RESEARCH LETTER

An MTA phosphorylase gene discovered in the metagenomic library derived from Antarctic top soil during screening for lipolytic active clones confers strong pink fluorescence in the presence of rhodamine B

Hubert Cieśliński[1], Anna Długołęcka[1], Józef Kur[1] & Marianna Turkiewicz[2]

[1]Department of Microbiology, Chemical Faculty, Gdańsk University of Technology, Gdańsk, Poland; and [2]Institute of Technical Biochemistry, Technical University of Łódź, Łódź, Poland

Correspondence: Hubert Cieśliński, Department of Microbiology, Chemical Faculty, Gdańsk University of Technology, Narutowicza 11/12, 80-952 Gdańsk, Poland. Tel.: +48 58 347 16 05; fax: +48 58 347 18 22; e-mail: hcieslin@pg.gda.pl

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Abstract

In this work, we present the construction of a metagenomic library in *Escherichia coli* using the pUC19 vector and environmental DNA directly isolated from Antarctic topsoil and screened for lipolytic enzymes. Unexpectedly, the screening on agar supplemented with olive oil and rhodamine B revealed one unusual pink fluorescent clone (PINKuv) out of 85 000 clones. This clone harbored a plasmid, pPINKuv, which has an insert of 8317 bp that has been completely sequenced. Further analysis of the insert showed eight ORFs. Three ORFs among these exhibited similarities to *Psychrobacter arcticus* genes. A nucleotide sequence designated as ORF4 encoded a protein with 93% identity to the methylthioadenosine phosphorylase of *P. arcticus*. This protein was responsible for the observed pink fluorescence of the PINKuv clone in the presence of rhodamine B. We found that colonies of recombinant *E. coli* TOP10F′/pUC19-ORF4 strain showed pink fluorescence under UV illumination on the Luria–Bertani agar supplemented with rhodamine B after culturing at 25, 30 and 37 °C. The same effect was achieved using other *E. coli* strains such as DH5α, LMG194, JM101 and BL21(DE3) pLysS. The results presented here will provide the basis for further studies on the use of the discovered gene as a new reporter gene for molecular biology applications.

Introduction

The power of metagenomics is the access, without prior nucleotide sequence information, to the unculturable majority, which is estimated to be > 99% of the prokaryotic organisms (Amann *et al.*, 1995; Rappe & Giovannoni, 2003; Schloss & Handelsman, 2003; Daniel, 2004; Streit *et al.*, 2004). Many studies related to an activity-based strategy have demonstrated that the metagenomic approach allows for finding various biocatalysts such as lipases, esterases, amylases, cellulases, xylanases, proteases and nitrilases (DeSantis *et al.*, 2002; Gupta *et al.*, 2002; Yun *et al.*, 2004; Kim *et al.*, 2006, 2008; Hardeman & Sjoling, 2007; Park *et al.*, 2007; Hu *et al.*, 2008; Jeon *et al.*, 2008; Wei *et al.*, 2008) and novel antibiotics, such as turbomycin A and B (Gillespie *et al.*, 2002).

In this study, we constructed an Antarctic soil metagenomic library and screened for lipolytic active clones. Several clones with lipolytic activity were detected. One of the clones obtained lacked lipolytic activity, but it surprisingly showed a pink fluorescent phenotype in the presence of rhodamine B. We identified the gene responsible for this phenotype.

Materials and methods

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 1.

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Table 1. Bacteria strains and plasmids used in this study

Bacteria or plasmids	Relevant characteristics	Source
Bacteria		
E. coli TOP10F′	F′{*lac*Iq Tn*10*(Tet^R)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ* ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G,	Invitrogen
E. coli DH5á	F$^-$ ø80d*lacZ* ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 λ$^-$ *thi*-1*gyr*A96 *rel*A1	Invitrogen
E. coli LMG194	F$^-$ Δ*lacX74 galE galK thi rpsL* Δ*phoA* (PvuII) Δ*ara714 leu*::*Tn10*	Invitrogen
E. coli JM101	F′ *tra*D36 *pro*A$^+$B$^+$ *lac*I^q Δ*(lacZ)*M15/Δ*(lac-pro*AB*) gln*V *thi*, general cloning strain	NEBioLabs
E. coli BL21 (DE3) pLysS	F$^-$ *omp*T *hsd*SB (rB$^-$ mB$^-$) *gal dcm* λ(DE3)pLysS T1R	Novagen
Plasmids		
pUC19	Cloning vector, Amp^R, ColE1 *ori*, *lacZ*	Fermentas
pPlNKuv	pUC19 containing 8311-bp Sau3AI fragment of metagenomic DNA, Amp^R	This work
pPlNKuvKpnI	pPlNKuv with deleted 3593-bp KpnI fragment, Amp^R	This work
pPlNKuvKpnISacI	pPlNKuvKpnI with deleted 3027-bp SacI fragment, Amp^R	This work
pPlNKuvKpnISalI	pPlNKuvKpnI with deleted 1834-bp SalI fragment, Amp^R	This work
pUC19-ORF4	pUC19 containing 918-bp XbaI–BamHI fragment with ORF4 sequence from pPlNKuv plasmid, Amp^R	This work
pUC19-RSFP	Encoding recombinant form of RSFP protein without His-tag domain, cloning vector	This work
pUC19-His-tag-RSFP	Encoding recombinant form of RSFP with His-tag placed at the C-terminal of the protein, cloning vector	This work
pUC19-RSFP-His-tag	Encoding recombinant form of RSFP with His-tag placed at the N-terminal of the protein, cloning vector	This work
pET22b(+)	Expression vector, Amp^R	Novagen
pET22b-RSFP	Encoding recombinant form of RSFP protein without His-tag domain, expression vector	This work
pET22b-His-tag-RSFP	Encoding recombinant form of RSFP with His-tag placed at the C-terminal of the protein, expression vector	This work
pET22b-RSFP-His-tag	Encoding recombinant form of RSFP with His-tag placed at the N-terminal of the protein, expression vector	This work

Sampling

The analyzed soil sample was collected from Adelie penguin rookery (62°09′46″S, 58°27′42″W), which is located in the neighborhood of Henryk Arctowski Polish Antarctic Station at King George Island. The sampling depth was 0–5 cm. The soil sample was characterized by alkaline pH (8.9), hygroscopic water (∼35%) and the amount (% of dry weight) of organic carbon (1.52 ± 0.42), nitrogen (0.81 ± 0.24), phosphorus (4.13 ± 1.5) and sulfur (0.42 ± 0.08).

Next, the soil sample was sieved to remove penguin dung debris and particulate matter > 2 mm and was kept without disturbance at 4 °C until analysis.

General DNA manipulations

Restriction enzymes, T4 DNA ligase and alkaline phosphatase were purchased from Fermentas (Lithuania). Restriction enzymes and other DNA-modifying enzymes were used according to the manufacturer's recommendations. The reagents for PCR and various oligodeoxynucleotides were purchased from DNA-Gdansk II (Poland).

Metagenomic library construction and screening for lipolytic activity

DNA from soil samples was extracted based on a method described previously (Zhou *et al.*, 1996) with some modifications. First, 25 μL of lysozyme (10 mg mL^{-1}, A&A Biotechnology, Poland) was added to the DNA extraction buffer [100 mM Tris-HCl (pH 8.0), 100 mM sodium EDTA (pH 8.0), 100 mM sodium phosphate (pH 8.0), 1.5 M NaCl, and 1% CTAB]. Second, 50 μL of proteinase K (20 mg mL^{-1}, A&A Biotechnology) was added to the sample incubated at 37 °C with horizontal shaking at 225 r.p.m. for 30 min. Finally, the extracted DNA was further purified using the Genomic Mini kit (A&A Biotechnology), followed by final purification with the Genomic AX Bacteria kit (A&A Biotechnology). The plasmid library of the purified DNA was constructed using the following procedures. DNA samples from Antarctic soil were used to construct the genomic DNA library following partial digestion with Sau3AI, optimized to maximize fragments in the 1–5-kbp size range. The fragments of 1–5 kbp resolved in an agarose gel were excised and concentrated. The BamHI-digested and phosphatase-treated pUC19 DNA was ligated using T4 DNA ligase (Epicentre) to the genomic DNA library digested with Sau3AI, and *Escherichia coli* TOP10F′ (Invitrogen) were transformed with the ligation products. The lipase activities of the transformants were tested on Luria–Bertani (LB) agar containing 100 μg mL^{-1} of ampicillin, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 2% (v/v) olive oil and 0.001% (w/v) rhodamine B (the fluorescent dye). This LB agar was designed as an LB-lipRB medium. Colonies were incubated for 1 day at 37 °C, followed by incubation for 2

days at 25 °C. Orange fluorescent halos around lipase-positive *E. coli* strains could be seen when these plates were exposed to a UV light of 312 nm (Kouker & Jaeger, 1987). Ampicillin, IPTG and rhodamine B were purchased from Sigma-Aldrich.

Analysis of the pink fluorescent *E. coli* clone

The *E. coli* clone showing pink fluorescence was inoculated into a 250-mL flask containing 50 mL of LB medium (100 μg mL^{-1} ampicillin). After overnight incubation at 37 °C, the cells were harvested by centrifugation at 5000 ***g*** for 15 min and washed twice with sterile distilled water. Next, the plasmid DNA was isolated using the Plasmid Mini isolation kit (A&A Biotechnology), retransformed into *E. coli* TOP10F′ and the new clones were examined on the LB-lipRB medium for lipase activity. The plasmid DNA from one of the positive clones (pink fluorescence at UV light 312 nm) was isolated as described above and designated as pPINK-UV. Next, a few samples with plasmid pPINK-UV DNA were digested with selected restriction enzymes to create restriction maps of the examined plasmid. The nucleotide sequence of the DNA insert from the pPINK-UV plasmid was determined using an ABI 3730 xl/ABI 3700 sequencing instrument (Agowa DE, Germany). The ORFs were detected using the ORF FINDER (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) provided by the National Center for Biotechnology Information (NCBI). The predicted function of ORFs was annotated using BLASTX search against the NCBI nonredundant protein database (Altschul *et al.*, 1997).

Identification and isolation of the *rsfp* gene

The identification of the *rsfp* gene (ORF4 from pPINKuv plasmid) scheme is presented in Fig. 1. The putative *rsfp* gene was amplified from the pink fluorescent-positive clone (pPINKuv) using F_19 and R_19 primers (Table 2). The amplified DNA was digested with BamHI and XbaI enzymes and then ligated into BamHI and XbaI double-digested pUC19 (Fermentas). Finally, *E. coli* TOP10F′ cells were transformed with the pUC19-ORF4 construct. The transformed cells were grown at 37 °C for 24 h on LB-lipRB medium and exposed to a UV light of 312 nm. Finally, the pUC19-ORF4 plasmid DNA obtained from one of the positive clones (pink fluorescence at UV light of 312 nm) was isolated and sequenced using ABI 3730 xl/ABI 3700 sequencing technology (Agowa DE).

Characterization of the UV fluorescent recombinant strain of *E. coli* TOP10F′/pUC19-ORF4

The fluorescence of the recombinant *E. coli* TOP10F′/pUC19-ORF4 strain was examined: (1) during growth at 18, 25, 30 and 37 °C on LB-lipRB medium; (2) during growth at 18, 25, 30 and 37 °C on LB-lipRB medium without 2% (v/v) olive oil; (3) during growth at 37 °C on LB agar containing 100 μg mL^{-1} of ampicillin, 0.001% (w/v) rhodamine B with or without 0.1 mM IPTG; and (4) during growth at 37 °C on LB agar containing 100 μg mL^{-1} of ampicillin, 0.1 mM IPTG and 2% (v/v) olive oil without 0.001% (w/v) rhodamine B. After incubation, the Petri dishes with visible *E. coli* colonies were exposed to a UV light of 312 nm for fluorescence examination. These experiments were carried out simultaneously with the *E. coli* TOP10F′/pUC19 and the *E. coli* TOP10F′/pPINKuv strains used as a negative and positive fluorescence controls, respectively. All experiments were triplicated independently.

Characterization of UV fluorescent recombinant strains of *E. coli* transformed with pUC19-ORF4 plasmid DNA

The fluorescence of selected *E. coli* strains (Table 1) transformed with pUC19-ORF4 plasmid DNA was examined during growth at 37 °C for 24–48 h on LB-lipRB medium. After incubation, the Petri dishes with visible *E. coli* colonies were exposed to a UV light of 312 nm for fluorescence examination. These experiments were carried out simultaneously with the *E. coli* TOP10F′/pUC19 and the *E. coli* TOP10F′/pPINKuv strains used as negative and positive fluorescence controls, respectively. All experiments were triplicated independently.

Expression and purification of recombinant RSFP protein in *E. coli*

For expression of RSFP, His-tag-RSFP and RSFP-His-tag proteins, the *rsfp* gene was amplified by PCR using appropriate primers (Table 2). The amplified DNA fragments were digested with XhoI enzyme, and then inserted into the pUC19 vector. The resulting recombinant plasmids were designed as pUC19-his-tag-RSFP, pUC19-RSFP-his-tag and pUC19-RSFP. The correctness of the constructed plasmids was confirmed by DNA sequencing using ABI 3730 XL sequencing technology (Genomed, Poland). Next, the expression plasmids were constructed in a three-step procedure. Firstly, the pUC19-his-tag-RSFP, pUC19-RSFP-his-tag and pUC19-RSFP were digested with NdeI and XhoI enzymes. Secondly, the resulted DNA fragments were separated on a 1.0% agarose gel, extracted from the gel using Gel-out (A&A Biotechnology) and then inserted into the NdeI and XhoI sites of pET-22b(+) expression vector. The resulting expression plasmids, named pET22b-RSFP, pET22b-His-tag-RSFP and pET22b-RSFP-His-tag (Table 1), were used for production of the wild type and N–His-tagged RSFP proteins, respectively.

Overproduction of the RSFP proteins was performed in the BL21(DE3)pLysS (Novagen) cells carrying the

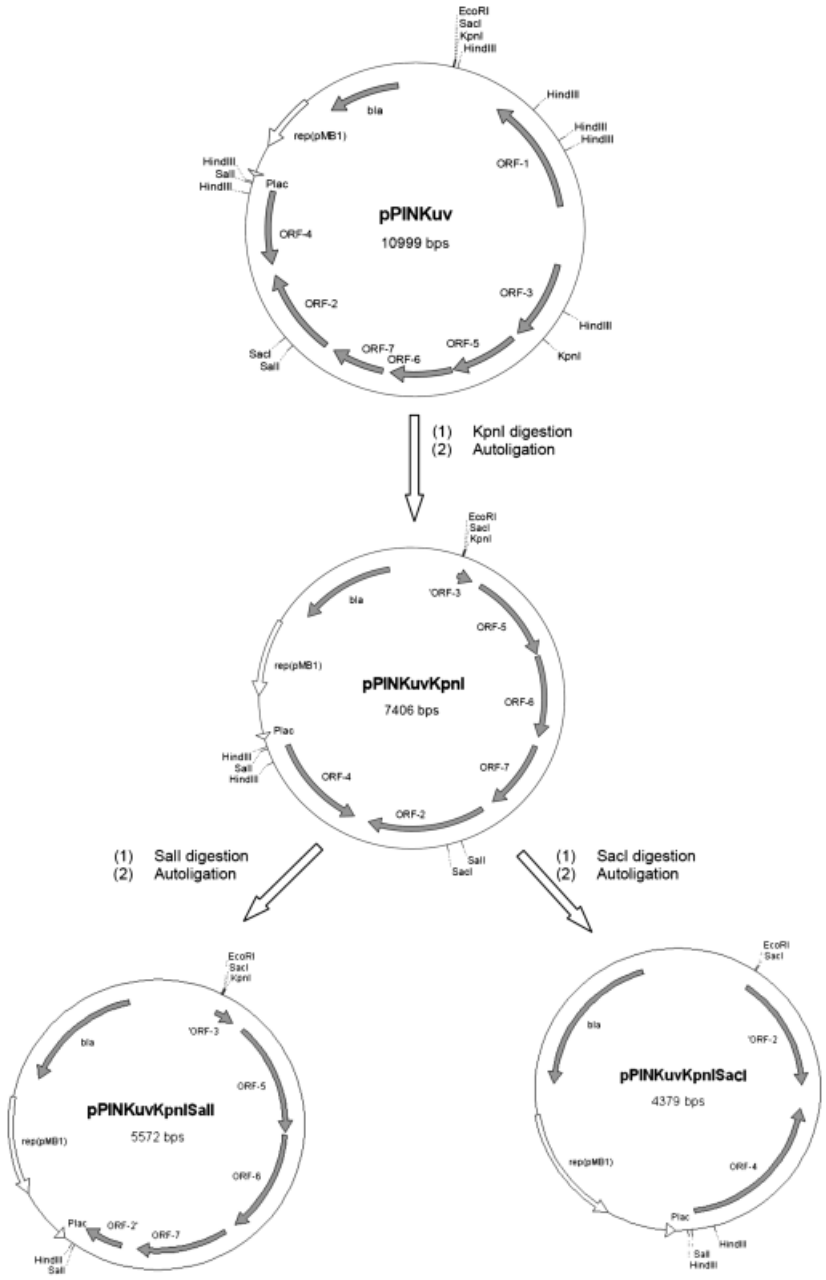

Fig. 1. Identification scheme of the *rsfp* gene.

pET22b-RSFP, pET22b-His-tag-RSFP or pET22b-RSFP-His-tag plasmids. Cells were grown at 30 °C in LB medium (1% peptone K, 0.5% yeast extract, and 1% NaCl) containing 20 μg mL^{-1} of chloramphenicol and 100 μg mL^{-1} of ampicillin. At $OD_{600\,nm} = 0.5$, IPTG was added to a final concentration of 1 mM and cultivation was continued for 5 h. Subsequently, cultures were centrifuged (5000 ***g***, 15 min, 4 °C) and pellets were resuspended in buffer A (50 mM Tris-HCl, pH 8.0). Samples were sonicated, and the cell debris and inclusion bodies were collected by centrifugation at 10 000 ***g***, for 20 min, at 4 °C. The precipitate containing inclusion bodies was solubilized and RSFP protein was refolded according to the previously described procedure (Cieśliński *et al.*, 2007).

His-tag RSFP proteins were purified using His-Bind Resin (Novagen) with Ni^{2+} ions according to the standard procedure. The purity of the proteins was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Laemmli (1970). The proteins' concentrations were measured using the method of Bradford (1976).

Table 2. Sequences of PCR primers used for PCR products amplification in this study

Primer pair	Primer name and sequence (5′–3′)	Source
A	F_19: GACTCTAGAGGATCATTTGGAGCCCAATATG	This work
	R_19: AATGGATCCTACGGAAGTAATGCTGCAAGT	This work
B	rsfp-His6-for*: AAACTCGAGCATATG*CACCATCATCATCATCATCA*CATGACTTCAACCGCAG	This work
	rsfp-rev: TTTCTCGAGCTACGGAAGTAATGCTGCAAGTCTTGTCT	This work
C	rsfp-for: AAACTCGAGCATATGACTTCAACCGCAGTAGAAATTACGGT	This work
	rsfp-His6-rev*: TTTCTCGAGCTA*GTGATGATGATGATGGTG*CGGAAGTAATGCTGCAAGTC	This work

*The DNA sequence (italic) of rsfp-His6-for and rsfp-His6-rev primers encoding His-tag domain.

Table 3. Sequence analysis of the ORFs encoded in metagenomic insert of pPINKuv

No. of ORF	Length (amino acid)	Putative function (most similar homologue)	Putative source organism	Accession number	% Identity/similarity	*E*-value
1	477	Hypothetical protein MDG893_17282	*Marinobacter algicola* DG893	ZP_01895580	55/66	$2e^{-139}$
2	351	Methyltransferase	*Psychrobacter arcticus* 273-4	YP_264441	95/97	0
3	322	Hypothetical protein Psyc_1160	*Psychrobacter arcticus* 273-4	YP_264445	97/98	0
4	297	Methylthioadenosine phosphorylase	*Psychrobacter arcticus* 273-4	YP_264440	93/96	$2e^{-163}$
5	269	Hypothetical protein Psyc_1159	*Psychrobacter arcticus* 273-4	YP_264444	90/94	$4e^{-143}$
6	246	Glycosyl transferase	*Psychrobacter arcticus* 273-4	YP_264443	82/91	$2e^{-117}$
7	212	Hypothetical protein Psyc_1157	*Psychrobacter arcticus* 273-4	YP_264442	86/92	$2e^{-98}$

The pink fluorescence assay

Ninety microliters of cell debris of *E. coli* BL21(DE3)pLysS/pET-RSFP after insoluble bodies separation (or 90 μL insoluble bodies' solution after refolding and dialysis to buffer A) were mixed with 10 μL of rhodamine B solution in buffer A (0.1 μg mL^{-1}). Simultaneously, 90 μL cell debris of *E. coli* BL21(DE3)pLysS/pET22b(+) (negative control) and 90 μL of buffer A were mixed with 10 μL rhodamine B solution in buffer A (0.1 μg mL^{-1}) (blank sample). Afterwards, the fluorescence of the samples was observed under UV (312 nm) and documented by photographs using a Versadoc model 1000 (Biometra). The same procedure was used for purified His-tag-RSFP and RSFP-His-tag proteins.

Nucleotide sequence accession number

The sequence of the *rsfp* gene was deposited in the GenBank database with accession number GQ202582.

Results

Construction and screening of a metagenomic library

A metagenomic library consisting of ~85 000 clones was constructed using DNA isolated from a soil sample collected from Adelie penguin rookery, which is located in the neighborhood of Henryk Arctowski Polish Antarctic Station at King George Island. To screen for lipase-producing clones, the pUC19-derivated clones were plated on an LB-lipRB medium. Several clones with lipolytic activity were detected. Unexpectedly, one of the obtained clones showed unusually strong pink fluorescence under UV illuminations without the expected orange fluorescent halo around it. Further examination revealed that this clone showed no lipolytic activity. This unusual clone was designated as pPINKuv.

Molecular analysis of pPINKuv

The insert of the recombinant plasmid recovered from *E. coli*/pPINKuv strain was sequenced. The nucleotide sequence analysis revealed the presence of seven ORFs longer than 100 codons. Three ORFs among those exhibited similarities to genes annotated with predicted functions (Table 3). Moreover, the results from the Table 3 showed that the metagenomic DNA insert is probably derived from genomic DNA of cold-adapted bacteria belonging to the *Psychrobacter* genus.

In order to identify the gene(s) responsible for the pink fluorescence, the metagenomic insert was partially digested with selected restriction endonucleases and the restriction fragments were subsequently cloned into pUC19 (Fig. 1). *Escherichia coli* TOP10F′ were transformed with the resulting constructs. The recombinant *E. coli* strains were screened for pink fluorescence again on LB-lipRB medium. This strategy was successful for two constructed plasmids designated as pPINKuvKpnI and pPINKuvKpnISacI. Two corresponding *E. coli* strains, *E. coli*/pPINKuvKpnI and *E. coli*/pPINKuvKpnISacI, were indistinguishable from the original

clone with respect to the observed pink fluorescence. In contrast to these results, the colonies of the *E. coli*/pPINKuvKpnISalI strain growing on the LB-lipRB medium did not show any pink fluorescence under UV illumination.

The positive pPINKuvKpnISacI clone with the smallest insert (1710 bp) contained the full sequence of the ORF4 and the partial sequence of the ORF2 from the pPINKuv clone (Fig. 1). Moreover, ORF4 was cloned in the orientation that allowed to express the ORF4 from the pLac promoter present in the pUC19 plasmid. We decided to clone the DNA fragment containing the ORF4 and its 25-bp upstream flanking region from the pPINKuv plasmid to the pUC19 vector. The metagenomic DNA fragment with the ORF4 sequence was amplified by PCR and inserted into pUC19 downstream of the pLac promoter. The 25-bp DNA sequence between the pLac promoter and the ATG starting codon of the ORF4 sequence was the same as in the metagenomic DNA. *Escherichia coli* TOP10F′ were transformed with the resulting pUC19-ORF4 plasmid, and the *E. coli* TOP10F′/pUC19-ORF4 colonies showed pink fluorescence on LB-lipRB medium under UV illumination.

In the next stage of our study, *E. coli* TOP10F′ were transformed with pUC19-ORF4, pPINKuv and pUC19 to screen for colony pink fluorescence after growth at 18, 25, 30 and 37 °C on LB-lipRB medium with or without supplementation of the olive oil. The *E. coli*/pPINKuv and the *E. coli*/pUC19-ORF4 colonies showed pink fluorescence after growth at 25, 30 and 37 °C. The colonies of the same strains did not show any pink fluorescence when cultured at 18 °C. We did not observe distinguishable differences in the fluorescence of the *E. coli*/pPINKuv and the *E. coli*/pUC19-ORF4 colonies (Fig. 2). Moreover, the colonies of both the strains analyzed showed comparable pink fluorescence after growth on media with and without oil olive supplementation (Table 4).

The *E. coli*/pUC19-ORF4 and *E. coli*/pPINKuv colonies showed weaker intensities of fluorescence after growth on the LB agar plates supplemented with ampicillin and rhodamine B than on the LB agar plates supplemented with ampicillin, IPTG and rhodamine B. This result confirmed that IPTG induced the expression of the RSFP protein from the pLac promoter from both the pUC19-ORF4 and the pPINKuv plasmid in *E. coli*.

The colonies of *E. coli*/pUC19-ORF4, *E. coli*/pPINK-UV and *E. coli*/pUC19 did not show any pink fluorescence on the LB agar plates supplemented with ampicillin, IPTG and oil olive but without rhodamine B. The presence of rhodamine B in culture media was crucial for the pink fluorescence of the recombinant *E. coli*/pUC19-ORF4 colonies.

The following *E. coli* strains: LMG194, JM101, DH5α and BL21(DE3)pLysS (Table 1) were transformed with pUC19-ORF4, pPINKuv or pUC19 plasmids, and examined for pink fluorescence. The examined strains transformed with pUC19-ORF4 or pPINKuv plasmids showed pink fluorescence under screening conditions.

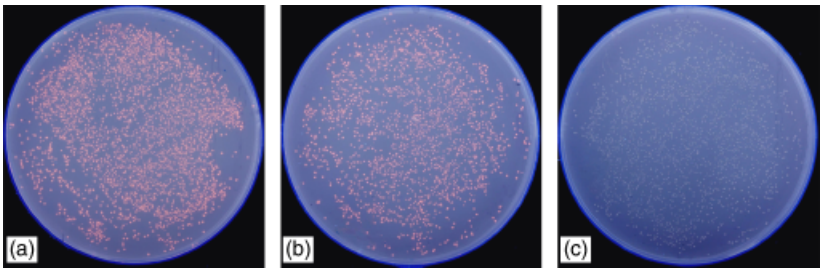

Fig. 2. Fluorescence of the *Escherichia coli* TOP10F′/pPINKuv (a), *E. coli* TOP10F′/pUC 19-ORF4 (b) and *E. coli* TOP10F′/pUC19 (c) colonies on the LB-lipRB plates without supplementation of olive oil under UV illumination (312 nm) after growth at 37 °C.

Table 4. The fluorescence of the *Escherichia coli* TOP10F′/pUC19, *E. coli* TOP10F′/pUC19-RSFP and *E. coli* TOP10F′/pPINKuv colonies after growth at different temperatures

	Colonies fluorescence at UV 312 nm							
	LB-lipRB medium with olive oil				LB-lipRB medium without olive oil			
Strains	18 °C	25 °C	30 °C	37 °C	18 °C	25 °C	30 °C	37 °C
E. coli TOP10F′/pUC19	−	−	−	−	−	−	−	−*
E. coli TOP10F′/pUC19-RSFP	−	+	+	+	−	+	+	+*
E. coli TOP10F′/pPINKuv	−	+	+	+	−	+	+	+*

+, presence of pink fluorescence; −, lack of pink fluorescence (background fluorescence).
*The results presented in Fig. 2.

Sequence analysis of the ORF4 product

The results reported above showed that the ORF4 product was responsible for the observed pink fluorescence. A nucleotide sequence of ORF4 encoded a protein with 93% identity to the methylthioadenosine phosphorylase of *Psychrobacter arcticus* 273-4 (GenBank accession no. AAZ19006). On this basis, we decided to name the ORF4 as an *rsfp* gene (rhodamine B, screening for lypolytic active clones, pink fluorescence of *E. coli* clones at UV light and methylthioadenosine phosphorylase (MTA phosphorylase).

Expression and purification of recombinant forms of the RSFP protein

To characterize whether the *rsfp* gene product interacts with rhodamine B, we heterologously expressed the *rsfp* gene in *E. coli*. For this, we constructed three recombinant strains: *E. coli* BL21(DE3)pLysS/pET-RSFP, *E. coli* BL21(DE3)pLysS/pET-His-tag-RSFP and *E. coli* BL21(DE3)pLysS/pET-RSFP-His-tag that overexpressed recombinant RSFP proteins without modification of the amino acid sequence and with the polyhistidine-tag at the N- or the C-terminus of the protein, respectively. Unfortunately, in each case, most of the RSFP proteins were found to be insoluble (data not shown). Furthermore, the refolded RSFP protein and the His-tag-RSFP and RSFP-His-tag proteins before and after purification using metal-affinity chromatography did not show any pink fluorescence in the presence of rhodamine B (the pink fluorescence assay). In contrast to this, the cell debris of the tested strains revealed strong fluorescence in the presence of rhodamine B (Fig. 3). However, the fluorescence disappeared after approximately 1 h of incubation of cell debris at 37 °C or 16 h at 4 °C. The addition of dithiothreitol (1 mM) to cell debris prolonged the time of observed pink fluorescence for analyzed samples.

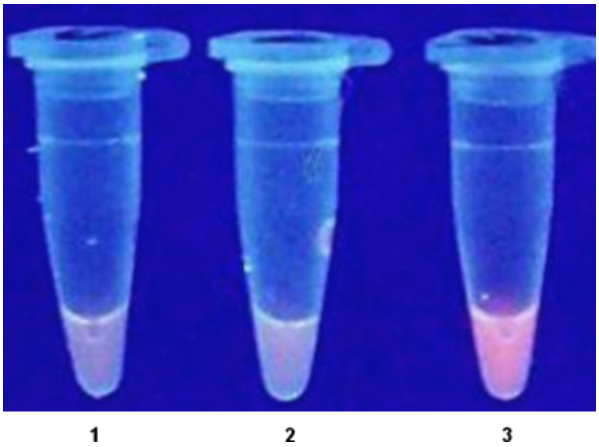

Fig. 3. The pink fluorescence assay: tube 1, the rhodamine B solution in buffer A (blank); tube 2, the cell debris of *Escherichia coli* BL21(DE3)-pLysS/pET22b (negative control); and tube 3, the cell debris of *E. coli* BL21(DE3)pLysS/pET22b-RSFP (analyzed sample).

Discussion

This is the first report describing the pink fluorescence phenomenon of the recombinant *E. coli* cells expressing the MTA phosphorylase gene growing on media containing rhodamine B.

Rhodamine B is a fluorescent dye used in a variety of biological studies, for example, as a marker and tracer in animal and insect studies (Lindsey, 1983; Blanco *et al.*, 2006), as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential (Reungpatthanaphong *et al.*, 2003), as a marker in multi-drug-resistant protein activity determination (Smital & Kurelec, 1998), as an extremely specific stain for cornification (Liisberg, 1968) and as a fluorescent dye in the complex with free fatty acids in a plate assay for bacterial lipases (Kouker & Jaeger, 1987).

The *rsfp* gene product shows strong similarity to the methylthioadenosine phosphorylase from *P. arcticus* 273-4 (Table 3). The gene coding for MTA phosphorylase has been identified in many organisms, in all three kingdoms: Archaea, Bacteria and Eukarya. MTA phosphorylase is responsible for the first step in the methionine salvage pathway after the transfer of the amino acid moiety from *S*-adenosylmethionine (Sekowska *et al.*, 2004). A human MTA phosphorylase is known as a ubiquitously expressed housekeeping gene in normal human tissues and cells. This enzyme has also been found to be deficient in a variety of human cancers (Behrmann *et al.*, 2003; Berasain *et al.*, 2004; Subhi *et al.*, 2004; Hellerbrand *et al.*, 2006; Marcé *et al.*, 2006). However, to our knowledge, there is no information about the participation of MTA phosphorylases in rhodamine-dependent fluorescence of the bacterial cells under UV illumination.

We found that the expression of the methylthioadenosine phosphorylase encoded by the ORF4 (*rsfp* gene) of the pPINKuv plasmid in *E. coli* cells and the presence of rhodamine B in the screening media were crucial for the observed pink fluorescence phenomena. The differences in the genetic background of the analyzed *E. coli* strains and a copy number of recombinant plasmid with the *rsfp* gene have no influence on the molecular mechanism responsible for the pink fluorescence observed. We constructed recombinant *E. coli* clones harboring low copy number recombinant pACYC184-rsfp plasmids that show the same intensity of pink fluorescence (data not shown).

The constructed *E. coli* strains showed pink fluorescence at 25, 30 and 37 °C, but not at 18 °C. At this stage of research, we are unable to explain the lack of fluorescence of the colonies grown at 18 °C without the elucidation of the

roles of the MTA phosphorylase and rhodamine B in the pink fluorescence phenomena. To do this, we obtained recombinant RSFP proteins, partially purified from insoluble bodies. However, these proteins did not show any fluorescence in the pink fluorescence assay. In contrast to this, the cell debris of recombinant *E. coli* strains showed fluorescence. These results suggest that besides the RSFP protein and rhodamine B, an additional factor is required for pink fluorescence phenomena. At the present stage of our research, we do not have enough data to explain the mechanism of the observed fluorescence phenomenon. Further studies will be necessary to clarify the relationship between the presence of rhodamine B in media and the MTA phosphorylase identified in this mechanism. The results presented in this paper encourage us to test the *rsfp* gene as a new marker gene (*in vivo*), for example, as a marker gene for a new set of promoter-trapping vectors for psychrotrophic or mesophilic bacteria.

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