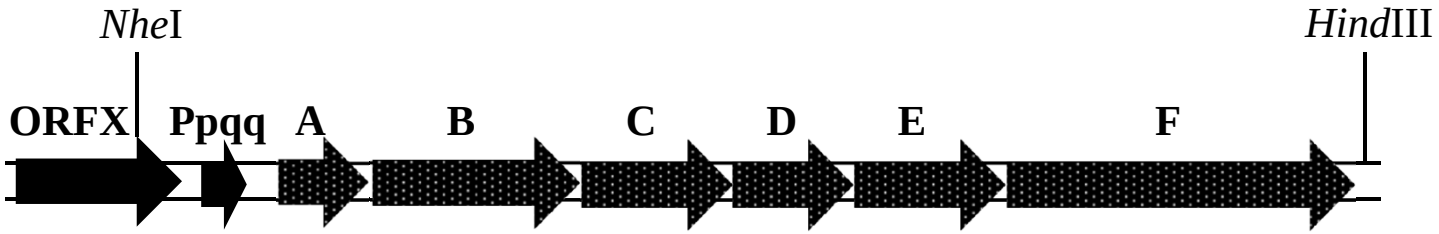
Fig. 1

a



Glutamate PQQ Tyrosine

b

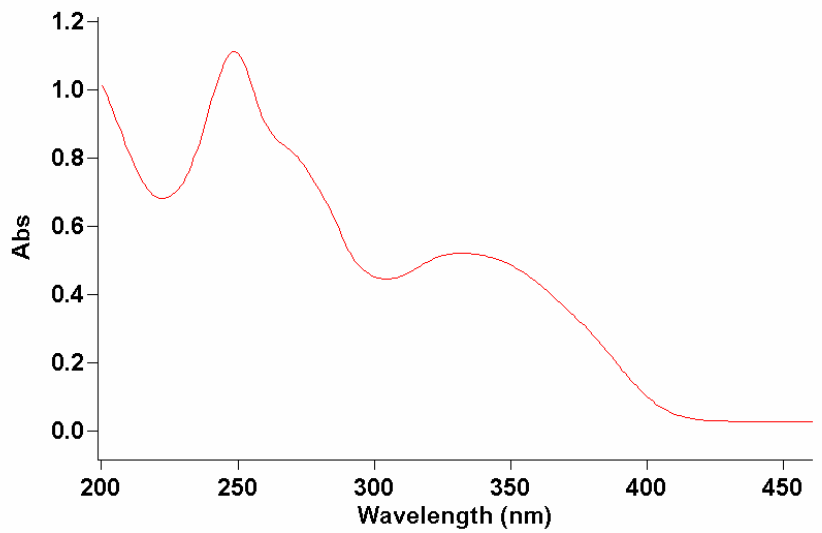


pBCP165 PQQ operon region

6700bp

Fig. 2

a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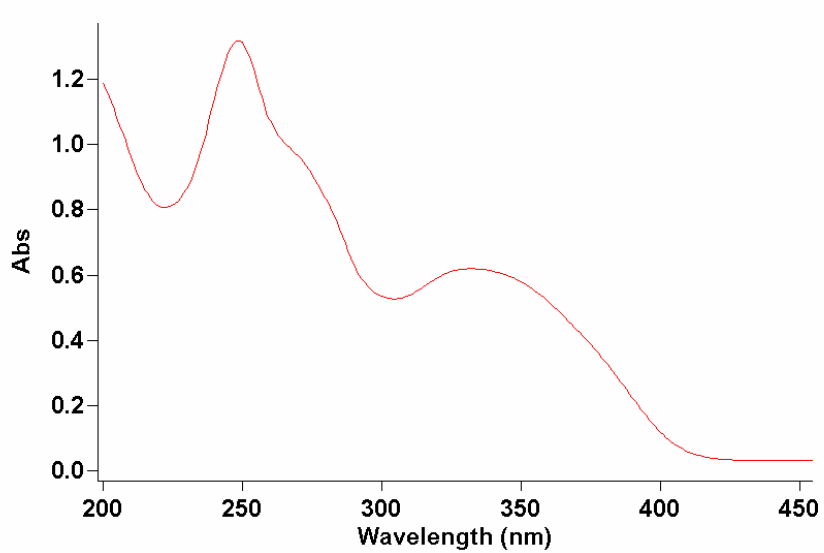
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SHORT COMMUNICATION

Co-expression of lupanine hydroxylase and pyrroloquinoline quinone leads to assembled and active recombinant lupanine hydroxylase in the *Escherichia coli* periplasm

Pavlos Stampolidis[1] • Naheed N. Kaderbhai[2] • David Bryant[2] • Ana Winters[2], Joseph Gallagher[2] • Mustak A. Kaderbhai*

[1]Department of Molecular Biology, Max Planck Institute of Biochemistry, 82152, Martinsried, Germany

[2]Institute of Biological, Environmental and Rural Sciences, Plas Gogerddan Campus, William Davies Building, Aberystwyth University, Aberystwyth, Ceredigion, SY23 3EE, United Kingdom

*Dr Mustak A. Kaderbhai is deceased

Correspondence: Naheed N. Kaderbhai; Tel: +44 1970 622294; Fax: +44 1970 823242; e-mail: nnk@aber.ac.uk

Keywords: pyrroloquinoline quinone, lupanine hydroxylase, *Escherichia coli,* periplasmic space, quinohaemoprotein, protein export

ABSTRACT

Lupanine hydroxylase (LH) is a quinohaemoprotein responsible for the conversion of the alkaloid, lupanine to 17-hydroxylupanine. Previous attempts to express the enzyme in *Escherichia coli* required *in vitro* addition of the co-factor pyrroloquinoline quinone (PQQ) and posed some impediments on subsequent structural studies for further characterization of the enzyme. An *E. coli* clone with LH and cytochrome *c* maturation operon was transformed with a third plasmid containing the PQQ operon from *Klebsiella pneumoniae*, *luh* gene and resulted in the production of periplasmically-targeted, correctly folded, PQQ and haem inserted active enzyme.

Interestingly, LH was less active than the *in vitro* incorporated PQQ-LH, presumably due to the incorporation of PQQ precursors in the periplasm. This is a first report of an active LH enzyme with *in vivo* incorporation of PQQ in *E. coli* and provides the necessary tool for further enzyme structural characterization.

Introduction

Pyrroloquinoline quinone [4,5-dihydro-4,5-dioxo-1*H*-pyrrolo-[2,3-*f*] quinoline-2,7,9-tricarboxylic acid (PQQ) is an important non-covalently bound redox cofactor in enzymes such as dehydrogenases, oxygenases and decarboxylases (Anthony 2001) along with NAD(P) and flavins (Duine and Jongejan 1989a). It is an aromatic, tricyclic, *ortho*-quinone and is a crucial link between compound oxidation and the respiratory chain (Duine and Jongejan 1989b). Enzymes containing PQQ as a cofactor are divided into two classes; Class I quinoproteins contain PQQ and Ca^{2+} as cofactors and class II quinohaemoproteins, in addition to PQQ and Ca^{2+} also carry haem at their C-terminus (Stoorvogel et al. 1996). In Class I quinoproteins, catalytic conversion of the substrate results in PQQ accepting two electrons ($PQQH_2$) which are then transferred to another redox protein (Davidson 2004), whereas Class II quinohaemoproteins transfer electrons from PQQ and then relay onto the haem moiety within the molecule before being donated to an external acceptor (Oubrie et al. 2002).

In eukaryotes, the presence of PQQ has so far been confirmed in mammalian tissues and milk, however, no enzymatic reaction which directly necessitates PQQ has been reported yet (Steinberg et al. 2003).

Studies in micro-organisms have revealed that a number of PQQ maturation chaperones are required for its synthesis and the genes coding for these factors are all organized into clusters that exhibit a high level of sequence homology (Meulenberg et al. 1990). Examples are *Methylobacterium extorquens* possessing seven PQQ maturation chaperones, *Klebsiella pneumonie* has six and *Acinetobacter calcoaceticus* has only four chaperones (Puehringer et al. 2008).

Interestingly, there is an absence of PQQ in some prokaryotes as a number of bacterial species are reported to express quino-enzymes lacking PQQ. An example in *Escherichia coli* is expression of glucose dehydrogenase minus PQQ (Matsushita et al. 1997) and the reason as to why *E. coli* would produce an enzyme whilst unable to produce its cofactor remains a mystery. One theory is that *E. coli* is in possession of an alternative pathway of PQQ production with a yet unidentified inducer (Biville et al. 1991). A second theory is that *E. coli* scavenges PQQ from its environment and the finding that mobile *E. coli* strains move chemo-tactically towards PQQ reinforces this argument (DeJonge et al. 1996). Although the complete biosynthetic pathway of PQQ remains elusive, tyrosine and glutamate (Fig. 1a) are the two main precursors (Magnusson et al. 2004).

LH is a class II, type I quinohaemoprotein which contains equimolar amounts of PQQ, Ca^{2+} and haem as co-factors (Stampolidis et al. 2009). It is a 72 kDa monomeric enzyme responsible for the initial conversion of the alkaloid lupanine to 17-hydroxylupanine (Toczko et al. 1963). Heterologous expression of the *luh* gene in *E. coli* also necessitated co-expression of the cytochrome *c* maturation machinery (Thony-Meyer 2003), responsible for haem insertion into the apoform of the enzyme (Stampolidis et al 2009). Based on these factors, we investigated the co-expression and *in vivo* incorporation of a third plasmid coding for PQQ into the above *E.coli* clone co-expressing LH and cytochrome *c* maturation machinery.

Materials and methods

Bacterial strains, growth conditions and plasmids

Starter cultures of *E. coli* TB1 [F^- *ara* Δ(*lac-proAB*) ((ϕ*80dlac* Δ(*lacZ*)*M15*) *rpsL*(*Str*^{r}) *thi hsdR*] (New England BioLabs) clones harbouring plasmids pEC86 (cytochrome *c* maturation operon), pEV-LH32 (untagged LH) and pINK-LH-His_4 (tetra-His tagged LH) were cultured in Luria Bertani broth (1% (w/v) Tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl) containing 75μg mL^{-1} ampicillin and/or 50μg mL^{-1} chloramphenicol were grown to saturation for 16h at 30^0C and applied as 2% (v/v) inoculum for batch cultivation in the MOPS medium (Karim et al. 1993) with the appropriate antibiotics and orbital agitation at 125 rev min^{-1} for 18h at 21^0C. The clone pK-187-PQQ, pEC86/ pK187-PQQ and pEC86/ pINK-LH-His_4/ pK187-PQQ for PQQ synthesis, was induced in MOPS in the presence of 1% (w/v) Na-gluconate, 50μM IPTG and kanamycin at 50μg mL^{-1} and grown as above.

Plasmid pBCP165 was provided by J. C. Arents (Velterop et al. 1995) and plasmids pEV-LH32, pINK-LH-His_4 and pEC86 were from Dr M. A. Kaderbhai laboratory.

Periplasmic extraction from clones

Periplasmic extract was prepared by the osmotic shock method as described previously (Kaderbhai et al. 2012) and was recovered by centrifugation at 10,000*g* for 5min and stored frozen at -80^0C.

His-tagged LH purification using Qiagen Ni-NTA

His-tagged LH was routinely purified from periplasmic extracts of clone pEC86/pINK-LH-His_4 via passage through Qiagen Ni-NTA column and eluted with 100mM imidazole

(pH 8.0) and 100mM EDTA. Untagged LH was purified from the pEC86/pEV-LH32 clone on DE-52-DEAE-cellulose columns as described previously (Stampolidis et al. 2009).

PQQ purification using DEAE Sepharose Fast Flow

A 10mL volume of periplasmic extract from 2L culture of pKK-187-PQQ (5ml) was mixed with DEAE Sepharose slurry (10ml) and was gently shaken overnight at 4^0C. The suspension was packed in a column, washed twice with 0.5M KPO_4 (pH 7.4) and eluted in 2mL total volume of 2M NaCl in 25mM Tris-HCl (pH 8). The salt was removed from the eluent by PQQ binding to a 2mL bed volume of C18 reverse phase column followed by two washes with 5mM HCl (pH 2.0) and final elution with 20% (v/v) methanol. PQQ was detected by diode-array detection and ESI-MS as described in Comont et al. (2012).

Identification of PQQ

Presence of PQQ in the isolates was detected spectrophotometrically at 257nm absorbance.

Measurement of *in vivo* and *in vitro* LH enzyme activity

LH was routinely activated on addition of 4mM $CaCl_2$ and incubation at room temperature for 1h. Activation of LH apoform *in vitro* necessitated addition of 200µM PQQ. LH was assayed at 25^0C using horse heart cytochrome *c* as the electron acceptor by increase in absorbance at 550nm due to its reduction as described by Stampolidis et al.

(2009). Assays were performed in duplicates, the reaction rate being linear during this time.

Results

Construction and expression of PQQ in *E. coli*

Plasmid pBCP165 comprising a 6.7 Kbp PQQ operon under the transcriptional control of a *K. pneumonie pqq* promoter was cleaved with *Nhe*I (blunted) and *Hind*III restriction endonucleases (Fig. 1b). The resulting fragment allowed closer proximity of the operon to the exogenous *lac* promoter present in the host plasmid. Colony PCR with primers, For-PQQ-Screen: 5'-GCCATCCTGCGGCAGC-3' and Rev-PQQ-Screen: 5'-CCCCCGGCCATTAATCCC-3' using part of the PQQ operon (For-PQQ-Screen) and part of the plasmid vector (Rev-PQQ-Screen) along with gene mapping with *Eco*RI and *Hind*III confirmed the presence of the entire PQQ operon in a positive clone designated as pK187-PQQ.

A red eluted band was obtained from the periplasmic extract of pKK187-PQQ after passage through a DEAE Sepharose Fast Flow column and gave a spectral scan with a major peak at 257nm, characteristic of PQQ. Comparison of this spectrum with a control spectrum of PQQ from *Methylophilus methylotrophus* showed no significant difference indicating that the PQQ produced in pKK187-PQQ was of good quality and purity (Fig. 2b).

The PQQ from pKK187-PQQ showed a major peak that eluted at 17 minutes following separation by C_{18} reverse phase chromatography and had a molecular mass of 331.1 *m/z* $[M+H]^+$ using ESI-MS (supplementary data 1).

Co-expression of PQQ and LH in *E.coli*

A fully functional LH with *in vivo* PQQ incorporation was obtained with the construction of a three plasmid system. Isolated plasmid pK187-PQQ (Kam^r), LH encoding pINK-LH-His_4 (Amp^r) and cytochrome *c* maturation machinery encoding pEC86 (Cm^r) were co-transformed into *E. coli* TB1 cells to generate clone pEC86/ pINK-LH-His_4/ pK187-PQQ. The TB1 strain, a JM83 derivative, carries the *hsdR* mutation which facilitates transformation efficiency and is reported to provide plasmid stability in conjunction to protein expression and purification (Yanisch-Perron et al. 1985; Belo et al. 1996). Successful transformants were expressed in the MOPS phosphate-limited medium containing 1% (w/v) Na-gluconate and 50μM IPTG in the presence of the three antibiotics (see Materials and Methods) for 18h at 21^0C. Periplasmic extracts from osmotically shocked cells were prepared as described in the Materials and Methods section.

Comparative activity of *in vivo* PQQ incorporated LH apoenzyme and *in vitro* activated LH apoenzyme

Comparison of tetra-His-tagged LH activity with the pEC86/pEV-LH32 untagged LH showed no significant difference in enzymatic activity (Table 1). Comparison of the purified LH activity from clone pEC86/ pINK-LH-His_4/ pK187-PQQ with LH from clone pEC86/ pINK-LH-His_4 showed *in vivo* activated form exhibited 10% of the activity of *in vitro* activated form. Periplasmic extracts from clones pEC86, pK187-PQQ and pEC86/

pK187-PQQ were also assayed for LH activity as controls and gave no significant enzyme activity.

Interestingly, although *in vivo* form of LH exhibits only 10% activity of its *in vitro* counterpart, comparison of the PQQ levels of production between the two respective host clones appeared to be similar.

Discussion

As a result of the inability of *E. coli* to endogenously produce PQQ, initial efforts to express quinoproteins, heterologously, in a simple and inexpensive way were impeded. In an effort to tackle this issue, cloning of the entire operon for PQQ from *Klebsiella pneumonia* to *E. coli* was carried out (Meulenberg et al. 1990). Expression of the 6.7 kbp operon consisting of genes *pqq A*, *B*, *C*, *D*, *E* and *F* resulted in *in vivo* incorporation of PQQ into glucose dehydrogenase and subsequent optimization by Velterop and co-workers (Velterop et al. 1995) with incorporation of plasmid pBCP165 in *E. coli* strain *JA221* resulted in greater yields in the region of 180nM.

Periplasmic targeting of LH in an earlier study (Stampolidis et al. 2009) resulted in homogeneous production of the apoform of the enzyme and was readily transformed into holoform upon subsequent *in vitro* addition of PQQ. However, this approach posed some impediments to further work in characterization of the enzyme. Preliminary attempts to examine the effect of replacing Ca^{2+} in the active site of the enzyme with other ions proved inconclusive due to micro quantities of other ions in PQQ preparation. Moreover, X-ray diffraction data from crystals of *in vitro* correctly folded LH apoform failed to provide meaningful diffraction patterns.

In this study, co-expression of the engineered LH and cytochrome *c* with PQQ resulted in the production of active LH with successful *in vivo* incorporation of PQQ. The 10% activity of the *in vivo* incorporated PQQ-LH might be due to the incorporation of PQQ precursors into the active site of the enzyme. This occurrence of PQQ precursors is not uncommon and has been observed in alcohol dehydrogenase from *Pseudomonas testosteroni* with similar effects on the enzyme activity and no change in the quaternary structure of the protein (Jongejan et al. 1989). Another probable cause for this phenomenon could be extracellular targeting of PQQ which limits cofactor availability in the periplasm for apoform incorporation.

Future attempts to produce LH crystals suitable for X-ray diffraction will facilitate studies on the structure of quinohaemoproteins and increase our understanding on the catalytic breakdown of the alkaloid, lupanine. The outcome of our approach is a first report of *in vivo* recombinant LH apoenzyme synthesized in *E. coli* and provides the necessary tools for further LH enzymology studies.

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Conflict of interest:

The authors declare no conflict of interest.

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Figure Legends

Fig. 1: Generation of PQQ in *E. coli*.

a) Precursors of PQQ biosynthesis. Glutamate and tyrosine have been identified as the two precursors of PQQ. PQQ maturation requires at least four different chaperones

b) The PQQ operon in pBCP165. ORFX is schematic diagram of an open-reading frame coding for an unidentified protein (not essential for PQQ expression), Ppqq; PQQ promoter and A-F; PQQ maturation genes.

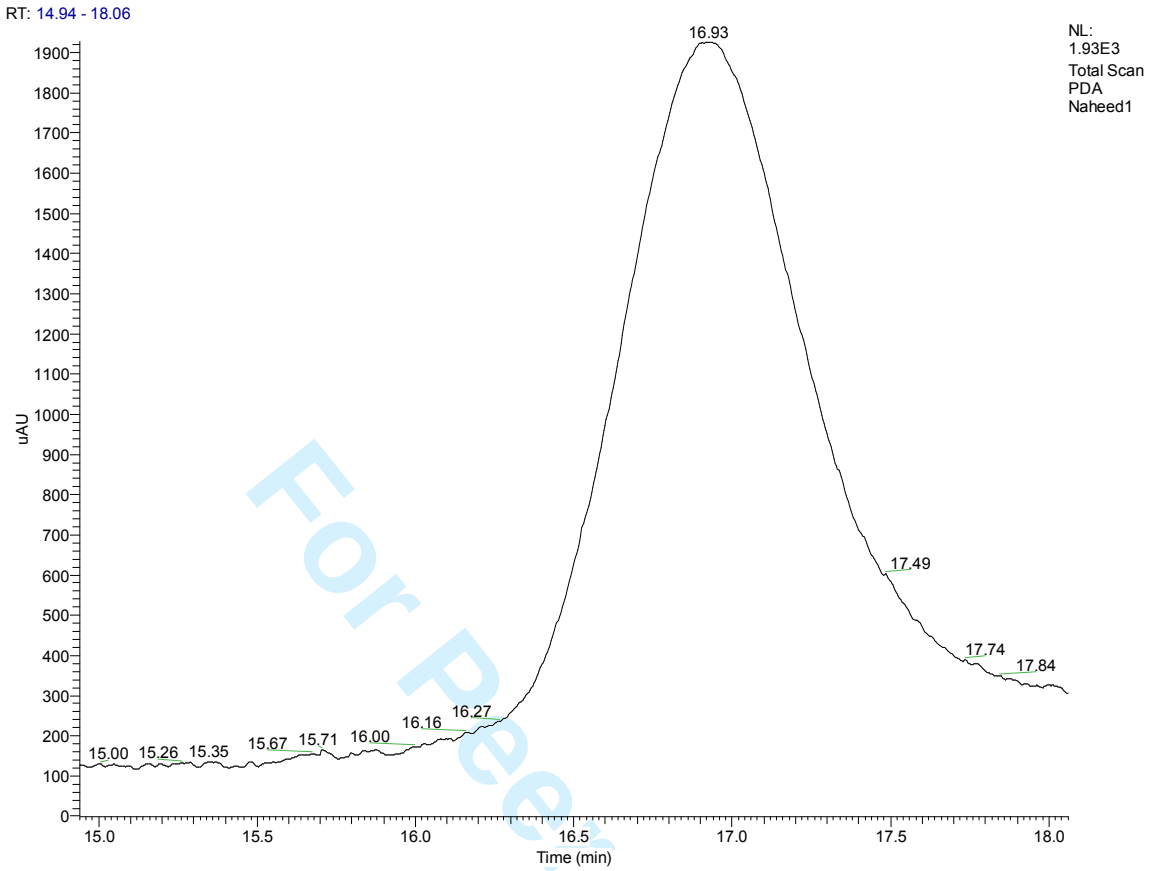
Fig. 2: PQQ spectra.

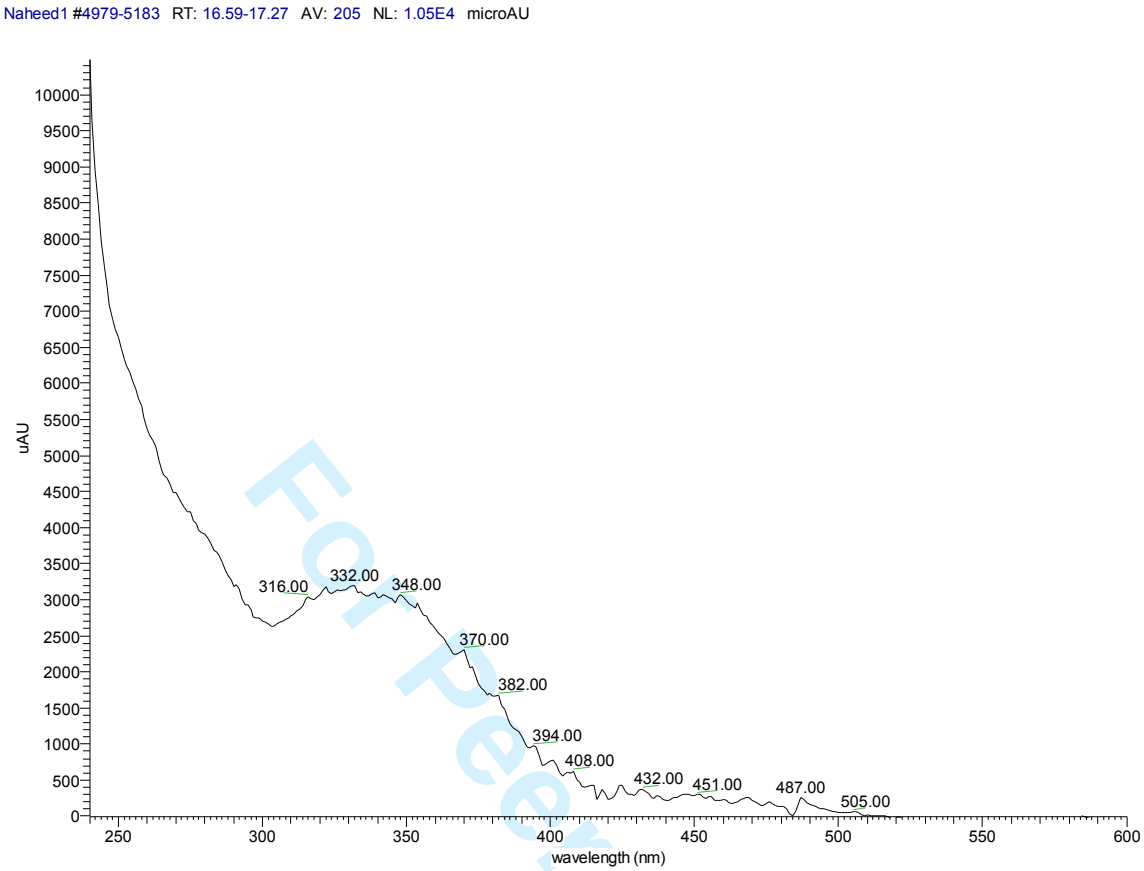
Spectral comparison of PQQ purified preparation from **a)** *Methylophilus methylotrophus* (control) and **b)** *Escherichia coli* pK187-PQQ.

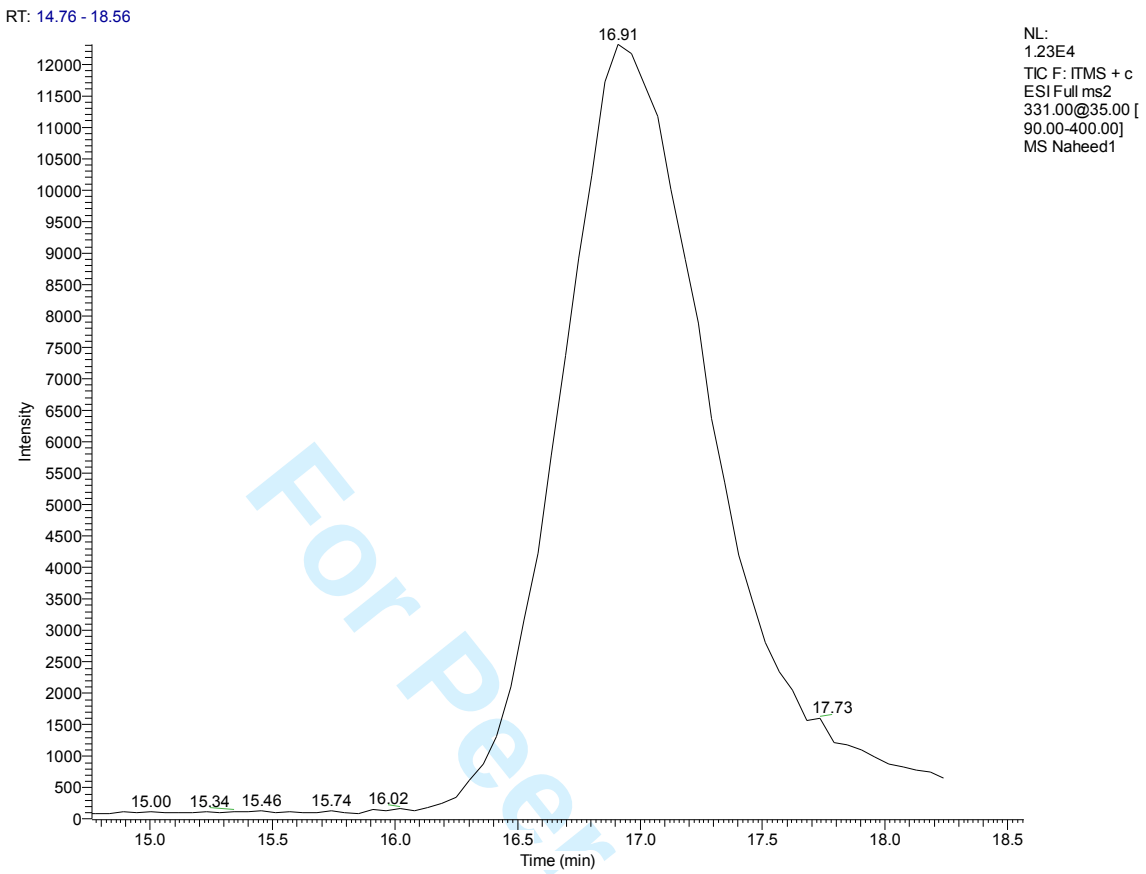
Table 1: Lupanine hydroxylase (LH) enzyme activity in *Escherichia coli* clones in the presence and/or absence of PQQ and Cyt*c* operons.

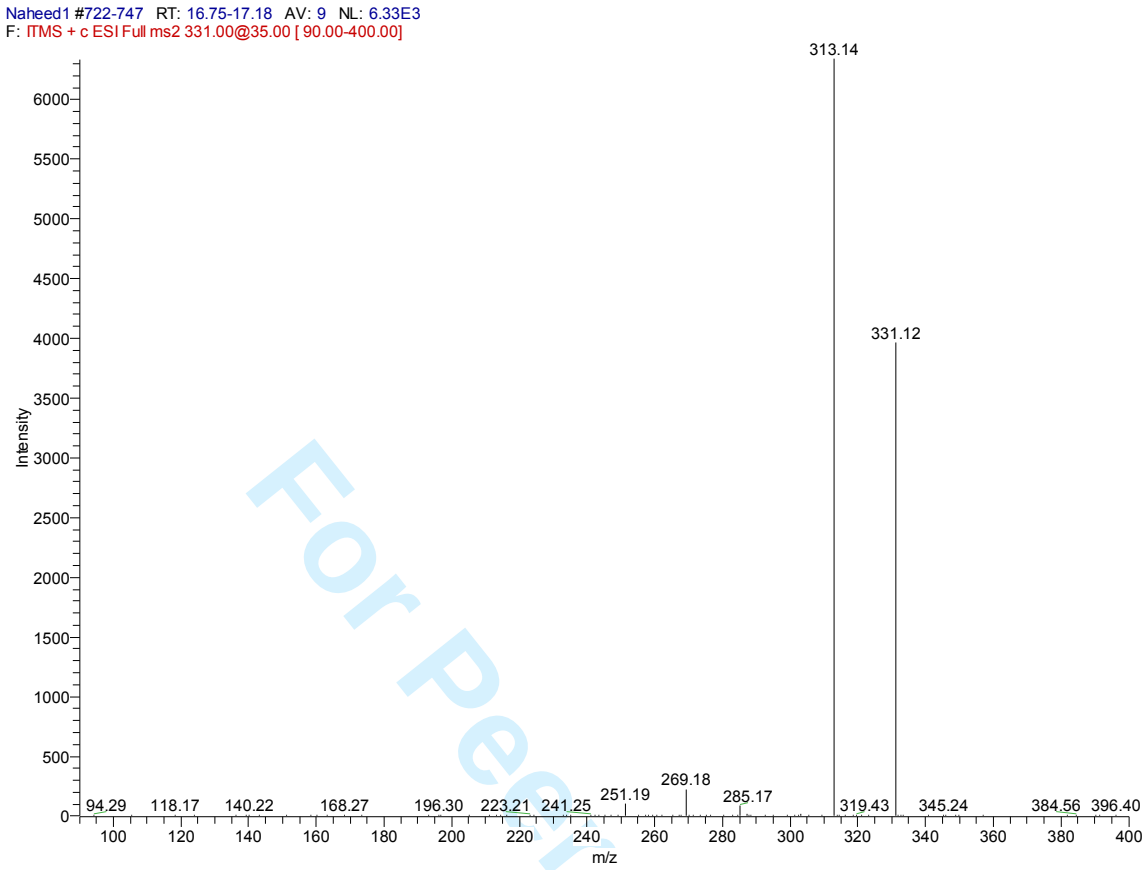
Clone name and description	LH expression	PQQ production	LH activity (units/mg periplasmic protein)
pEC86/pEV-LH32-untagged LH and Cyt*c* operon	+	-	**66**
pEC86/pINK-LH-His_4 tagged-Cyt*c* operon	+	-	**54**
pEC86/pINK-LH-His_4 tagged /pK187-PQQ and Cyt*c* operons	+	+	**6.8**
pEC86-Cyt*c* operon	-	-	**0.15**
pK187-PQQ operon	-	+	**0**
pEC86/pK187-PQQ and Cyt*c* operons	-	+	**0.30**

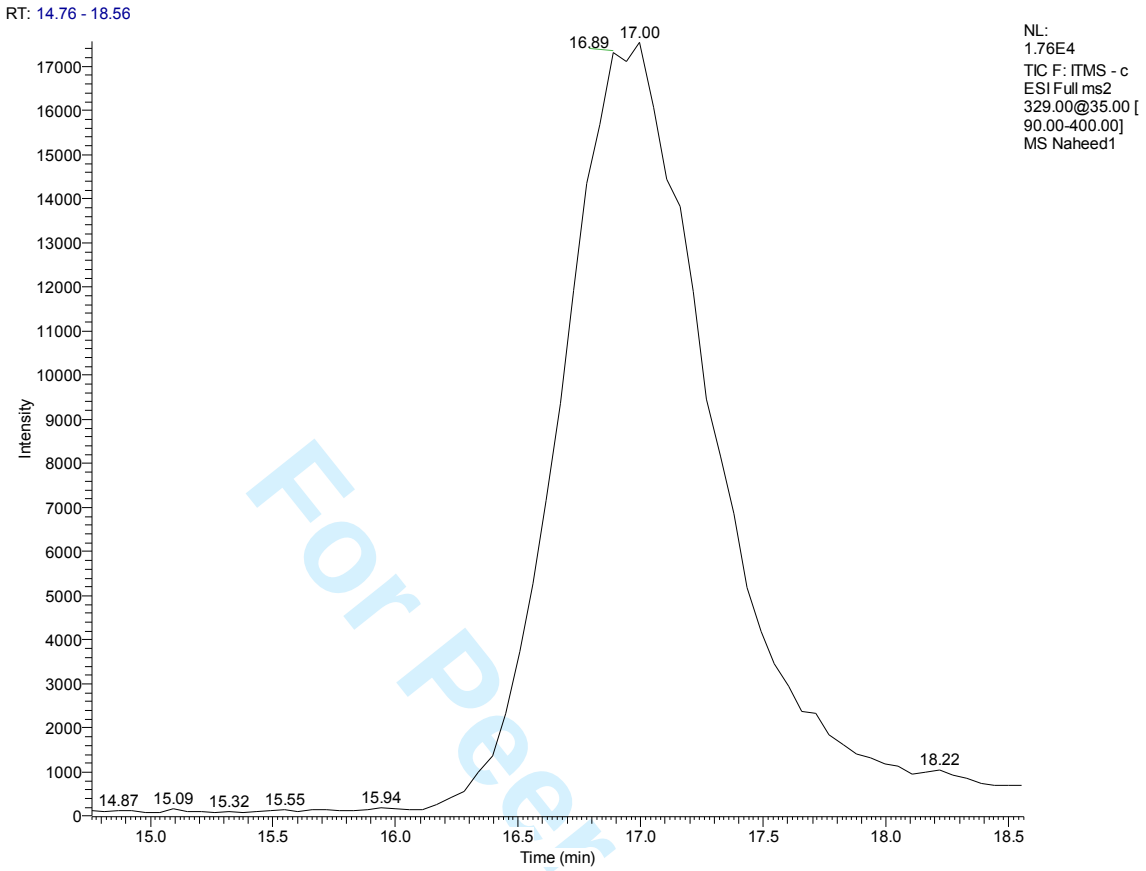
Signs represent presence (**+**) or absence (**-**) of LH expression and PQQ production.

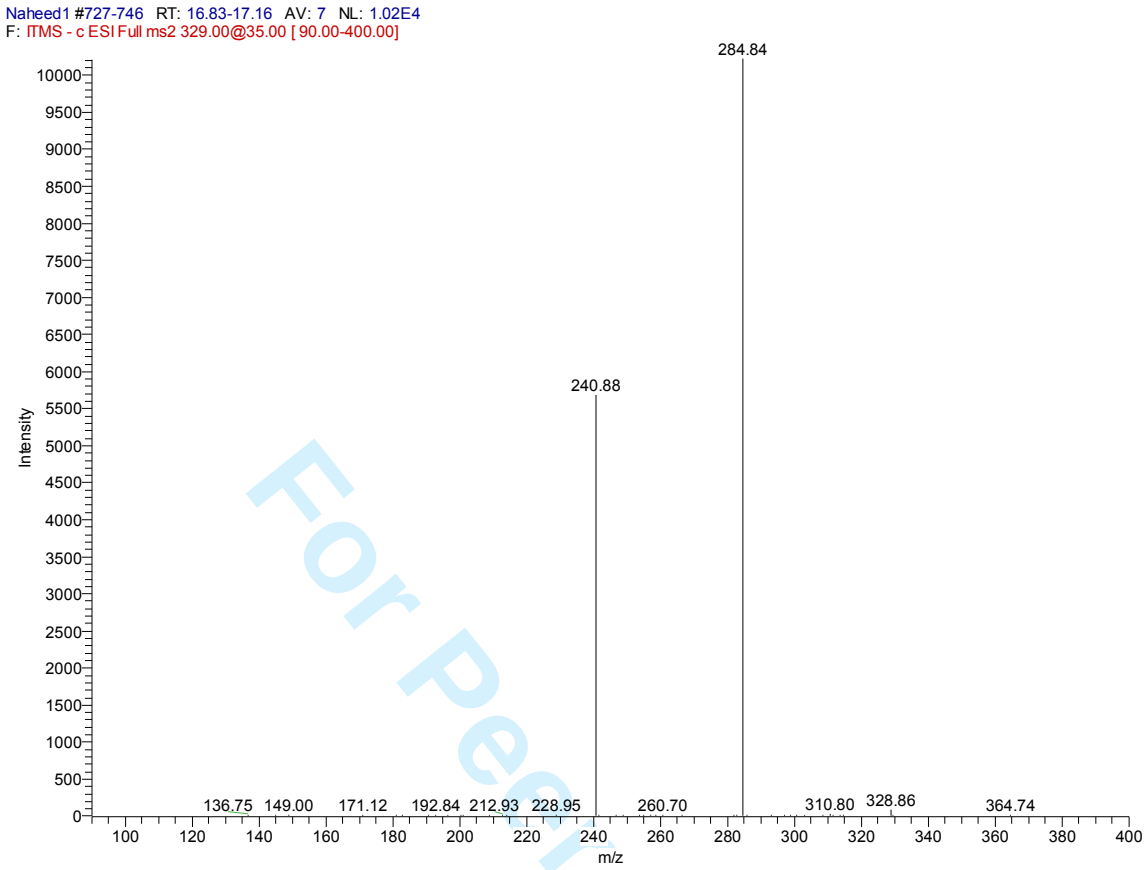












15% SDS-PAGE OF pEC86, pKK-187, pEC86/pEVLH32 (1), pEC86/pINK-LH-His_4 (2)