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Proceedings of

Humboldt Kolleg :

SYNERGY, NETWORKING AND THE ROLE OF FUNDAMENTAL RESEARCH DEVELOPMENT IN SOUTH EAST ASIA

in conjunction with :

THE INTERNATIONAL CONFERENCE ON NATURAL SCIENCES (ICONS) 2011

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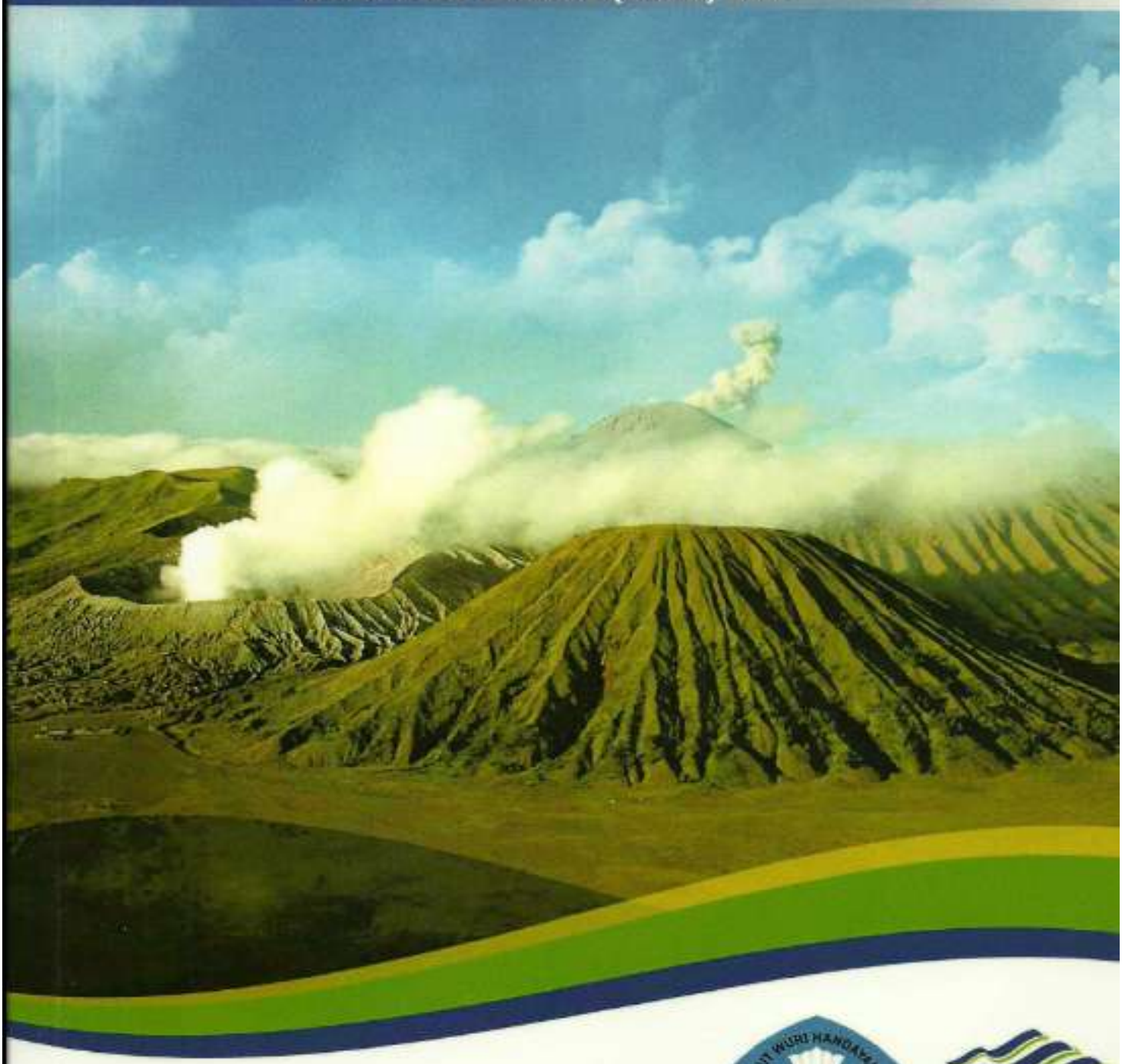

The Bureau of Planning and International Cooperation, Ministry of National Education

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FOREWORD

The First International Conference on Natural Sciences, July 9-11, 2011 in Batu, East Java, Indonesia, brought together scientists from nine countries from South-East Asia, Germany and Japan. South-East Asia is extremely rich in natural resources, many of them still untapped, but has also extremely densely populated areas that have to cope with the ensuing problems including infrastructure measures, intensive agri- and aquaculture, waste management, and nature preservation. Study, use and development of existing resources and coping with the aforementioned problems, requires interdisciplinary cooperation. Based on a network of Alexander von Humboldt alumni, the conference aimed at linking the wide professional expertise, at making the best use of existing equipment and pinpointing gaps, and at integrating basic and applied research.

This book is a mosaic of the impressive oral and poster presentations of the conference. It reflects the scientific diversity, existing contacts, and areas of promising new joint ventures. Editing such a wide scope of subjects was fascinating and challenging, allowing at the same time to reflect the many discussions during the meeting that encompassed a world of science. We trust that the book may serve a similar function among the participants, as well as for a wider scope of readers.

Thanks to all who contributed: Irfan Tri Raharjo as coordinator, our co-editors helping to review the submissions, the Alexander von Humboldt Foundation who gave financial and logistic support, and, last but not least, the Rector, Leenawaty Limantara, and the staff of Ma Chung University who had already organized the meeting so well and now relieved us of many formal and administrative tasks involved in making the book.

May this seed grow and bear rich fruit!

Malang, 15 March 2012

Hugo Scheer
Editor-in-Chief
Ludgwig Maximilians University, Germany

VORWORT

Die "First International Conference on Natural Sciences" brachte vom 9.-11. Juli 2011 Wissenschaftler aus neun Ländern Südostasiens, Deutschland und Japan nach Batu in Ost-Java, Indonesien. Südostasien ist außergewöhnlich reich an natürlichen Ressourcen, von denen noch viele unangetastet sind. Es hat gleichzeitig außerordentlich dicht bevölkerte Gebiete, in denen die daraus erwachsenden Probleme der Infrastruktur, der intensiven Landwirtschaft und Aquakultur, und des Naturschutzes gelöst werden müssen. Exploration, Nutzung und Entwicklung der vielfältigen Ressourcen und die Lösung der häufig konfliktträchtigen Probleme ist nur durch interdisziplinäre Kooperationen möglich. Es war das Ziel dieser Konferenz, auf der Basis eines Netzwerks "Südostasien" der Alexander von Humboldt Alumni einschlägige Erfahrungen zu bündeln, vorhandene technische Ausrüstung Labor-übergreifend zu nutzen, Lücken zu definieren, und Wissenschaftler aus der Grundlagenforschung und aus technologischen Anwendungen zusammenzuführen.

Das vorliegende Buch gibt, als ein Mosaik, die thematisch breit gefächerten und beeindruckenden Vorträge und Poster wieder, die auf der Konferenz vorgestellt wurden. Es zeigt die Vielfalt der Forschung, bereits bestehende Kontakte, und Möglichkeiten zu neuen Kooperationen. Die Herausgabe eines solch weiten Spektrums von Arbeiten war zugleich beeindruckend und fordernd, es gab uns gleichzeitig die Gelegenheit, noch einmal die vielen Diskussionen zu erinnern, die eine Welt der Wissenschaft umfassten. Wir hoffen, dass dieses Buch bei allen Teilnehmern diese Funktion erfüllen wird, und die Themen und Teilnehmer einem weiteren Kreis von Lesern nahebringt.

Wir danken allen Beteiligten: Irfan Tri Raharjo für die Koordination des Buches, den Mitherausgebern für die kritische Durchsicht und Kommentierung der Manuskripte, der Alexander von Humboldt Stiftung für finanzielle und logistische Unterstützung. Nicht zuletzt danken wir der Rektorin der Ma Chung Universität, Leenawaty Limantara, und ihren Mitarbeitern; nach der ausgezeichneten Organisation der Konferenz haben sie uns durch Entlastung von vielen formalen und administrativen Aufgaben auch die Herausgabe dieses Buches leicht gemacht.

Möge dieses Samenkorn gut anwachsen und reiche Ernte bringen!

Malang, 15. März 2012

Hugo Scheer
der Chefredakteur
Ludwig-Maximilians-Universität, Deutschland

Messages

MESSAGE FROM THE SECRETARY GENERAL OF THE ALEXANDER VON HUMBOLDT FOUNDATION

Contribution of the Humboldt Foundation to the "Proceedings" on the occasion of the Humboldt Kolleg in Indonesia in 2011

Knowledge creates development – this is not only the case in developing and emerging countries, but in industrialized countries, too. The major issues in areas like resource conservation, global warming, sustainable energy supplies and healthcare, as well as access to water can only be tackled jointly, which means across both borders and disciplines. The key to this are highly-qualified academics in the natural sciences and engineering as well as in the humanities and social sciences. By selecting and promoting the best researchers and creating and developing self-supporting networks the Alexander von Humboldt Foundation has set itself the task of making a significant contribution to development and thus to the improvement of living conditions. It does not base its selection on country, subject, religious belief or gender, but purely on academic eligibility. The Foundation sponsors individuals involved in both applied and basic research. The best chance of successfully addressing the problem areas described above lies in precisely this complementarity.

Good research results alone are not enough. What is crucial is whether they have been achieved in different cultural contexts, are introduced into different social contexts and have a long-term impact when they are implemented. Mutual trust is required if this is to be achieved. For this reason, the Alexander von Humboldt Foundation places great emphasis on engendering trust: the academics it sponsors and their families are embedded in a culture of mentoring and counseling not only during their stay in Germany but also after their return to their own countries. The foundation provides platforms for cross-disciplinary, cross-border networking; it offers its alumni a whole range of sponsoring opportunities that allow them to continue the research projects they have started in Germany in their own countries.

It is important to keep upgrading the portfolio of programmes. To this end, the Alexander von Humboldt Foundation regularly organizes round-table discussions with fellows, alumni and their hosts in order to tailor their programmes to genuine needs. The International Climate Protection Fellowships, which were introduced recently, are one example of this process. They seek to address the global challenge of climate change in the context of cross-border, international cooperation. Up to 20 of these fellowships are available every year for potential leaders from non-European emerging and developing countries in the field of climate protection and resource conservation.

The first Humboldt Kolleg in Indonesia under the heading "Synergy, Networking and the Role of Fundamental Research Development in ASEA", together with the International Conference on Natural Sciences (ICONS 2011) and the research results that were presented and discussed there, constitute an important response to surmounting cross-border challenges.

Dr. Klaus Manderla
Head of Division for Asia
Alexander von Humboldt Foundation

MESSAGE FROM THE CHAIRWOMAN OF HUMBOLDT KOLLEG IN CONJUCTION WITH ICONS 2011

Welcome to Humboldt Kolleg Synergy, Networking and the Role of Fundamental Research Development in South-East Asia in conjunction with the International Conference on Natural Sciences 2011

The year 2011 is a monumental year for the Humboldt Fellow Indonesia for the success of the first Humboldt Kolleg in Indonesia which was held in conjunction with the International Conference on Natural Sciences 2011. Owing to the excellent cooperation between Humboldt Club Indonesia under the leadership of Dr. L. T. Handoko and Ma Chung University, the program was successfully and officially opened on 8th July, 2011 (for Humboldt fellows) and on 9th July, 2011 (for public). The program intended for Humboldt fellows, academics, and scientists from South-East Asia, was created in the form of a plenary lecture, invited lectures, oral presentations, a poster session, and an excursion to Mount Bromo in East-Java. The program consisted of four themes: (1) the role of natural sciences in conserving natural resources, (2) the role of natural sciences in overcoming global warming, (3) the role of natural sciences in developing science and technology, and (4) the role of natural sciences in improving human welfare. Through the three-day activities, three outcomes could be secured: (1) two conference proceedings to be published by Shaker-Verlag, Germany, and an Indonesian publisher; (2) a book entitled *Humboldtians in South-East Asia: Research Interests and Future Prospects*, and (3) the declaration of Malang Humboldt Resolution. Humboldt Kolleg I in Indonesia was attended by 23 Humboldt fellows from the 55 Humboldt Fellows invited, and 129 researchers and academics representing Germany, Japan, Indonesia, Singapore, the Philippines, Malaysia, Korea, and Vietnam.

The proceedings book was created to compile all the International Conference on Natural Sciences (ICONS) activities held as a single unit of activities of the first Humboldt Kolleg in Indonesia. In practice, several writers withdrew their articles because their articles were successfully published in national and international journals so that they are not mentioned in the proceedings.

We sincerely hope that this book can be used as a scientific reference for many scholars.

Malang, 15 March 2012

Leenawaty Limantara

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PRELIMINARY STUDY OF IRON SULFUR FLAVOPROTEIN FROM *METHANOSARCINA ACETIVORANS*

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ABSTRACT - M. acetivorans involved in the anaerobic conversion of biomass to methane which is essential to the global carbon cycle. Most of the methane is produced by a two-step process in which complex organic matter is fermented to acetate that is further converted to methane and carbon dioxide. The same process is also a key to the conversion of renewable plant biomass to methane as an alternative energy source. Fundamental science in energy conservation during methanogenesis in M. acetivorans is poorly understood. This proses involved a sequential activity of redox proteins. Iron sulfur flavoproteins (Isf) are a redox protein with unclear function with an iron sulfur cluster and an FMN as prosthetic groups. The genome sequence of M. acetivorans has been completed and published. Interestingly, a total 19 homolog of Isf from M. acetivoran were identified from its genome sequence. Isf was suggested to play a role in oxidative stress and as an adapter in electron transfer systems from one electron carrier to two-electron carrier. However, the exact function and the presence of multicopy Isf are unknown. This research aims to initiate a study of structure and function of Isf from M. acetivorans. In order to have enough material, one of the Isf genes (MA0327 locus) was overexpressed in Escherichia coli as a His-tag protein. The purified protein was reconstituted in vitro. The UV-Vis spectra of reconstituted protein showed the typical iron-sulfur flavoprotein signature. Further analysis showed the iron-sulfur:FMN ratio of 1:1 per protein molecule. The result showed that the Isf has been overexpressed and reconstituted, however further activity studies need to be done to confirm whether the protein is active as a redox protein

Keywords: *iro-sulfur, flavin*

I. INTRODUCTION

The carbon cycle affects all life on Earth. Carbon is neither created nor destroyed, so the recycling of carbon-containing compounds in the environment is essential to the continuation of life. Microbes play important roles in all aspects of the global carbon cycle and are a very important consideration when studying climate change. Methane-producing microbes contribute to methane emission which is potent a greenhouse gas. However, methane is also a potential energy source to meet future energy needs. *Methanosarcina acetivorans* is a marine isolate of methane producing archaea, which has the largest genome among known *Archaea* [1]. While carbon flow from carbon source into methane in Archea is well understood, electron transfer during methanogenesis still needs to be studied [2]. Following the completion of the genome sequence of *M. acetivorans*, functional genomic gets a big attention to understand the functions of all genes in *M. acetivorans* [3-5].

Electron transfer processes generally are carried out by redox proteins. *M. acetivorans* has 19 copies of open reading frames annotated as iron-sulfur flavoproteins (Isf) with unknown function [1]. An Isf homolog from *Methanosarcina thermophilla* has been studied and crystallized. On the basis of its prosthetic group composition, *M. thermophilla* was suggested as an adapter to switch electron transfer from one to two electron carriers [6]. An in vitro study suggested that Isf might also play a role as oxygen scavenger during oxidative stress [7]. In order to study further Isf in *M acetivorans*, one of the Isf (Isf1) which is encoded by MA0327 locus was overexpressed in *E. coli*. This paper communicates the genome sequence analysis, overexpression, purification, and initial characterization of Isf1.

2. MATERIAL AND METHODS

2.1 *Overexpression of Isf1*

The ORF (MA0327) encoding Isf1 was amplified by PCR from genomic DNA of *M. acetivorans* and cloned into a pTYB11 vector (New England Biolabs Inc) at SapI and PstI multicloning sites. Using the pTYB11 vector allows overexpression of Isf as C-terminal fusion with intein tag. A pair of primers, sense (5'GATGATTGCTTCCAACATGAAAGTCATTGC) and antisense (5'GGTGGTCTGCAGTTATCAATTTTTTCTTCAG), was used to amplify the gene. SapI site was introduced in sense primer while PstI site was introduced in antisence primer. The PCR reaction was done using the Fast Start High Fidelity PCR system (Roche). The PCR product and pTYB11 were then double digested using SapI and PstI. Digested products were ligated to produce a recombinant plasmid. The recombinant plasmid was subsequently used to transform NovaBlue Single Competent Cells (EMD Biosciences). The recombinant plasmid containing the DNA insert was confirmed by DNA sequencing and transformed into *E. coli Rosetta*™ (DE3) pLysS Competent Cells (EMD Biosciences) that was cultured at 37°C in LB medium containing 100 ug/ml ampicillin. Production of Isf1 was induced by the addition of 0.5mM IPTG when the OD_{600} reached 0.7. The culture was then incubated for another 16 h at 16°C.

2.2 *Purification of Isf1*

The purification was conducted anaerobically under 5% hydrogen and 95% nitrogen atmosphere. Approximately 15g of thawed cells were resuspended in 20mM Hepes pH 7 containing 500mM NaCl. 10mM and 0.25mM phenylmethanesulfonyl fluoride were then added and the cells lysed by being passed twice through a French pressure cell at 110MPa. Cell debris and membranes were removed by centrifugation at 10000*g* for 45 min at 4°C. The supernatant was loaded onto a chitin column equilibrated with 20mM Hepes pH 7 containing 500mM NaCl. The column was then washed with 10 column volumes of the equilibration buffer. Subsequently the column was washed with quickly washed with cleavage buffer (with 20mM Hepes pH 7, 500mM NaCl, 50mM DTT), and incubated at room temperature overnight. Tagged Isf1 was eluted from the chitin column using the cleavage buffer. Eluted solution was concentrated using pressure filtration technique with Vivacell 70, 10,000 MWCO (Sartorius).

2.3 *Iron Sulphur and Flavin Reconstitution*

The reconstitution method used was a modified version of the method described previously [6, 7]. To 100 ml of anaerobic 50mM hepes pH 7.0, 800 µl of β-mercaptoethanol was added dropwise while the preparation was gently stirred. After 10 min, 2.5 to 10 mg of pure protein (eluted from the chitin column) was added. Then 300 µl of 60mM ferric chloride, 300 µl of 60mM sodium sulfide, and 300µl of 15mM FMN (all in 50mM hepes pH 7) were added stepwise at 10-min intervals. The mixtures were incubated anaerobically at 4°C overnight and then concentrated by using pressure filtration technique with Vivacell 70, 10,000 MWCO (Sartorius). Excess Fe, S, and FMN were removed by several by passages through a desalting column.

2.4 *Prosthetic group determinations*

Noncovalently bound flavin was released from the Isf1 by either adding trichloroacetic acid to a final concentration of 5% or boiling for 5 min. The precipitated protein was removed by centrifugation and the supernatant was filtered using an Ultrafree-MC 5000 NMWL filter unit (Millipore). The filtrate was then neutralized by adding 2M K2HPO4. The flavin type was determined by TLC using a silica gel matrix (Fluka) with n-butanol/acetic acid/water (4: 1: 5) as the mobile phase. The free flavin concentration was determined spectroscopically using an extinction coefficient of 12.2mM-1 cm-1 at 452 nm (oxidized form). Iron was determined colorimetrically as the ferene-complex [8] and acid labile sulphur was determined by a method described in [9]. The protein concentration was quantified by the bicinchoninic assay (Pierce).

2.5 *Miscellaneous*

Total RNA was isolated from methanol-grown *M. acetivorans* cells and RT-PCR was performed as described in ref[10] except that RT-PCR was carried out with Access RT-PCR kit (Promega, Madison, WI). PCR was done at an annealing temperature of 50°C, and electrophoresis was done using 1.2% agarose. Iron and sulphur determinations were done according to [6] and flavin determination was done according to [2].

RESULTS AND DISCUSSIONS

Initial results of transcriptional mapping indicated that Isf1 is encoded in an novel operon of 5 genes (Figure 1). The operon consists of MA0326, MA0327, MA0328, MA0329, and MA0330 which annotated as gene encoding quinone reductase, iron sulfur flavoprotein, flavoredoxin, hypothetical protein, and NADPH flavoreductase. Quinone reductase [11] and flavoredoxin [2] had been overexpressed and partially characterized. Quinone reductase has been reported a NADPH reductase containing 1 FAD molecule per protein and flavoredoxin is an FMN containing protein which reducible by ferredoxin. Up to know, the genes has not communicated, and the function of the operon is also unknown. Isf has a similarity to the CagG gene which is part of the C-1027 genetic locus. The gene is involved in the conversion of chorismate to the benzoxazolinate moiety in biosynthesis of enediyne antitumor antibiotic in *Streptomyces globisporus* [12]. It is possible that the novel operon is involved in a similar biological process. This initial result needs to be carried at different experimental condition to confirm whether the operon only contains five genes or the upstream and downstream of these genes are also part of the operon.

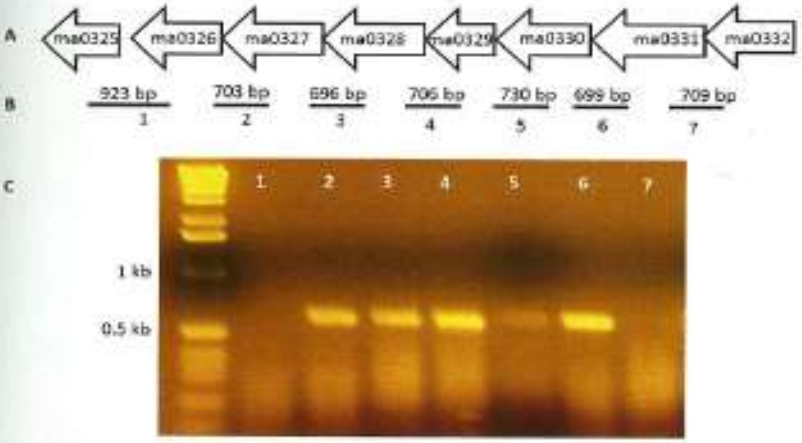

Figure 1. Trascriptional mapping of the isf1 genome region. (A) Map of genes upstream and downstream of Isf1 (B) Predicted RT-PCR results (C) RT-PCR results.

Figure 2 shows a multiple sequence alignment of MA0327 with the putative iron sulfur proteins from *Methanosarcina mazei*, *Methanosarcina barkeri*, and identified iron sulfur flavoprotein from *Methanosarcina thermophila*.

```
M.mazei Goel        MKVVAFNGSPRKEGNTASLIKPVLAELDKEGIETEIVQIGGKSVHGCTAC
M.barkeri fusaro    MKVVAFNGSPRKDGNTAALIKPVLAELEKEGIETEVVQIGGKKIHGCTAC
M.acetivorans C2A   MKVIAFNGSPRKEGNTVTLIKHILAELEKEGIETEMVQIGGKSVHGCTAC
M.thermphilla       MKITGISGSPRKGQNCEKIIGAALEVAAERGFETDTVFISNKEVAPCDAC

M.mazei Goel        SKCFEMRDRKCVIDNDIVKECIEKMLEADGIILASPTYFADLTPELKALI
M.barkeri fusaro    GKCYEKMDKKCVIDKDIVKECIEKMLEADGIILASPTYFADLTPELKALI
M.acetivorans C2A   AKCYEKMDKKCVIDKDIVKECIEKMLEADGIILASPTYFSDLTPELKALI
M.thermphilla       GACRDQDF--CVIDDD MDEIYEKMRAADGIIVAAPVYMGNYPAQLKALF

M.mazei Goel        DRAGFVAKANSEMFSYKVGAAVVAVRRAGSIHVFDSINHFFTISQMIIPG
M.barkeri fusaro    DRAGFVAKANSEMFSHKVGAAVVAVRRAGSIHVFDSINHFFTISQMIIPG
M.acetivorans C2A   DRAGFVAKANSEMFRYKVGAAVVAVRRAGSVHVFDSINHFFTISQMVIPG
M.thermphilla       DRSVLLRRRNFALKEKVGAALSVGGSRNGGQEKTIQSIHDMMHIHGMIVV

M.mazei Goel        SSYWKNGIGRAEGDVEKDDEGIRTMQILGQNMAWLLKKLKE
M.barkeri fusaro    SSYWKNGMGLAEGEVEKDEEGIQTMQTLGQNIAWLLKKLNV
M.acetivorans C2A   ASYWKIGIGLAEGEVEKDEEGIRTMQVLGQNMAWLLKCDA
M.thermphilla       GDNSEFGGITKGFAEEDTVGMQTVSETAKKLCVLELIQKNRDK
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Figure 2. Multiple sequence alignment of Isf. The highlighted amino acid residues indicated the predicted binding motif for a 4Fe-4S cluster.

Sequence analysis shows a conserved $CX_2CX_2CX_{3-7}C$ motif which has been known as a conserved [illegible] motif for iron 4Fe-4S sulfur cluster [6; 13]. Although crystal structures of Isf from *M. thermophila* and *Archaeoglobus fulgidus* have been resolved, no FMN binding motif is concluded. In *M. thermophila* [illegible] FMN in M. thermophila is surrounded by positively charged amino acid residues, while in *A. fulgidus* [illegible] surrounded by hydrophobic amino acid residues. The FMN binding motif in *M. acetivorans* cannot be derived from both crystal structures because the 3D folding of the protein is unknown and no satisfactory computational method is available for predicting 3D structure.

Isf1 was succesfully overexpressed in *E.coli* and and purified into homogeneity using a one step purification procedure. SDS-PAGE gives an apparent Isf1 molecular mass of 22 ± 0.5 kDa which is close to Isf theoretical molecular mass of 21 kDa (Figure 3A). The Slight difference between apparent and theoretical molecular mass presumably is due to charge differences between Isf1 and standard proteins. In order to maximise iron-sulfur and flavin incorporation into Isf one, the purified protein was reconstituted. UV-Vis spectrum of reconstituted Isf1 shows a typical of iron-sulfur flavoprotein with peaks around 380 and 450 nm (Figure 3B). This spectra is different compared to those of flavin containing proteins or iron sulfur flavoproteins alone. Isf UV-Vis spectrum is a sum of iron sulfur anf flavin spectra.

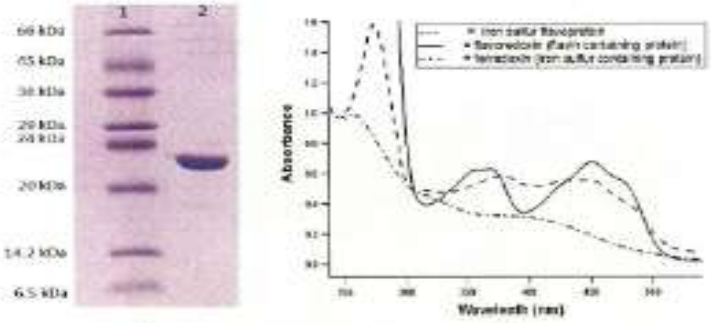

Figure 3. SDS-PAGE (A) and UV-Vis spectra of purified Isf1 (B)

Qualitative analysis using Thin Layer Chromatography showed Isf1 contains a flavin with the retention time similar to Flavin mononucleotide (FMN) which concluded that Isf contains FMN. Furthermore, Iron sulfur determination of reconstituted protein showed Fe:S:FMN ratio of 3.9:3.6:0.94 which suggested that Isf contains one of 4Fe-4S cluster and one FMN.

Based on the results, a novel operon is proposed to encode enzymes which might function in a novel organic compund conversion such as antibiotic biosynthesis. Isf as a part of this operon is an iron sulfur flavoprotein containing one 4Fe-4S cluster and one FMN molecule. The structue of the iron sulfur needs to be furthur confirmed with other methods such as Electron Paramagnetic Resonance or Circular Dichroism.

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