



The Synthetic Cell: From the Mind to Life

to Space

By:
Michael Montague

J. Craig Venter™
INSTITUTE

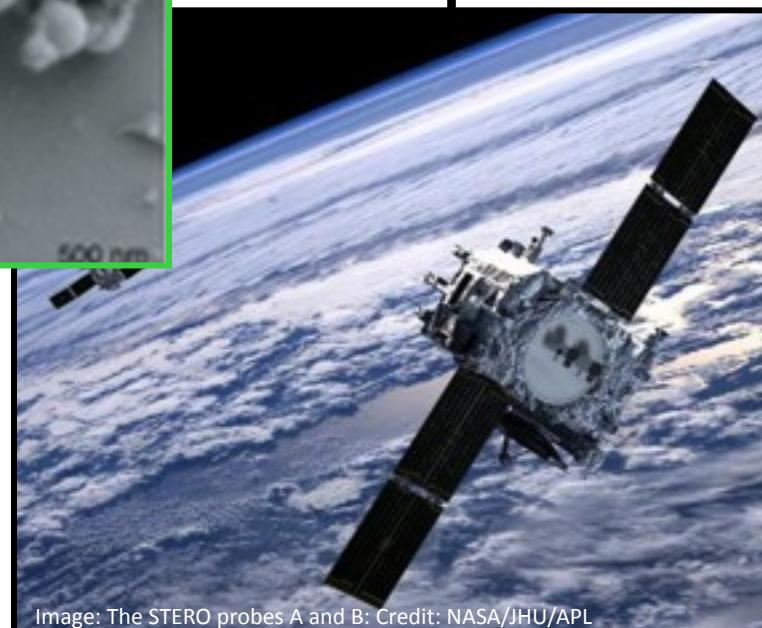
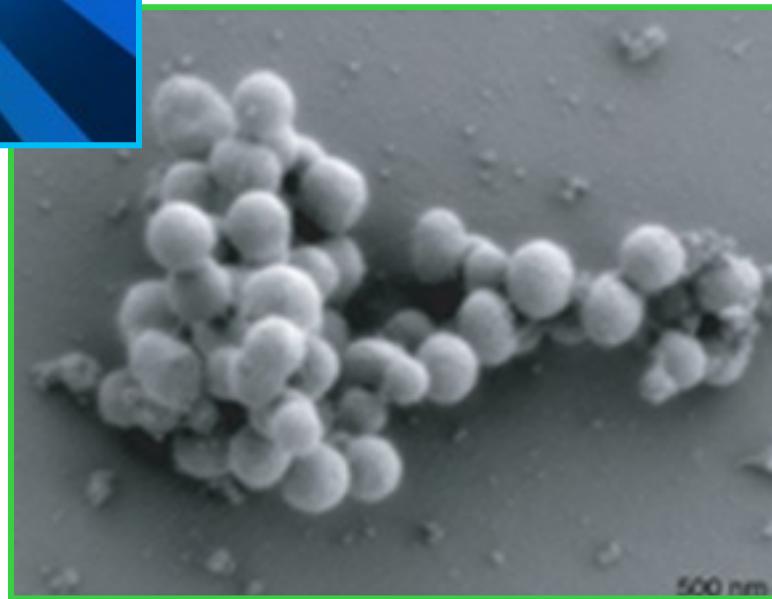


Image: The STEREO probes A and B: Credit: NASA/JHU/APL

The focus of Synthetic Biology at
the JCVI has been:

The Synthetic Cell



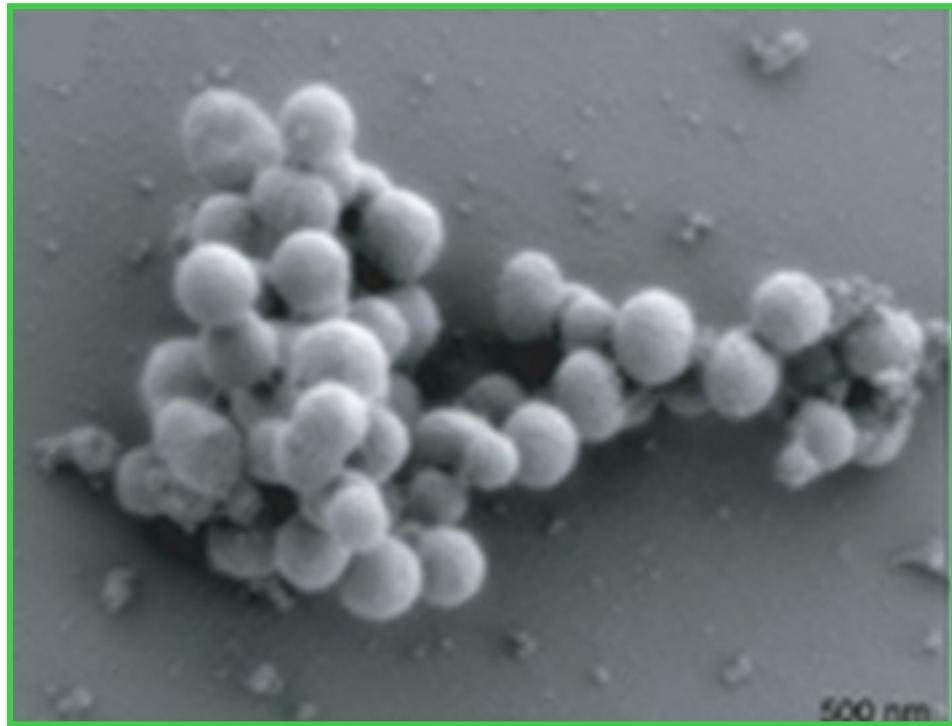
Image credit: Chidrin R Skary

"Synthetic"



"Artificial"

Synthetic Cell= A cell
operating off of a
genome that is 100%
the result of human
design decisions.



1995-2010

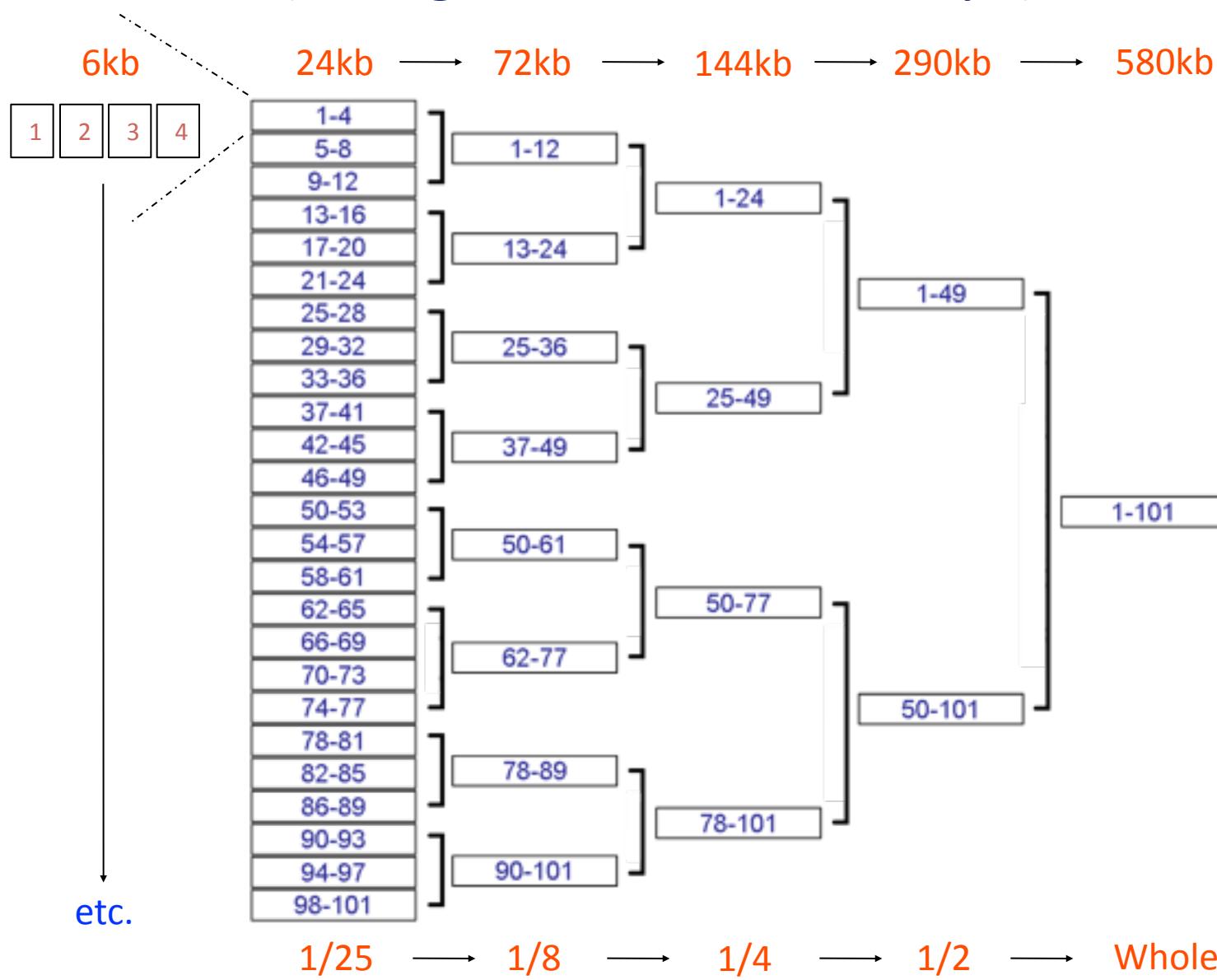
2050 ?



1887-1889

1930 The tower lost the title of the world's tallest structure when the Chrysler Building was completed in New York City.

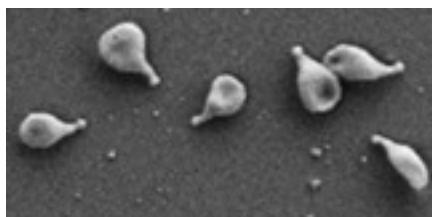
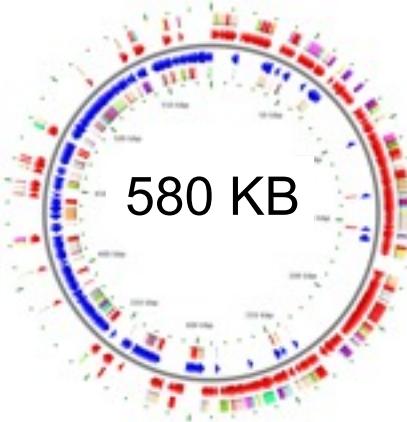
Initial Genome Assembly Strategy (Using “Gibson Assembly”)



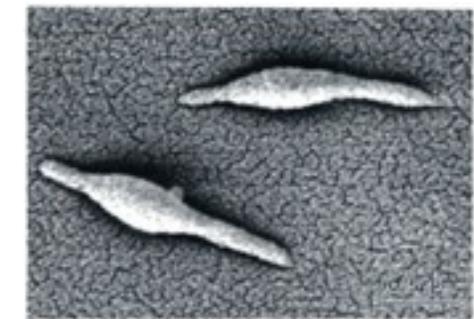
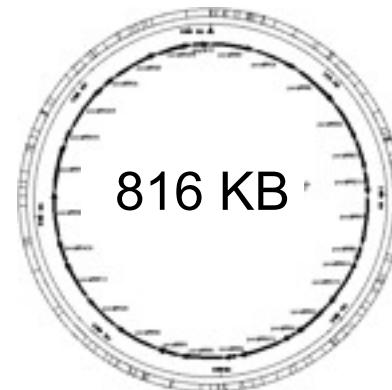
A Brief Introduction to Mycoplasma

- **Small Genomes**: 0.5-1.5 million base-pairs.
- **Alternate Genetic Code**: Instead of being a stop codon, UGA codes for Tryptophan.
- **No Cell Wall**
- Obligate Cellular **Parasites**, but can be grown in pure culture with very rich media.
- Very **limited genetic tools** available.

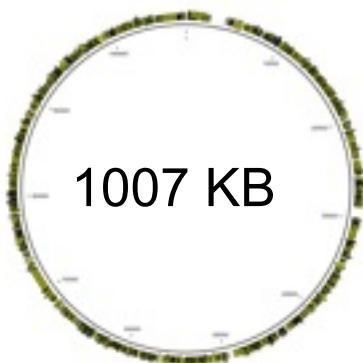
A Brief Introduction to Mycoplasma



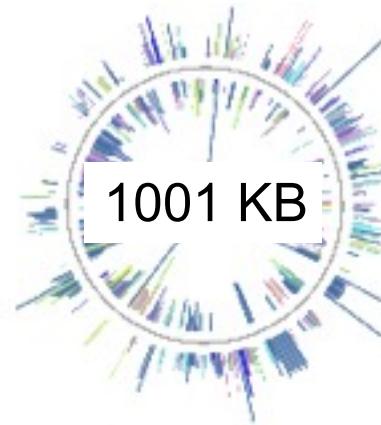
Mycoplasma genitalium



Mycoplasma pneumoniae

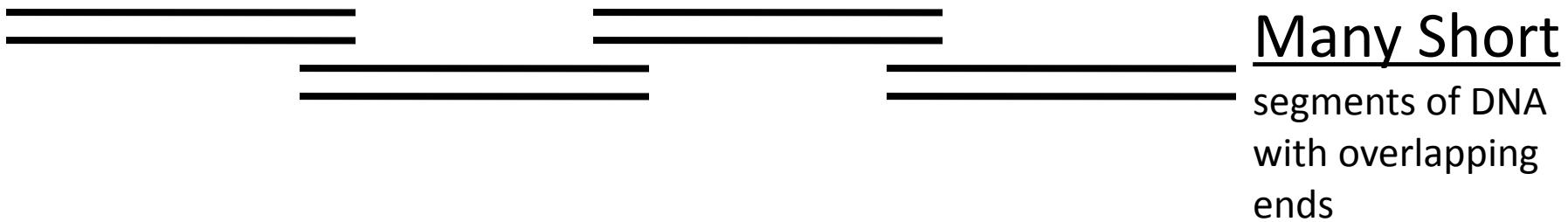


Mycoplasma mycoides capri



Mycoplasma capricolum

Isothermal Assembly AKA “Gibson Assembly”



Add:

- T5 exonuclease
 - Phusion DNA polymerase
 - Taq Ligase
 - Phusion buffer + dNTPs + PEG
- Incubate 50 °C 30 minutes



One Large Target Sequence

GTCTCTTGTCA GACTAGACGATGACTGATCGT CAGTGAAACCTACGAATCCG 3'
CAGAGAACAGTCTGATCTGCTACTGACTAGCA GTCACTTGGATGCTTAGGC 5'

3' GTCACTTGGATGCTTAGGC AGTCTCTTGTCA GACTAGACGATGACTGATCG
5' CAGTGAAACCTACGAATCCG TCAGAGAACAGTCTGATCTGCTACTGACTAGC

T5 Exonuclease Degrades 5' ends

GTCTCTTGTCA~~GACTAGACGATGACTGATCGT~~**CAGTGAAACCTACGAATCCG** 3'
CAGAGAACAGTCTGATCTGCTACTG 5'

3' **GTCACTTTGGATGCTTAGGC** AGTCTCTTGTCA~~GACTAGACGATGACTGATCGT~~
5' AGTCTGATCTGCTACTGACTAGC

single-stranded 3' ends can now anneal



"The Gibson Assembly Song"

The Cambridge iGEM Team for 2010

<http://www.cambridgeigem.org>

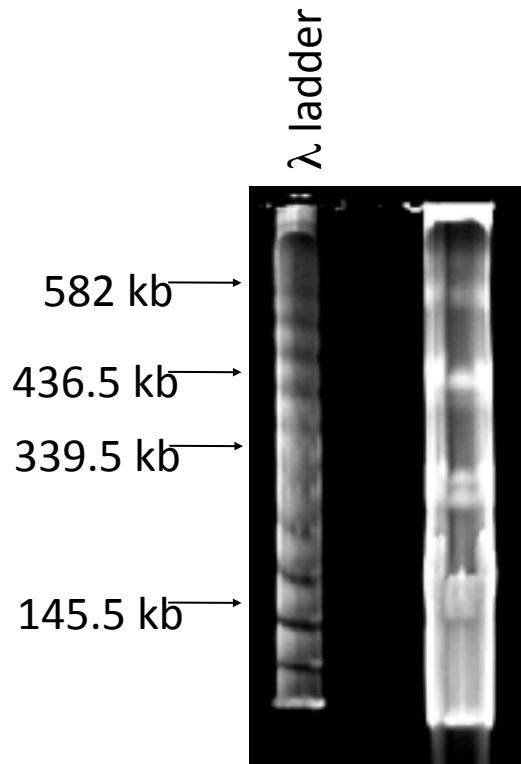
<http://www.gibthon.org>

<http://www.youtube.com/watch?v=WCWjJFU1be8>

GTCTCTTGTCA GACTAGACGGATGACTGATCGT CAGTGAAACCTACGAATCCG TCAGAGAACAGTCTGATCTGCTACTGACTAGC
CAGAGAACAGTC TGAATCTGCTACTGACTAGCA GTCACTTGGATGCCTAGGC AGTCCTCTGCA GACTAGAGATGACTAGC

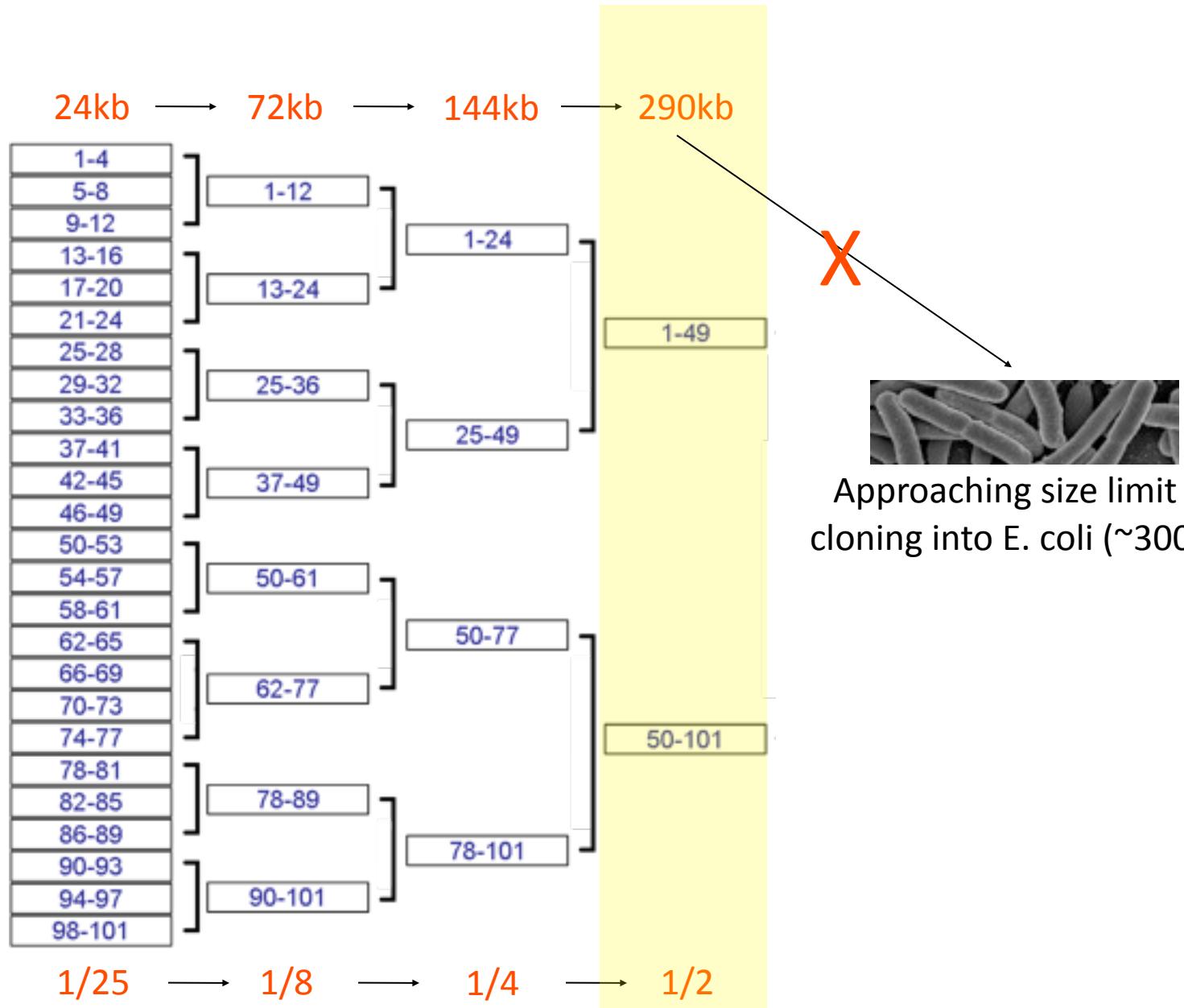
Phusion DNA polymerase extends the 3' ends to fill in the single stranded region.

Taq Ligase closes the remaining knicks.



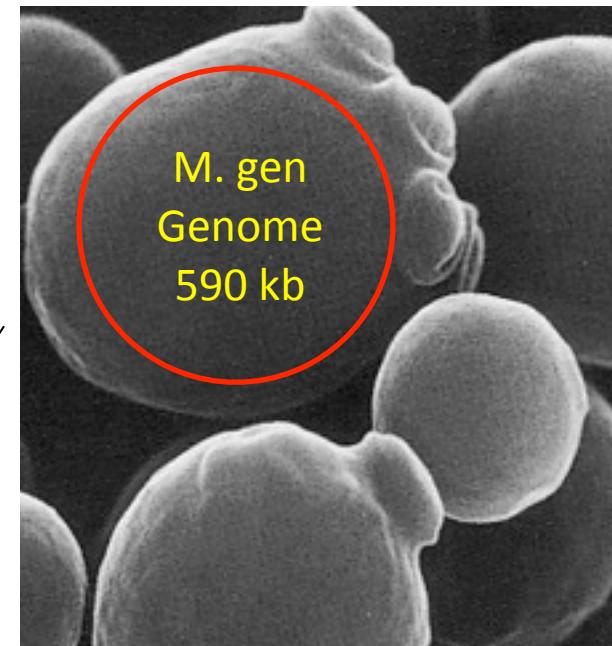
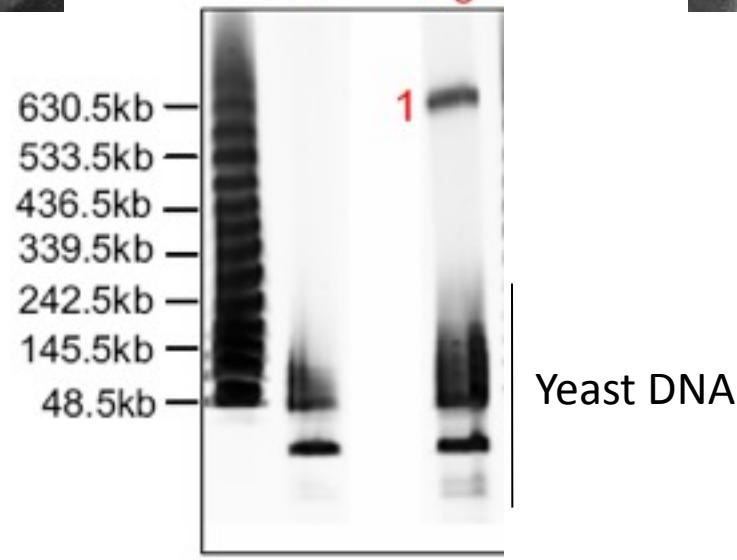
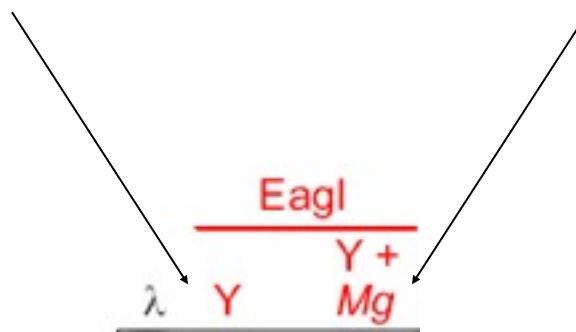
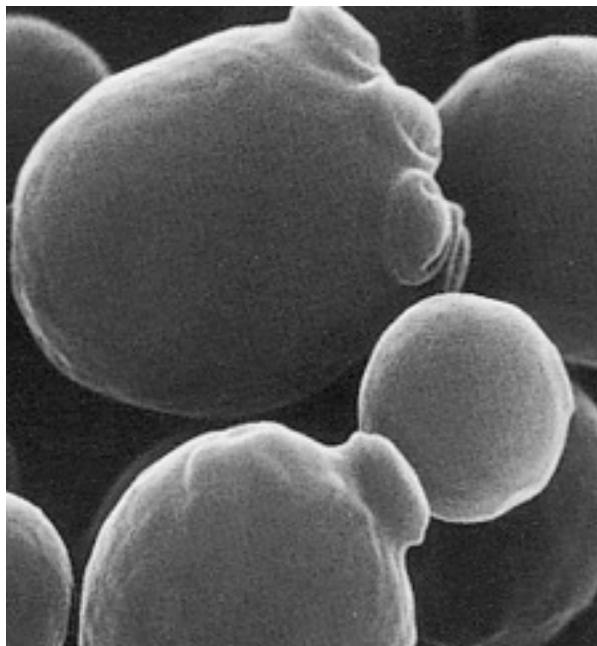
FIGE

$\frac{1}{2}$ molecule assemblies could not be cloned in E. coli



Approaching size limit of cloning into E. coli (~300kb)

Restriction digests show that 25 pieces can be assembled in yeast in one shot



Transformation-associated recombination (TAR) cloning.

BMC Genomics

Technology article
A general cloning system to selectively isolate any eukaryotic or prokaryotic genomic region in yeast
Madimir N Novak, Natalya Kropotina, Sun-Ho Lee, Ilya Ouspenski, I-Carl Barnett and Vladimir Larionov*

bioRxiv preprint doi: <https://doi.org/10.1101/2010.09.27.207020>; this version posted September 27, 2010. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Abstract
Background: Transformation-associated recombination (TAR) cloning is used as a strategy to clone a variety of large eukaryotic organisms or prokaryotic genomes. The major challenge of homologous recombination is the presence of heterologous DNA sequences that have been cloned. This study presents a general cloning system that has been developed to eliminate heterologous DNA and to facilitate the cloning of any genomic region.

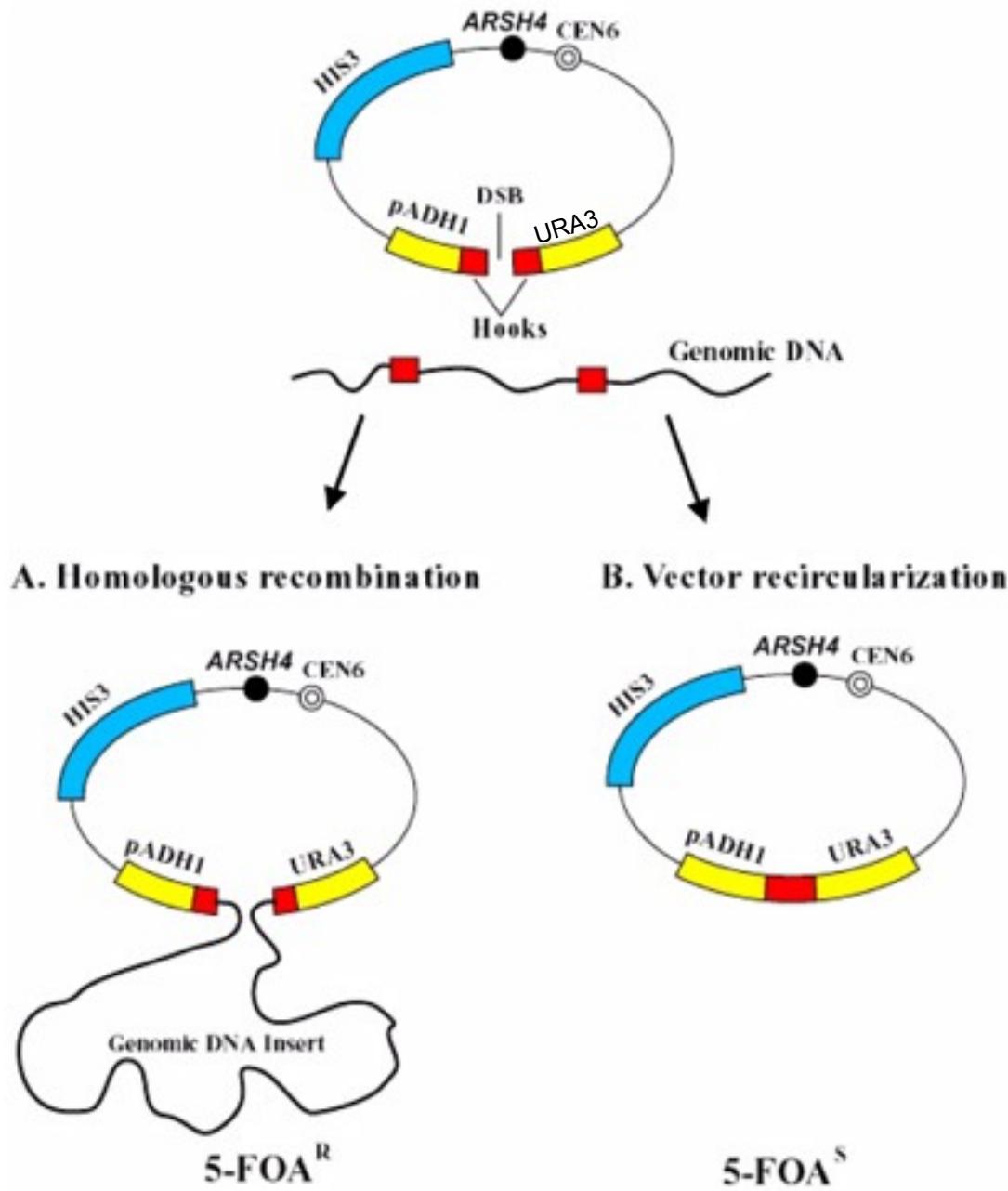
Results: TAR cloning requires that the cloned DNA fragment serve as a unique restriction enzyme (RE) site that can function as the target of recombination in yeast, which prevents wide application of the method. In this paper, we describe a new TAR cloning strategy that allows cloning of genomic regions containing RE sites. We show that the HIN-1001 protein, which contains HIN-1001 as a central regulatory module, facilitates the TAR cloning and the insertion of any RE site. The HIN-1001 protein is a very robust molecular tool that makes it possible to clone any genomic region in yeast. The HIN-1001 protein is also useful for screening and allowing selection of TAR cloning strains against background vector recombination events.

Conclusion: The new genetic system expands the area of application of TAR cloning by allowing cloning of genomic regions containing additional eukaryotic genomic regions or the presence of restriction-releasing enzymes.

Background
The development of biology requires that the used genetic tools progress from words to numbers and the next challenge often requires generation of a nucleic acid – requires the cloning of the gene between certain positions and boundaries of interest and which are unique but are poorly represented in libraries, and

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Page 1 of 11
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24kb → 580kb

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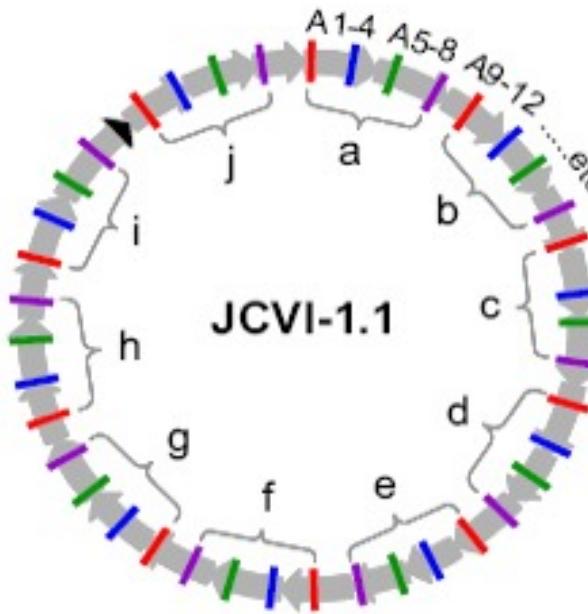
- 25 way assembly
- Hundreds of colonies
- Screen 10
- 1 of 10 correct

1/25

Whole

Amplicon size (bp)

	Set 1	Set 2	Set 3	Set 4
a	100	146	125	175
b	200	250	225	275
c	300	348	325	375
d	400	450	425	475
e	500	550	525	575
f	600	650	625	675
g	700	750	725	775
h	800	850	825	875
i	900	950	925	975
j	1000	1050	1025	1075



PNAS PNAS

One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic *Mycoplasma genitalium* genome

(Daniel G. Gibson^{1,2}, Jérôme M. Glass^{1,2}, Kwan-Hui Wu^{1,2}, Barbara E. Thompson^{1,2}, Michael A. Venter^{1,2}, Monica Aboim^{1,2}, Michael Q. Monteagudo^{1,2}, Craig Henrich^{1,2}, Matthew O. Smith^{1,2}, and Clyde H. Hutchison III^{1,2})

¹The J. Craig Venter Institute, Synthetic Biology Group, Rockville, MD 20850; ²The Institute for Genomic Research, Synthetic Biology Group, Rockville, MD 20850

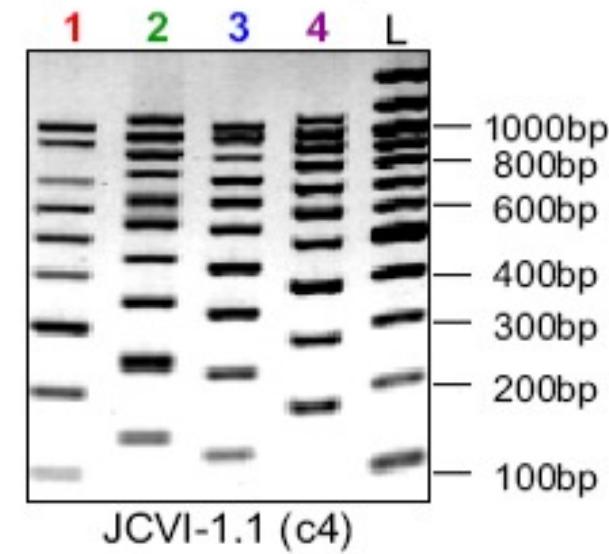
Contributed by J. Craig Venter, October 10, 2008; revised November 10, 2008; accepted November 10, 2008.

We previously reported assembly of 25 overlapping DNA fragments from bacterial genomes to complete genomes in a single step in yeast. We now report the assembly of 25 overlapping DNA fragments from one construction of the *Mycoplasma genitalium* genome. This genome was chosen because it is the smallest self-replicating genome known. The use of yeast recombination greatly simplifies the assembly of large DNA molecules from both synthetic and natural fragments.

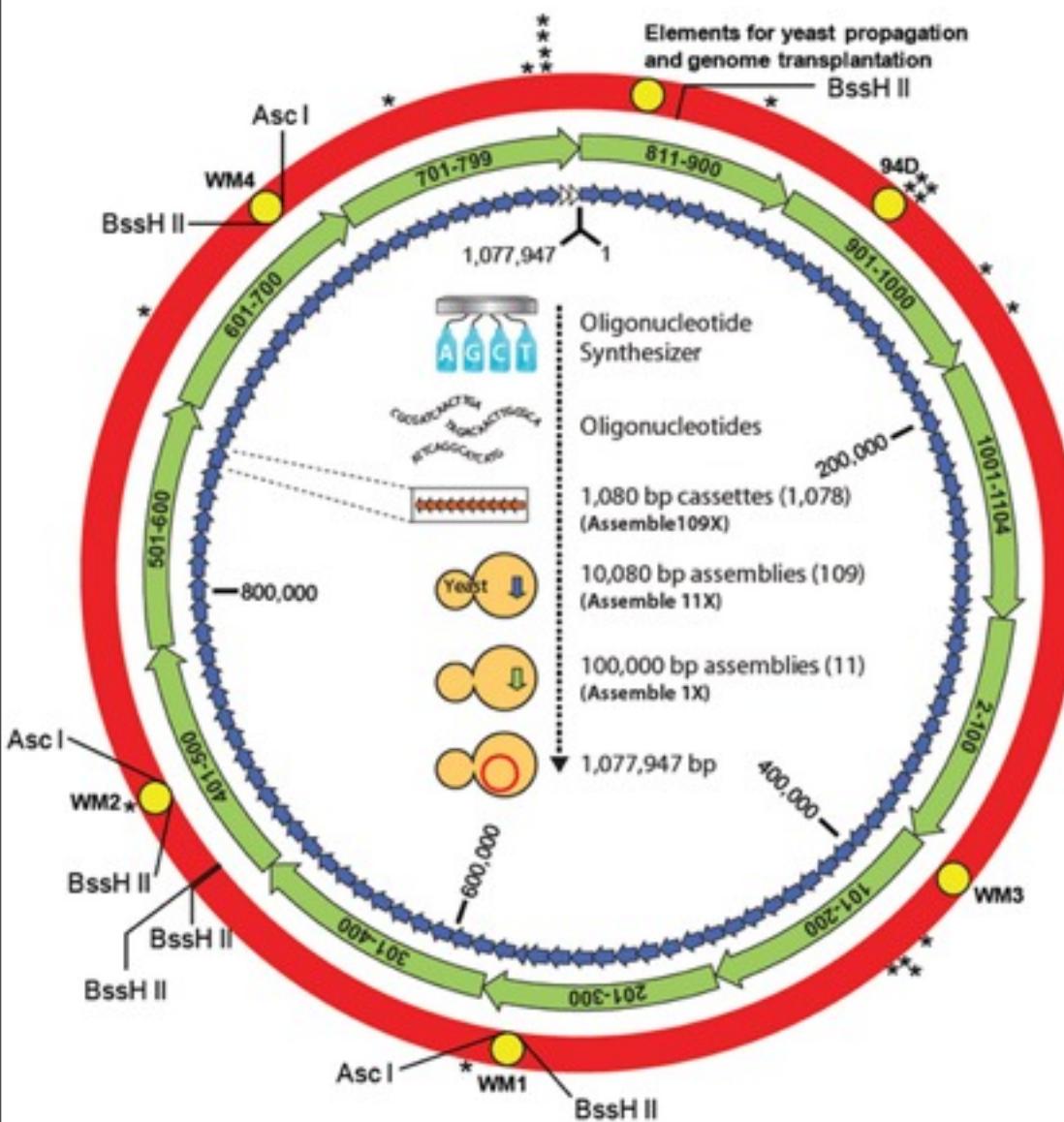
See also: Gibson et al., *Genome synthesis: combinatorial assembly of yeast transformation*; *Microplasmid*: *Synthetic biology*.

Yeast has long been considered a genetically tractable organism because of its ability to take up and incorporate DNA fragments. More than 30 years ago, Okamoto et al. (1) reported the transformation of yeast with a recombinant plasmid containing a fragment of *lambda* DNA (λL21) using a modified method involving saponin, CaCl₂, and PEG. More recently, Cho-Woo et al. (2) reported mechanistic studies demonstrating that DNA molecules

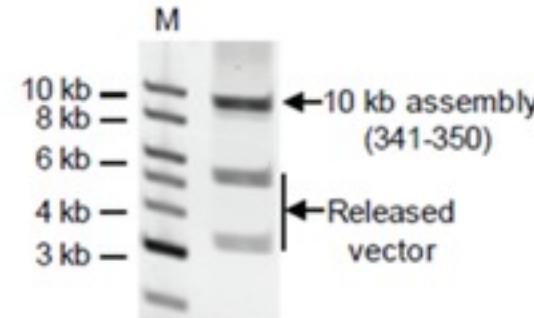
Primer set



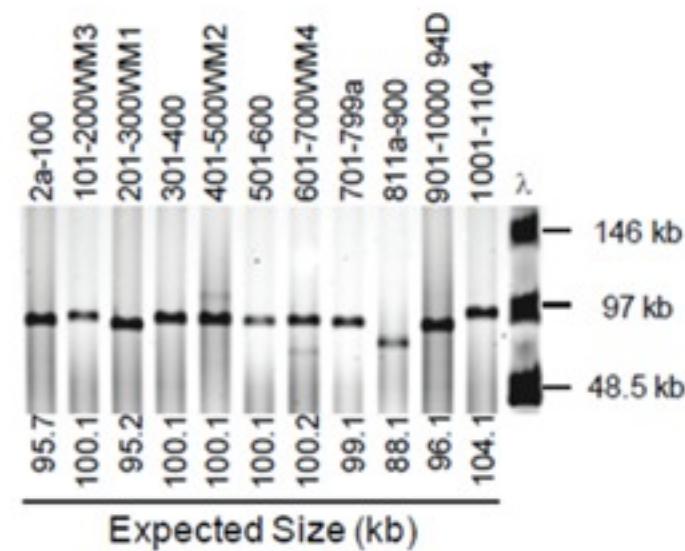
Schematic demonstrating the assembly of the synthetic *M. mycoides* genome in yeast



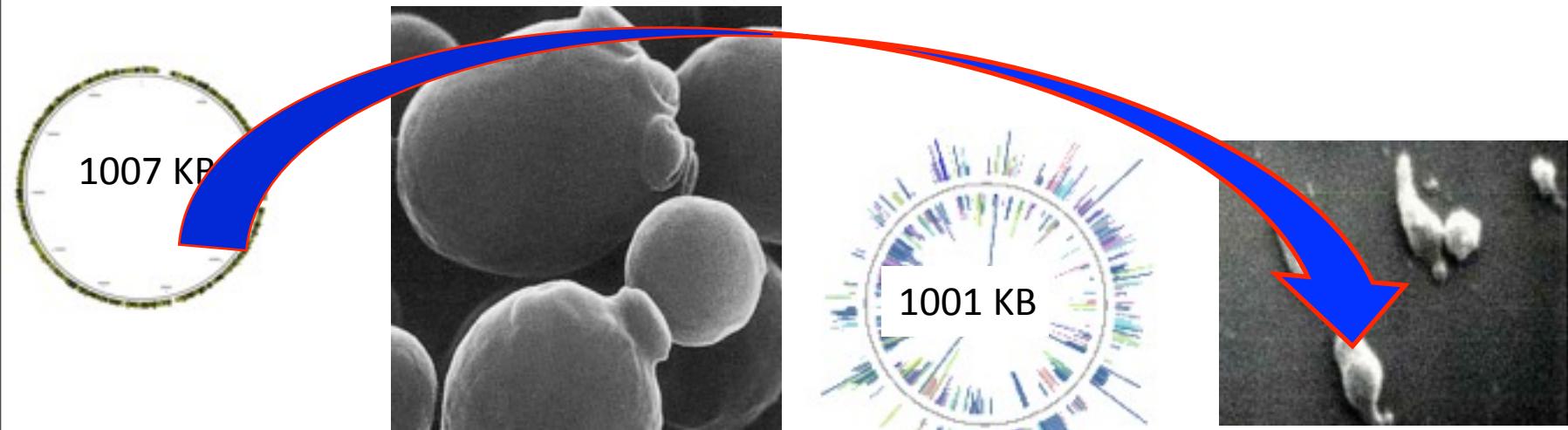
Example 10 kb sub-assembly



All eleven 100 kb sub-assemblies

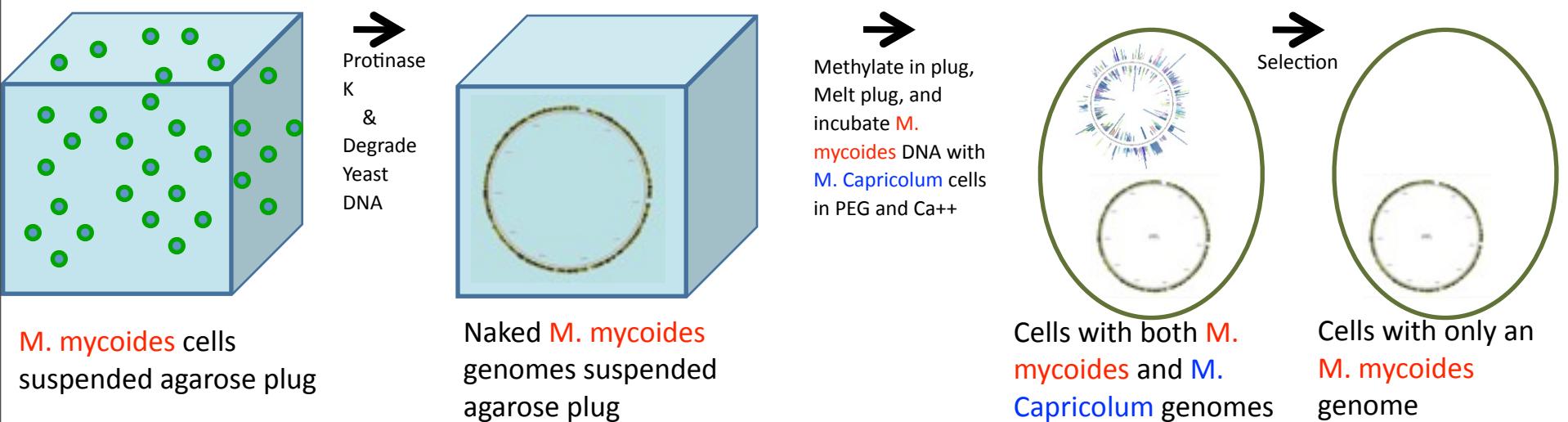


Whole Genome Transplantation.

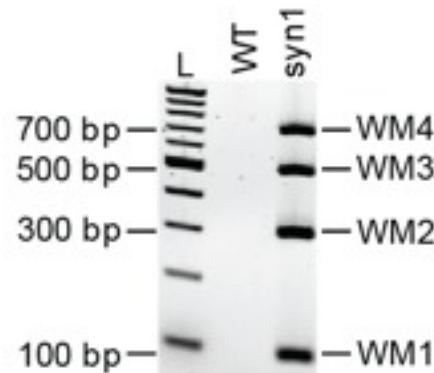
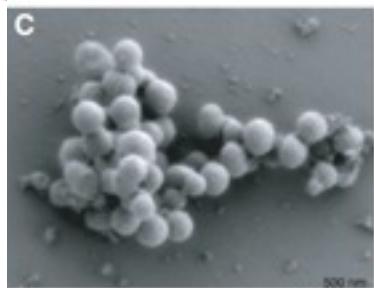
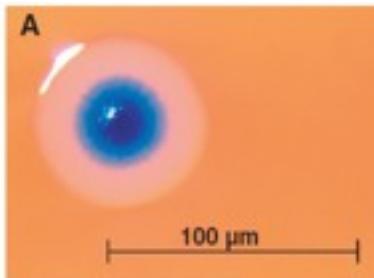


Mycoplasma mycoides
capri... IN YEAST

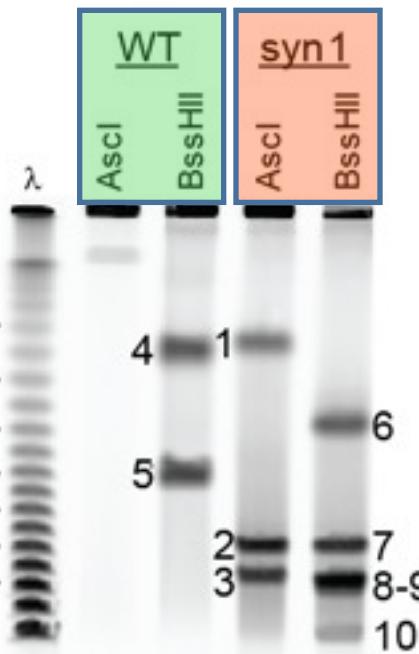
Mycoplasma capricolum



Characterization of the JCVI-syn1.0 transplants

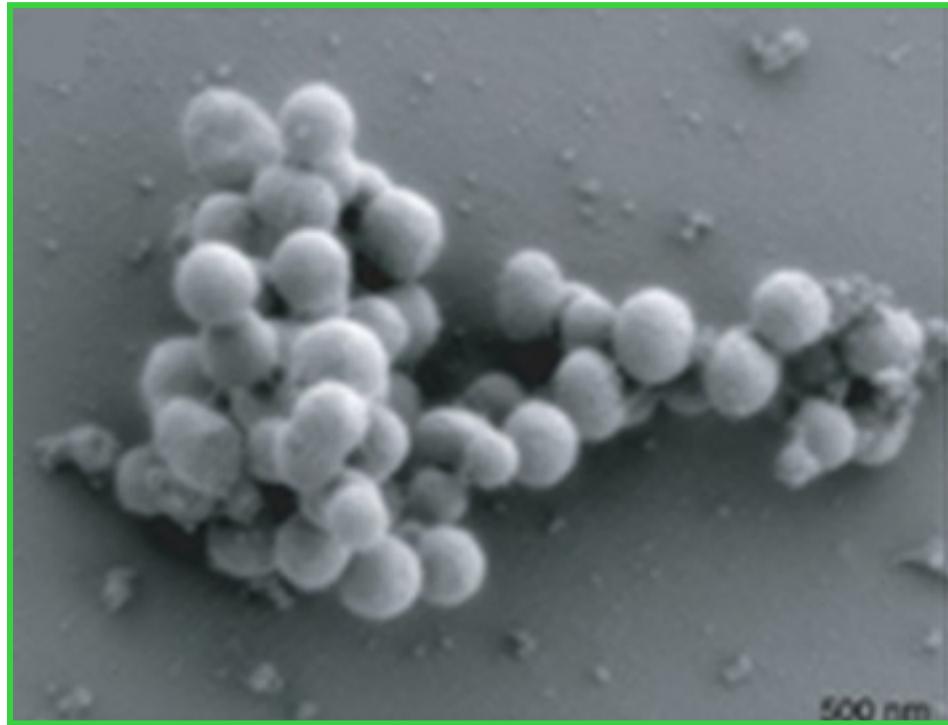


Multiplex PCR of Watermark Sequences



CHEF gel restriction analysis

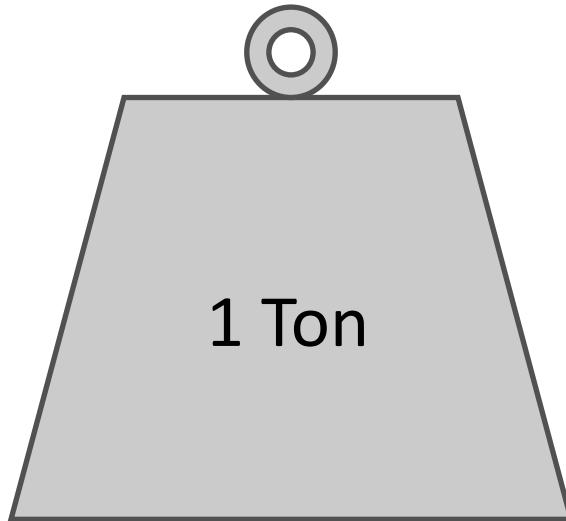
Strain	Digest	Fragment # and size (kb)
WT	Asci	No sites
WT	BssHII	(4) 668 (5) 419
Syn235	Asci	(1) 685 (2) 233 (3) 160
Syn235	BssHII	(6) 533 (7) 233 (8) 152 (9) 126 (10) 34



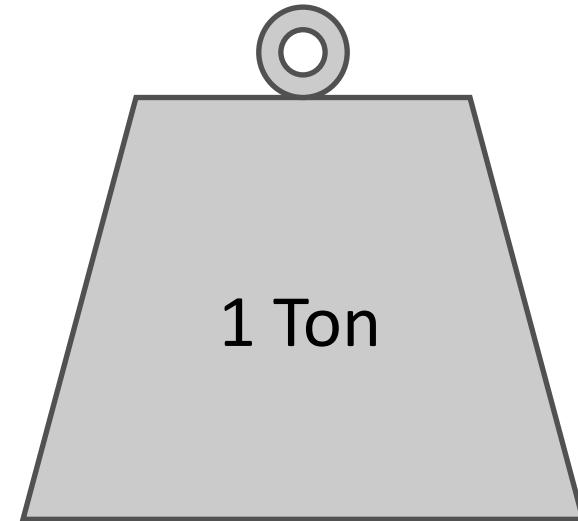
- DNA assembly by invitro recombination.
- DNA assembly by yeast recombination.
- Whole genome cloning in yeast.
- Genome Transplantation.

- Steel Lattice Construction.

The Apollo Equation



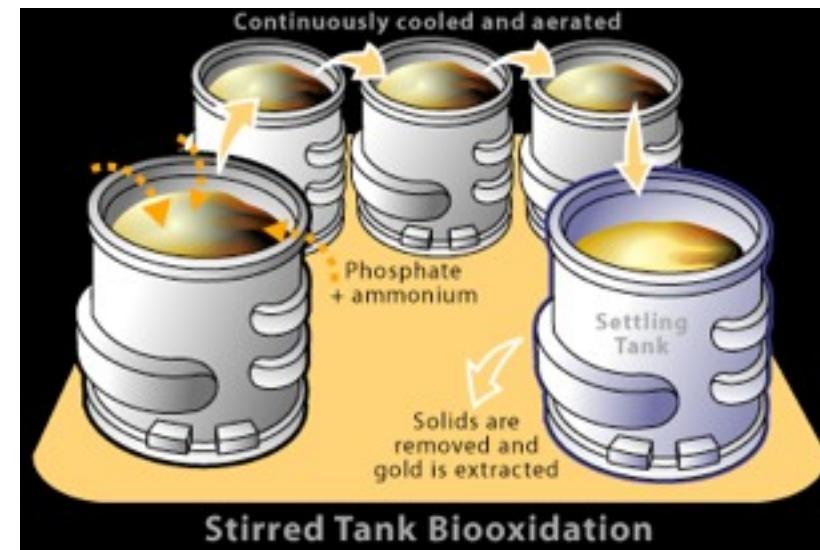
1 Ton of equipment
or consumables in
space



1 Ton of equipment or
consumables launched
from the Earth into
space

Metal Extraction: "Bio-Leaching"

- In Situ Resource Utilization is a way to break the Apollo Equation.
- Bio-Leaching USING NATURAL BACTERIAL COMMUNITIES has been used on Earth for decades for the extraction of Copper and Gold from low-grade ore.
- It is now being adapted to other elements such as Cobalt.



This picture is attributed to "BioMineWiki" and the author Anna Bauer.
http://wiki.biomine.skelleftea.se/wiki/index.php/Main_Page

This picture is attributed to "The Science Creative Quarterly" (scq.ubc.ca) and Jane Wang. This graphic file is protected under [the creative commons licence](http://creativecommons.org/licenses/by-sa/3.0/)

Bioleaching bacterial communities are amenable to metagenomic sampling.

- Acidophilic biofilm from the Richmond mine at Iron Mountain.
- Near-complete genomes recovered without culturing due to extremely small number of species present.

articles

Community structure and metabolism through reconstruction of microbial genomes from the environment

Gene W. Tyson¹, Jørn Pedersen^{2,3}, Philip Hugenholtz¹, Eric E. Allen¹, Rachna J. Ram¹, Paul H. Richardson¹, Victor V. Solov'yov⁴, Edward M. Rubin⁵, Daniel S. Rothman^{1,4} & Jillian F. Banfield^{1,2}

¹Department of Environmental Science, Policy and Management, ²Department of Earth and Planetary Sciences, and ³Department of Physics, University of California Berkeley, California 94720, USA
⁴Joint Genome Institute, Walnut Creek, California 94598, USA

Microbial communities are vital in the functioning of all ecosystems; however, most microorganisms are uncultivated, and their roles in natural systems are unclear. Here, using random shotgun sequencing of DNA from a natural acidophilic biofilm, we report reconstruction of near-complete genomes of *Leptospirillum* group II and *Ferrovibrio* type II, and partial recovery of three other genomes. This was possible because the biofilm was dominated by a small number of species populations and the frequency of genomic rearrangements and gene insertions or deletions was relatively low. Because each sequence read came from a different individual, we could determine that single-nucleotide polymorphisms are the predominant form of heterogeneity at the strain level. The *Leptospirillum* group II genome had remarkably few nucleotide polymorphisms, despite the existence of low-abundance variants. The *Ferrovibrio* type II genome seems to be a composite from three ancestral strains that have undergone homologous recombination to form a large population of mosaic genomes. Analysis of the gene complement for each organism revealed the pathways for carbon and nitrogen fixation and energy generation, and provided insights into survival strategies in an extreme environment.

The study of microbial evolution and ecology has been revolutionized by DNA sequencing and analysis^{1–4}. However, isolates have been the main source of sequence data, and only a small fraction of microorganisms have been cultivated^{5–8}. Consequently, focus has shifted towards the analysis of uncultivated microorganisms via cloning of conserved genes⁹ and genome fragments directly from the environment^{10–12}. To date, only a small fraction of genes have been recovered from individual environments, limiting the analysis of microbial communities as networks characterized by symbioses, competition and partitioning of community-existent roles. Comprehensive genomic data would resolve organism-specific pathways and provide insights into population structure, speciation and evolution. So far, sequencing of whole communities has not been practical because most communities comprise hundreds to thousands of species¹³.

Acid mine drainage (AMD) is a worldwide environmental problem that arises largely from microbial activity¹⁴. Here, we focused on a low-complexity AMD microbial biofilm growing hundreds of feet underground within a pyrite (FeS₂) ore body^{15–17}. This represents a self-contained biogeochemical system characterized by tight coupling between microbial iron oxidation and acidification due to pyrite dissolution^{18,19}. Random shotgun sequencing of DNA from entire microbial communities is one approach for the recovery of the gene complement of uncultivated organisms, and for determining the degree of variability within populations at the genome level. We used random shotgun sequencing of the biofilm to obtain the first reconstruction of multiple genomes directly from a natural sample. The results provide novel insights into community structure, and reveal the strategies that underpin microbial activity in this environment.

Initial characterization of the biofilm

Biofilms growing on the surface of flowing AMD in the five-way region of the Richmond mine at Iron Mountain, California¹⁵, were sampled in March 2000. Screening using group-specific

fluorescence *in situ* hybridization (FISH) revealed that all biofilms contained mixtures of bacteria (*Leptospirillum*, *Sulfobacillus* and, in a few cases, *Acidimicrobium*) and archaea (*Ferroplasma* and other members of the *Thermoplasmatales*). The genome of one of these archaea, *Ferroplasma acidarmanus* sp. 1, isolated from the Richmond mine, has been sequenced previously (<http://www.jgi.doe.gov/cgi-bin/blast/firr/faeroma/homepage.html>).

A pink biofilm (Fig. 1a) typical of AMD communities was selected for detailed genomic characterization (see Supplementary Information). The biofilm was dominated by *Leptospirillum* species, and contained *F. acidarmanus* at a relatively low abundance (Fig. 1b–c). This biofilm was growing in pH 0.85, 42 °C, 317 mM Fe, 14 mM Zn, 4 mM Cu and 2 mM As solution, and was collected from a surface area of approximately 0.03 m².

A pink ribosomal RNA gene clone library was constructed from DNA extracted from the pink biofilm, and 384 clones were analysed (see Supplementary Information). Results indicated the presence of three bacterial and three archaeal lineages. The most abundant clones are close relatives of *L. ferriphilum*¹⁷ and belong to *Leptospirillum* group II (ref. 18). Although 94% of the *Leptospirillum* group II clones were identical, 17 minor variants were detected with up to 1.2% 16S rRNA gene-sequence divergence from the dominant type. Tightly defined groups (up to 1% sequence divergence) related to *Leptospirillum* group III (ref. 18), *Sulfobacillus*, *Ferroplasma* (some identical to ref. 1), *G-plasma*²⁰ and *G'-plasma*²¹ were also detected. *Leptospirillum* group III, *G-plasma* and *G'-plasma* have only recently been detected in culture-independent molecular surveys. FISH-based quantification (Fig. 1c; see also Supplementary Information) confirmed the dominance of *Leptospirillum* group II in the biofilm.

Community genome sequencing and assembly

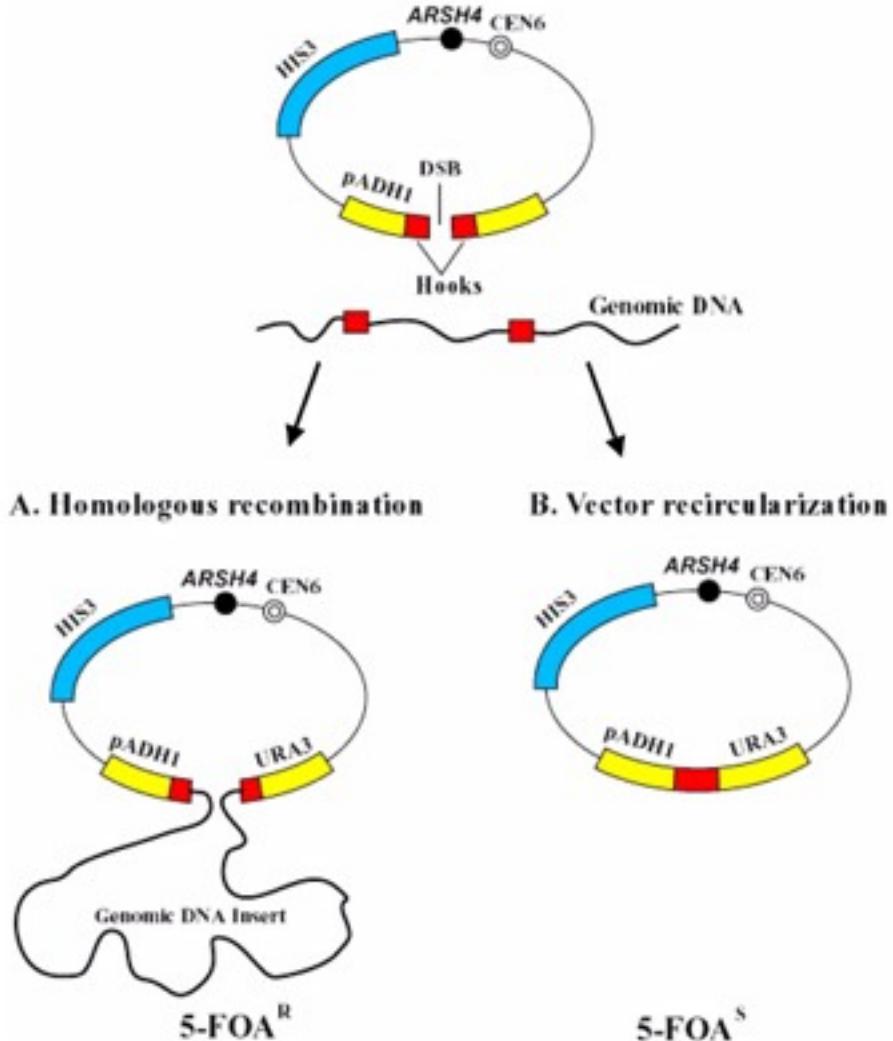
In conventional shotgun sequencing projects of microbial isolates, all shotgun fragments are derived from clones of the same genome. When using the shotgun sequencing approach on genomes from an

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Bioleaching bacterial communities are amenable to metagenomic sampling.



Whole genome cloning offers the possibility for massively up-scaled metagenomic sampling!

Proposed direction of experiments:

- 1.Using asteroidal and lunar stimulants, create a laboratory analog to tank bioleaching based mining operations in space.
- 2.Populate the laboratory analog(s) with undefined bacterial communities from nature.
- 3.Score different populations for their capability to liberate desired metals from the simulants.
- 4.Use TAR-cloning and metagenomic sampling to characterize and sequence the best-scoring populations.
- 5.Identify genes of interest that may be involved in the liberation of desired metals.



This was a massive team effort

- Algire, Mikkel
- Alperovich, Nina
- Assad-Garcia, Nacyra
- Baden-Tillson, Holly
- Benders, Gwyn**
- Chuang, Ray-Yuan
- Dai, Jianli
- Denisova, Evgeniya
- Galande, Amit
- Gibson, Daniel**
- Glass, John
- Hutchison, Clyde
- Iyer, Prabha
- Jiga, Adriana
- Krishnakumar, Radha
- Lartigue, Carole**
- Ma, Li
- Merryman, Chuck
- Montague, Michael
- Moodie, Monzia
- Moy, Jan
- Noskov, Vladimir
- Pfannkoch, Cindi
- Phang, Quan
- Qi, Zhi-Qing
- Ramon, Adi
- Saran, Dayal
- Smith, Ham
- Tagwerker, Christian
- Thomas, David
- Tran, Catherine
- Vashee, Sanjay
- Venter, J. Craig**
- Young, Lei
- Zaveri, Jayshree
- Johnson, Justin
- Brownley, Anushka
- Parmar, Prashanth
- Pieper, Rembert
- Stockwell, Tim
- Sutton, Granger
- Viswanathan, Lakshmi
- Yooseph, Shibu
- Segall-Shapiro, Thomas**
- Calvey, Christopher**
- Parmar, Prashanth**

Funding from
Synthetic Genomics Inc.
JCVI
DOE GTL program

Modular parts must be characterized in space!



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The BioBrick Foundation (BBF) is a non-for-profit organization founded by engineers and scientists from MIT, Harvard, and UCSD with significant experience in both non-profit and commercial biotechnology ventures. BBF encourages the development and responsible use of technologies based on BioBrick® standard DNA parts that encode basic biological functions.

Using BioBrick® standard biological parts, a synthetic biologist or biological engineer can, already, to some extent, program living organisms. In the case we a computer scientist can program a computer. The DNA sequence information and other characteristics of BioBrick® standard biological parts are made available to the public free of charge currently via BBF's Registry of Standard Biological Parts.

BioBrick™

Any individual or organization is welcome to design, improve, and contribute BioBrick® standard biological parts to the Registry. For example, in the summer of 2007, over 400 students and institutions at 60+ universities around the world are adding, sharing, and using BioBrick® standard biological parts as part of the International Genetically Engineered Machine (iGEM) competition.

The BBF supports an open technical standards setting process that is used to define BioBrick® standard biological parts, and other technical matters relevant to synthetic biology research and applications. Feel free to join or contribute to the work of the BBF Technical Standards Working Group if you are interested.

The BBF also supports a Legal Working Group that is developing the BioBrick® legal schema. Feel free to join or contribute to the BBF Legal Working Group if you are interested.

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Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq

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A comprehensive analysis of both the molecular genetic and phenotypic responses of any organism to the space flight environment has never been accomplished because of significant technological and logistical hurdles. Moreover, the effects of space flight on microbial pathogenicity and associated infectious disease risks have not been studied. The bacterial pathogen *Salmonella typhimurium* was grown aboard Space Shuttle mission STS-115 and compared with identical ground control cultures. Global microarray and proteomic analyses revealed that 167 transcripts and 73 proteins changed expression with the conserved RNA-binding protein Hfq identified as a likely global regulator involved in the response to this environment. Hfq involvement was confirmed with a ground-based microgravity culture model. Space flight samples exhibited enhanced virulence in a murine infection model and extracellular matrix accumulation consistent with a biofilm. Strategies to target Hfq and related regulators could potentially decrease infectious disease risks during space flight missions and provide novel therapeutic options on Earth.

microgravity | Space Shuttle | low shear modeled microgravity | rotating wall vessel | salmonella

Environmental conditions and crew member immune dysfunction associated with space flight may increase the risk of infectious disease during a long-duration mission (1–4). However, our knowledge of microbial changes in response to space flight conditions and the corresponding changes to infectious disease risk is limited and unclear. Elucidation of such risks and the mechanisms behind any space flight-induced changes to microbial pathogens holds the potential to decrease risk for human exploration of space and provide insight into how pathogens cause infections in Earth-based environments. Numerous logistical and technological hurdles exist when performing biological space flight experimentation, and an extremely limited number of opportunities to perform such research are available. Accordingly, comprehensive analysis of cells, including pathogenic microbes, at the molecular and phenotypic level during space flight offers a rare opportunity to examine their behavior and response in this environment.

Previous studies using the enteric bacterial pathogen *Salmonella enterica* serovar *Typhimurium* showed that growth in a ground-based space flight analog bioreactor, termed the rotating wall vessel (RWV), induced global genotypic and phenotypic changes in this organism (5–7). Specifically, *S. typhimurium* grown in this space flight analog culture environment, described

as low-shear modeled microgravity (LSMMG), exhibited increased virulence, increased resistance to environmental stresses (acid, osmotic, and thermal), increased survival in macrophages, and global changes in gene expression at the transcriptional and translational levels (5–7). Collectively, these results suggested the potential that the true space flight environment could globally alter bacterial genotypic and phenotypic responses. Thus, we designed an experimental approach to test our hypothesis, specifically to culture *S. typhimurium* during space flight and evaluate changes in microbial gene expression and virulence in response to this environment.

Our experiments were flown on Space Shuttle Atlantis Mission STS-115 (September 2006). In this experiment, cultures of *S. typhimurium* were activated to grow in space for a specific time period and then either fixed in an RNA/protein fixative or supplemented with additional growth media after this time period [supporting information (SI) Fig. 3]. At 2.5 h after landing at Kennedy Space Center, the culture samples were recovered and subsequently used for whole-genome transcriptional microarray and proteomic analysis (fixed samples) or for infections in a murine model of salmonellosis (media-supplemented samples). In each case, the flight culture samples were compared with culture samples grown under identical conditions on the ground at Kennedy Space Center using coordinated activation

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Abbreviations: LSMMG, low-shear modeled microgravity; RWV, rotating-wall vessel; RPA, fluid processing apparatus; Km, kanamycin.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17323.

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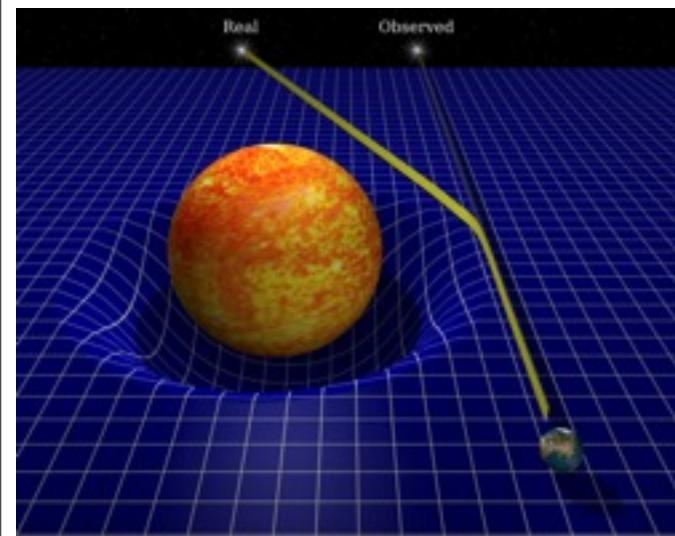
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