II.I.5 Hydrogen from Water in a Novel Recombinant O_2-Tolerant Cyanobacterial System

Qing Xu (Primary Contact) and
Hamilton O. Smith
J. Craig Venter Institute (JCVI)
9704 Medical Center Drive
Rockville, MD 20850
Phone: (301) 795-7248; Fax: (240) 268-4004
E-mail: qxu@jcvi.org

DOE Technology Development Manager:
Roxanne Garland
Phone: (202) 586-7260; Fax: (202) 586-2373
E-mail: Roxanne.Garland@ee.doe.gov

DOE Project Manager: Lea Yancey
Phone: (303) 275-4944; Fax: (303) 275-4753
E-mail: Lea.Yancey@go.doe.gov

Contract Number: DE-FC36-05GO15027

National Laboratory Collaborators:
Pin-Ching Maness and Jianping Yu
National Renewable Energy Laboratory (NREL), Golden, CO

Project Start Date: May 1, 2005
Project End Date: Project continuation and direction determined annually by DOE

Objectives

Develop an O_2-tolerant cyanobacterial system for sustained and continuous light-driven H_2-production from water.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section (3.1.4) of the Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan:

(AH) Rate of Hydrogen Production

(AI) Continuity of Photoproduction

Technical Targets

Characteristics	Current Status	2011 Target	2018 Target
Duration of continuous H_2 photoproduction in air	Zero to 30 seconds in air	Produce a cyanobacterial recombinant evolving H_2 through an O_2-tolerant hydrogenase	H_2 production in air for 30 min

Accomplishments

JCVI

- We have knocked out the native hydrogenase (Hox) in cyanobacterium *Synechococcus* 7942. This knockout mutant has been used as a recipient for transferring O_2-tolerant hydrogenases.
- We have purified *Thiocapsa* O_2-tolerant NiFe-hydrogenase from *Thiocapsa roseopersicina*. Polyclonal antibodies specific for this hydrogenase have been raised and used to verify heterologous hydrogenase expression.
- We established two efficient systems for transferring foreign hydrogenase genes into the cyanobacterial recipient. We have transferred totally nine structural and accessory genes of *Thiocapsa* hydrogenase into the recipient through homologous deoxyribonucleic acid (DNA) recombination. Hydrogenase expression in the host has been verified.
- Our study reveals that the novel environmental hydrogenase shares similar maturation mechanisms to those of *Thiocapsa* O_2-tolerant hydrogenase.
- We have searched global ocean sampling (GOS) metagenomic data for novel hydrogenases. One novel hydrogenase has been cloned from environmental DNAs and heterologously expressed in *T. roseopersicina*. The hetero-expressed hydrogenase is active and is highly O_2-stable. This represents the first report of identifying a novel O_2-tolerant NiFe-hydrogenase from environmental microbes through a metagenomic approach.

NREL

- We cloned the O_2-tolerant *Rubrivivax* hydrogenase genes in two plasmids suitable for expression in *Synechocystis*. One plasmid will integrate *Rubrivivax* genes into the genome of *Synechocystis* via the homologous recombination mechanism.

Genes cloned into the second plasmid will be expressed off plasmid. Expression from both plasmids is driven by a native promoter in *Synechocystis*.

- We generated antibodies and tested their specificity against the various subunits of the *Rubrivivax* hydrogenase. We have verified the transformation of four genes and the expression of two hydrogenase structural subunits.

Introduction

Photobiological processes are attractive routes to renewable H_2 production. With the input of solar energy, photosynthetic microbes such as cyanobacteria and green algae carry out oxygenic photosynthesis, extracting energy from water oxidation. The resulting energy can couple to a hydrogenase system yielding H_2. However, one major difficulty is that most hydrogen-evolving hydrogenases are inhibited by O_2, which is an inherent byproduct of oxygenic photosynthesis. The rate of H_2 production is thus limited. Certain photosynthetic bacteria are reported to have an O_2-tolerant evolving hydrogenase, yet these microbes do not split water, requiring other more expensive feedstock.

To overcome these difficulties, we propose to construct novel microbial hybrids by genetically transferring an O_2-tolerant hydrogenase from other bacteria into cyanobacteria. These hybrids will use the photosynthetic machinery of the cyanobacterial hosts to perform the water-oxidation reaction with the input of solar energy, and couple the resulting reducing equivalents to the O_2-tolerant bacterial hydrogenase, all within the same microbe. This addresses one of the key technological hurdles to cost-effective photobiological production of H_2, overcoming the sensitivity of the hydrogenase enzyme to O_2, which limits the production of hydrogen in current algal systems.

Approach

Our goal is to construct a novel microbial hybrid taking advantage of the most desirable properties of both cyanobacteria and other bacteria, to serve as the basis for technology to produce renewable H_2 from water. To achieve this goal, we use the following two approaches. The first approach is to transfer known O_2-tolerant hydrogenases from anoxygenic photosynthetic bacteria *Thiocapsa* and *Rubrivivax* to cyanobacteria. Since only a very limited number of O_2-tolerant hydrogenase is available, our second approach is to identify novel O_2-tolerant hydrogenases from environmental microbial communities and transfer them into cyanobacteria.

Results

Transferring O_2-Tolerant Hydrogenase Genes from Thiocapsa into Cyanobacteria

Our goal for this part of the work is to transfer an O_2-tolerant NiFe-hydrogenase (Hyn) from *T. roseopersicina* into cyanobacteria. We have knocked out a native hydrogenase (Hox) in cyanobacterium *Synechococcus* 7942. This knockout mutant was confirmed not to contain any hydrogenase activities and used as a recipient for transferring the hydrogenase. We previously reported cloning the structural (*hynS/L*) and accessory genes (*hynD/hupK/hypC1/hypC2*) of this hydrogenase and integrating them into the chromosome of *Synechococcus* through homologous DNA recombination, creating a recombinant cyanobacterial strain Hyn3. In the past year, we established two efficient systems to transfer additional accessory genes into the strain Hyn3. Using an expression vector with a different selection marker and flanking regions, we cloned accessory genes *hypF*, *hypE*, and *hypD*. Through homologous DNA recombination, we integrated these genes into the chromosome of the strain Hyn3 and created a new strain called Hyn4. To verify that Hyn4 is authentic, we prepared its chromosomal DNA and used it as template to perform polymerase chain reaction (PCR). Our results confirmed that *hypD/hypE/hypF* genes were indeed integrated into the chromosomes. We further performed Southern blotting to confirm correct genotype for *hypD/hypE/hypF*. Chromosomal DNA was prepared from Hyn4 (Hyn4-8) and wild type strains and digested with NdeI and HindIII (Figure 1). Plasmid pHyn4 (as positive control) was subjected to parallel digestion. A digoxigenin (DIG)-labeled probe corresponding to the entire *hypE* gene was made by PCR (Figure 1a) and used to hybridize the blot made from digested DNAs. Southern blotting results show presence of the entire 5 kb piece in the strain Hyn4, which was the same size as that of pHyn4 (Figure 1b), indicating the genotype of *hypD*, *hypE*, and *hypF* is accurate. We verified isopropyl β-D-1-thiogalactopyranoside-inducible expression of *Thiocapsa* O_2-tolerant hydrogenase by Western blotting using hydrogenase-specific antibodies. Overall, we have transferred nine structural and accessory genes of *Thiocapsa* hydrogenase into the cyanobacterium.

Purifying Thiocapsa O_2-Tolerant Hydrogenases from T. Roseopersicina

To further quantify its O_2 tolerance and to determine its electron mediator requirements in the cyanobacterial host, *Thiocapsa* hydrogenases needs to be purified from *T. roseopersicina*. We sought to purify the enzyme using column chromatography. A crude extract of *T. roseopersicina* was applied to a diethylaminoethyl

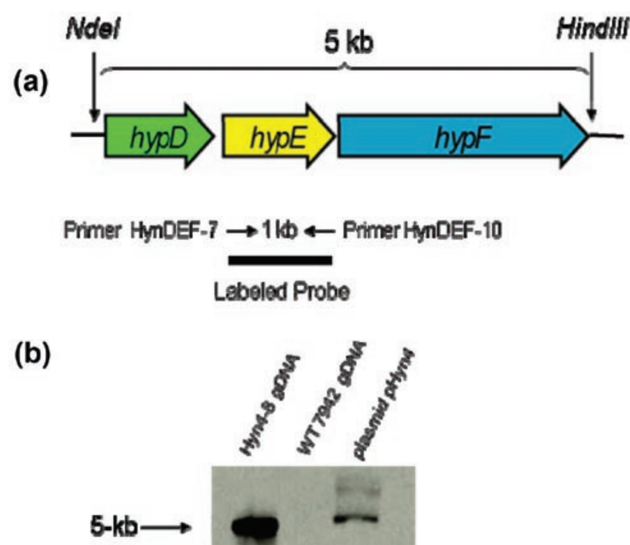

FIGURE 1. Southern blot confirmation of integration of *hypD, hypE,* and *hypF* genes of *Thiocapsaq* O_2-tolerant hydrogenase into the cyanobacterial strain Hyn4 (Hyn4-8). Panel A: Restriction endonuclease map of the *hypD/hype/hypF* gene locus in the strain Hyn4. Digestion of Hyn4's genomic DNA with NdeI and HindIII should release a 5 kb DNA fragment. A 1 kb DIG-labeled probe was made by PCR and used for Southern blot. PCR primers and genomic location of the probe are indicated. Panel B: Southern blot result of the strain Hyn4 (Hyn4-8). Plasmid pHyn4 and wild type PCC7942 genomic DNA were used as positive and negative controls, respectively. gDNA represents genomic DNA. The size of hybridized band, 5 kb, is indicated.

cellulose column, and the loaded column was then eluted with a linear gradient of 0-0.6 M NaCl (Figure 2a). Screening hydrogenase activity in eluted fractions by hydrogenase activity assay revealed that the *T. roseopersicina* hydrogenase was eluted from the column in the fraction #22 (Figure 2b). The enzyme in this fraction was further purified through Mono-S and Mono-Q columns. We are now ready to use purified enzyme for functional analysis.

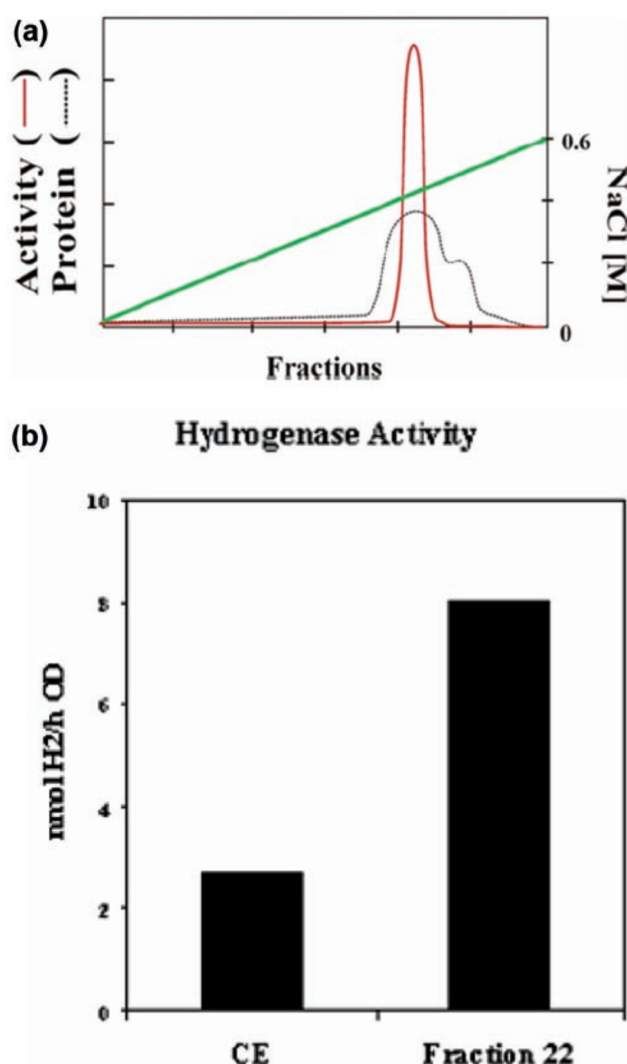

FIGURE 2. Purification of *Thiocapsa roseopersicina* O_2-tolerant hydrogenase. Panel A: Chromatography of *Thiocapsa* crude extract. The curve for H_2 evolution activity of eluted fractions was in red; the curve of protein concentration was in black; and NaCl gradient 0-0.6M in green. Panel B. H_2 evolution activity assay Purified *Thiocapsa roseopersicina* O_2-tolerant hydrogenase. CE: crude extract; Fraction 22: Purified hydrogenase

Identifying Novel O_2-Tolerant Hydrogenases from Marine Microbes in the Global Ocean

The goal for this part of the work is to identify novel O_2-tolerant hydrogenases from the ocean and transfer them into cyanobacteria. JCVI's GOS Expedition generated 6.3 billion bps of non-redundant DNA sequences, from which 6.12 million proteins were predicted. They nearly cover all known prokaryotic protein families and double the number of current proteins and, in addition, revealed 3995 GOS-only clusters that have no homology to known protein families. To take advantage of this huge amount of genomic information, we searched novel hydrogenases through analysis of the metagenomic data.

Totally, we found 76 large subunit and 52 small subunit sequences (Figure 3). The numbers of these sequences were further compared to those of hydrogenase sequences previously published in the public database Genbank (Figure 3). For the large subunit, there were 261 National Center for Biotechnology Information sequences (vs. additional 76 sequences we found); and for the small subunit, there were 234 NCBI sequences (vs. additional 52 sequences we found). These data indicate that the GOS study has made significant contribution to the collection of NiFe-hydrogenases in the database.

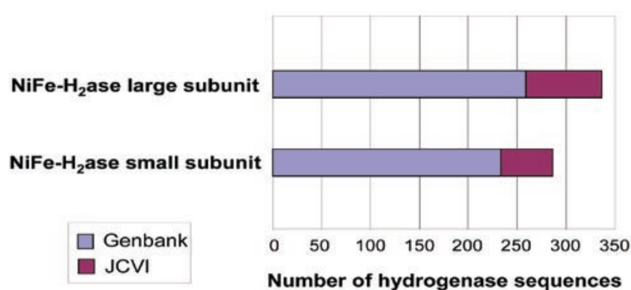

FIGURE 3. Comparison of the numbers of JCVI's novel hydrogenase sequences with those published in the public database GenBank. The numbers of JCVI's sequences are labeled as purple; and the numbers of GenBank's sequences are in blue.

We reported cloning of a novel NiFe-hydrogenases homologous to *Thiocapsa* O_2-tolerant hydrogenase from the environmental DNA samples. Genes of this novel hydrogenase (*hynS/L*, *hupH* and *hyaD*) were heterologously expressed in *T. roseopersicina* strain GB112131, a mutant strain with all three native hydrogenases knocked out. We functionally analyzed this heterologously expressed hydrogenase and found it was active with both H_2-evolution and H_2-uptake activities. We tested its oxygen-stability in the air and found it is extremely stable. Our results indicate that *T. roseopersicina* accessory proteins were able to complement environmental accessory proteins. We further compared the maturation machineries between the environmental hydrogenase and *T. roseopersicina* hydrogenases by performing complementation experiments. We introduced the environmental *hyaD* gene into *T. roseopercicina hynD* mutant strain DYDG2, in which the native *hynD* gene encoding an endopeptidase was knocked out. Western and hydrogenase assay results demonstrate that *hyaD* can fully restore cleavage and enzyme activities of *Thiocapsa* hydrogenase in the mutant DYDG2, despite the fact that HyaD shares only 37% identity to its *T. roseopersicina* counterpart HynD. Overall, our study reveals that the native endopeptidase and *Thiocapsa* hydrogenases share similar maturation mechanisms.

NREL: Transferring O_2-Tolerant Hydrogenase from Rubrivivax Gelatinosus to Cyanobacteria

The goal for this part of the work is to construct a cyanobacterial recombinant harboring the O_2-tolerant hydrogenase from *Rubrivivax gelatinosus*, using *Synechocystis* PCC6803 (S6803) as the model host. We used two plasmid vectors to accomplish this goal. Plasmid pPETE_sigD is provided by Prof. Robert Burnap (Oklahoma State University) that features (1) light-regulated transcription, (2) a spectinomycin antibiotic resistance marker, and (3) neutral site for homologous recombination. Four structural genes *cooLXUH* encoding the *Rubrivivax* O_2-tolerant hydrogenase (Figure 4a) have been successfully amplified and cloned into the pPETE_sigD plasmid by replacing its *sigD* gene (data not shown). A S6803 mutant lacking all of its native hydrogenase subunits (Hox^-; provided by Prof. Teruo Ogawa) served as the recipient with zero H_2-production background. After transformation, PCR analysis of several transgenic lines indicated the deletion of the *Rubrivivax cooL* gene (encoding hydrogenase small subunit) although the spectinomycin resistance gene *aadA* was integrated into the neutral site of the S6803 genome. Meanwhile we have generated and validated three peptide antibodies (CooH, CooX, and CooU) for detecting *Rubrivivax* protein expression in the above Hox^- transgenic lines. As shown in Figure 4b, *Rubrivivax* hydrogenase subunits CooH (catalytic subunit) and CooU (unknown function) were both expressed in two out of four tested LXUH transgenic lines, confirming the integration of both genes into the Hox^- genome. Since the transfer of up to 12 genes is likely needed to express a functional *Rubrivivax* hydrogenase, we employed a broad host range plasmid pRL1342 (provided by Prof. Peter Wolk, Michigan State University) to express additional genes. We constructed pRL1342_LA plasmid carrying codon-optimized *Rubrivivax cooL* and *hypA* (hydrogenase maturation) genes. PCR result indicated the successful transformation of both genes in two S6803 recipients, wild type and a mutant lacking the hydrogenase large-subunit ($HoxH^-$) (Figure 4c). The latter serves as a zero-background host allowing easy detection of *Rubrivivax* hydrogenase activity.

Conclusions and Future Directions

JCVI: From the JCVI's research, we conclude that environmental microbes have significant potential in carrying new genes or pathways that may improve efficiency of renewable energy production. From GOS sequences we found 76 large subunit and 52 small subunit sequences. We succeed in converting a piece of environmental DNA into a novel functional NiFe-hydrogenase and demonstrating its extraordinary O_2-stability. We studied maturation mechanisms of the hydrogenase, which refines our knowledge on [NiFe] hydrogenase assembly machineries. We also purified *Thiocapsa* O_2-tolerant hydrogenase from *T. roseopersicina*. Furthermore, we transferred two structural and seven accessory genes of this hydrogenase into *Synechococcus* and demonstrated that the *Thiocapsa* hydrogenase was expressed in the host.

NREL: The NREL research confirmed the expression in *Synechocystis* of two genes encoding subunits of the O_2-tolerant hydrogenase from *Rubrivivax*. Moreover, we transformed successfully a *Rubrivivax* hydrogenase maturation gene in *Synechocystis*. We have generated antibodies and verified their specificity to each of the *Rubrivivax*

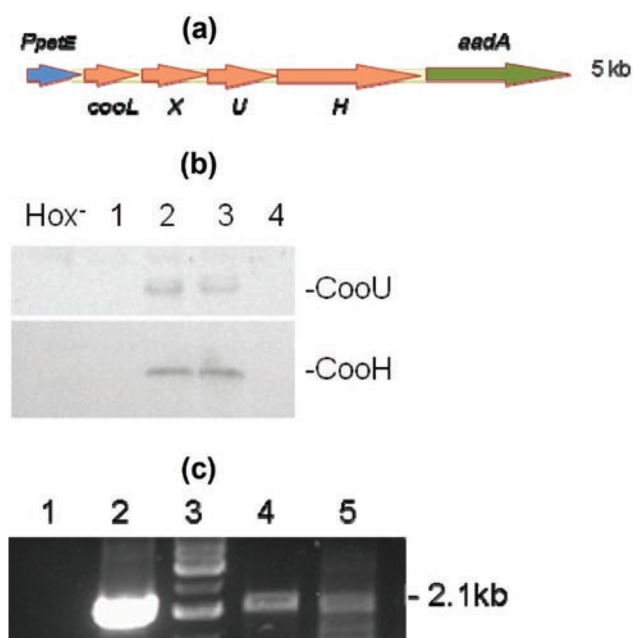

FIGURE 4. (a) *Rubrivivax* hydrogenase structural genes *cooLXUH* for integration into *Synechocystis.* PpetE: a light- and copper-regulated plastocyanin promoter. *aadA*: spectinomycin resistance gene (b) western blot showing expression of *Rubrivivax* hydrogenase proteins in *Synechocystis* Hox- host transformed with the pPETE_LXUH plasmid. Hox-: untransformed control; lanes 1-4: four transgenic lines with two lines (lanes 2 and 3) expressing *Rubrivivax* CooU and CooH proteins. (c) PCR verification of the presence of *Rubrivivax hypA* and *cooL* genes in *Synechocystis.* PCR primers encompass a DNA region with an expected 2.1 kb product containing three genes (chloramphenicol resistance gene *CAT, cooL,* and *hypA*). lane 1: wild type as negative control; lane 2: pRL1342_LA as positive control; lane 3: molecular weight marker; lanes 4 and 5: wild type and HoxH- transformed with pRL1342_LA plasmid.

hydrogenase subunits, which will be used to probe expression of additional subunits in future research.

Future Directions

- Verify hydrogenase functionality of novel environmental and *Thiocapsa* hydrogenases in oxygen (JCVI).
- Continue to transfer genes of the novel environmental hydrogenase into the cyanobacterium and analyze resulting recombinant cyanobacterial strains (JCVI).
- Continue to transfer more genes of *Thiocapsa* O_2-tolerant hydrogenase into the cyanobacterium and analyze resulting recombinant cyanobacterial strains (JCVI).
- Optimize conditions and transfer additional hydrogenase structural and maturation genes of *Rubrivivax* to express a functional O_2-tolerant hydrogenase in *Synechocystis* (NREL).

FY 2009 Publications/Presentations

1. G. Maróti, Y. Tong, S. Yooseph, H. Baden-Tillson, H.O. Smith, K.L. Kovács, M. Frazier, J.C. Venter, and Q. Xu. Discovery of a [NiFe]-hydrogenase in Metagenomic Sargasso Sea DNA: Cloning and Functional Study in *Thiocapsa roseopersicina. Applied and Environmental Microbiology*, **2009**, (Accepted for publication).

2. S.J. Williamson, Rusch D.B., Yooseph S., Halpern A.L., Heidelberg K.B., Glass J.I., Andrews-Pfannkoch C., Fadrosh D., Miller C.S., Sutton G., Frazier M., and J.C. Venter. The Sorcerer II Global Ocean Sampling Expedition: metagenomic characterization of viruses within aquatic microbial samples. *PLoS ONE*, **2008**, 23; 3(1):e1456.

3. Ghirardi, M.L., K. Sergey, P.C. Maness, S. Smolinski, and M. Seibert. Hydrogen production, algal. *Wiley Encyclopedia of Industrial Biotechnol*. **2009**, In press.

4. M.L. Ghirardi , P.C. Maness, and M. Seibert. "Photobiological Methods of Renewable Hydrogen Production," *in* Solar Generation of Hydrogen (K. Rajeshwar, R. McConnell, and S. Licht, Eds.) Springer, N.Y., **2008,** Chapter 8, pp 229-271.

5. M.L. Ghirardi, A. Dubini, J.P. Yu, and P.C. Maness. Photobiological hydrogen-producing systems. Chemical Society Reviews. **2009**, 38, 52-61.

6. P.C. Maness, J.P. Yu, C.E. Eckert, and M.L. Ghirardi. "Photobiological hydrogen production – processes and challenges", **2009,** Microbe 4: 275-280.

7. Q. Xu orally presented "construct a cyanobacterial recombinant for solar H_2 production" at Synthetic Genomics Inc., Aug. 4th, 2008, San Diego, CA.

8. Q. Xu and P.C. Maness orally presented "Update on JCVI and NREL Joint project", Hydrogen Production Technical Team Biological Hydrogen Production Review Meeting, August 7, 2008.

9. Q. Xu and P.C. Maness. DOE Annual Hydrogen Program Review Meeting, Washington, DC, May 18-21, 2009. Presentation pdp# 18.

10. P.C. Maness. Society for Industrial Microbiology Annual Meeting, August 10-14, 2008. San Diego, CA. Poster "Metabolic engineering of cyanobacterium *Synechocystis* PCC6803 for solar H_2 production".

11. Yu, J.P. and P.C. Maness. 18th Western Photosynthesis Conference. January 8-11, 2009. Pacific Grove, CA. Presented a poster "Expression of an oxygen tolerant evolving hydrogenase in *E. coli* and in the cyanobacterium *Synechocystis* 6803".