WHO'S THERE ANYWAY?…STRAIN LEVEL DIFFERENTIATION OF THE PROBIOTIC *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS*

Dairy Practices Council
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Robert F. Roberts, Ph.D.
Professor and Head
Department of Food Science
The Pennsylvania State University
rfr3@psu.edu

www.foodscience.psu.edu

Long-Term Goals of Probiotic Research in the Roberts' Lab

1. Identify and evaluate strategies to enhance the survival of probiotic bacteria in dairy and non-dairy food systems
2. Develop molecular methods to identify and differentiate probiotic bacteria
3. Capitalize on strengths in microbiology and dairy technology to generate clinical evidence of the efficacy and mode of action of probiotic bacteria

Before we get to far…Taxonomy Review

- Genus
  - ″one or more species with the same general phenotypic characteristics and which cluster together on the basis of 16s rRNA sequences″ (Brenner et al 2001)
- Species
  - ″a group of strains that are highly similar to each other and collectively have certain distinguishing characteristics″ (Colwell et al 1995)
- Subspecies
  - ″a group of strains within a species that consistently cluster on the basis of phenotypic and genotypic characteristics″ (Wayne et al 1987; Brenner et al 2001)

What is a "Strain"

- Strain
  - "any culture knowingly defined from the original strain" (De Vos and Truber 2000)
  - "Descendents of a single isolation in pure culture…ultimately derived from an initial single colony" (Brenner et al 2001)
- Issues
  - These definitions are stringent and require a good deal of knowledge of source and history
  - No mention of phenotype or genotype
  - No accounting for change over time
  - No accounting for the commercial relevance of specific characteristics

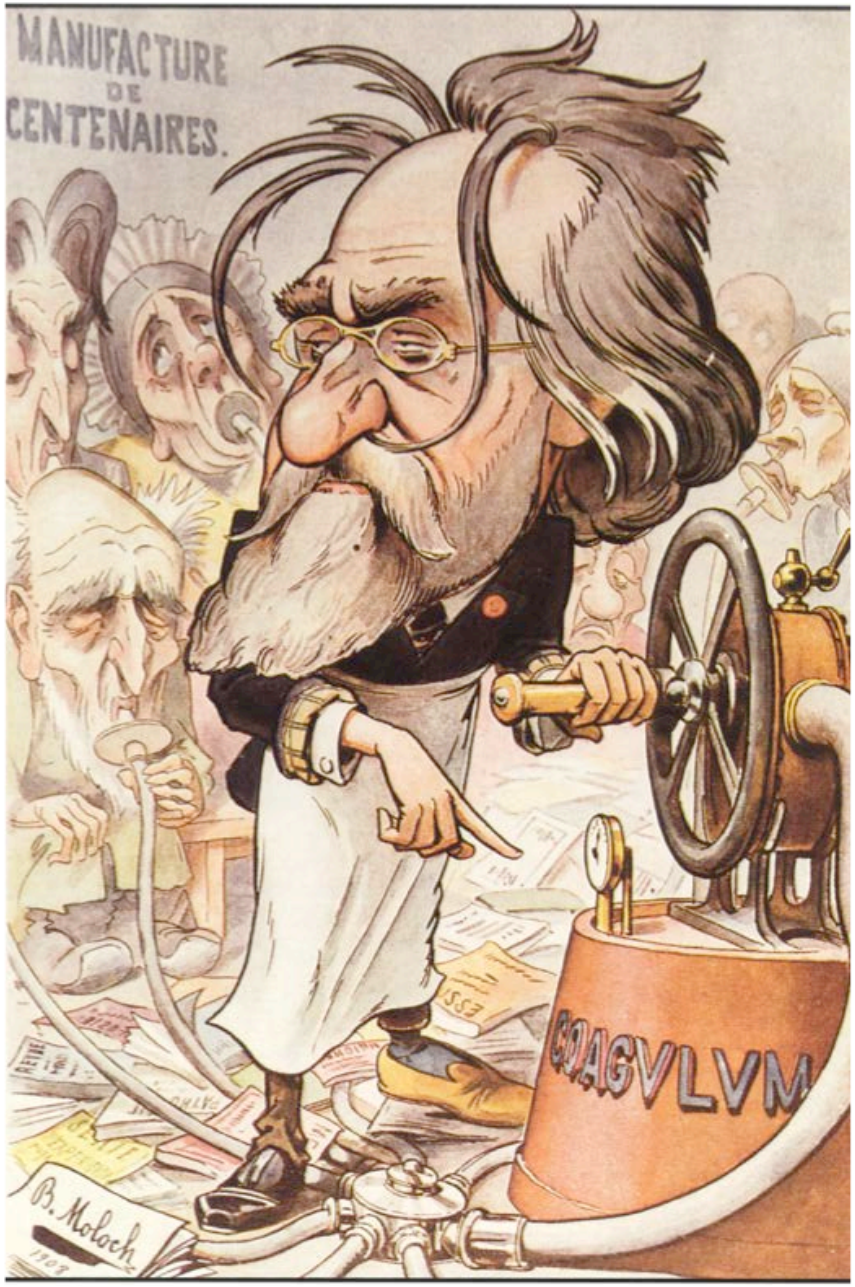
An "Operational" Definition of Strain

- "a strain is an isolate that can be differentiated from other isolates of the same genus, species and subspecies by at least one *(relevant)* phenotypic or genotypic characterisitic" (Tenover et al 1995; Dijkshoorn and Towner 2001)
- Considerations
  - What phenotypic or genotypic characteristics are significant enough to justify identifying an organism as belonging to a particular strain?
  - How many "differences" can be allowed within a strain?

Probiotics

“Live microorganisms which when administered in adequate amounts confer a health benefit on the host”

-(FAO/WHO 2002)

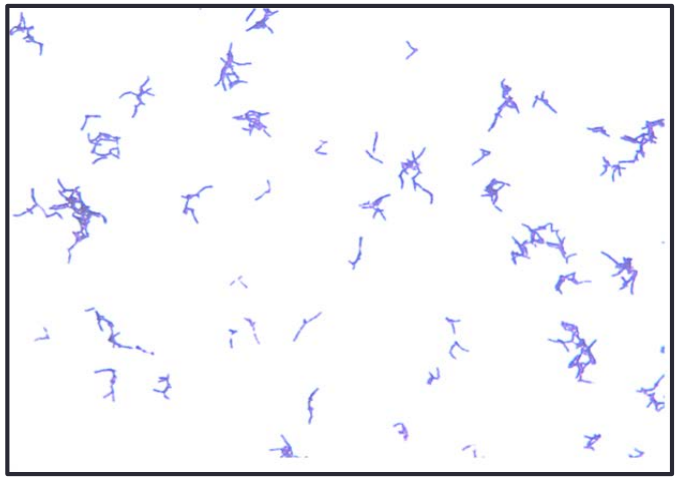


Probiotics

- ***Lactobacillus***
  - *L. acidophilus*
  - *L. casei*
  - *L. gasseri*
  - *L. johnsonii*
  - *L. reuteri*
  - *L. rhamnosus*
- ***Bifidobacterium***
  - *B. adolescentis*
  - *B. animalis* ssp. *animalis*
  - ***B. animalis* ssp. *lactis***
  - *B. bifidum*
  - *B. breve*
  - *B. longum* ssp. *infantis*
  - *B. longum* ssp. *longum*

Bifidobacterium animalis subsp. *lactis*

- First isolated and identified as a new species in 1997 (Meile *et al.* 1997)
- Most common (sub)species of bifidobacteria isolated from dairy products (Fasoli *et al.* 2003)
- Relevant Characteristics
  - Oxygen-tolerant (Simpson *et al*, 2005)
  - Acid-resistant (Matsumoto *et al*, 2004, Vernazza *et al*, 2006)
  - Bile-tolerant (Jayamanne and Adams 2006)
  - Growth observed in milk and milk based media (Masco *et al.* 2004)



DANNON
BIFIDUS REGULARIS
ACTIVIA

www.activia.com

For Probiotic Bifidobacteria…

Genus	*Bifidobacterium*
Species	*animalis*
Subspecies	*lactis*
Strain	*BB12*
	HN019
	DSMZ 10140
	YFS

- Since the health effects of probiotic bacteria are considered STRAIN SPECIFIC (FAO/WHO 2002)
- How do you know “who’s there?” in a product?

Significance of Strain Specific Identification

- Why do we care?
  - Health benefits are considered to be **strain-specific**
  - Allow manufacturers of probiotic formulations to identify their product
  - Allow verification of strains isolated from participants in clinical trials

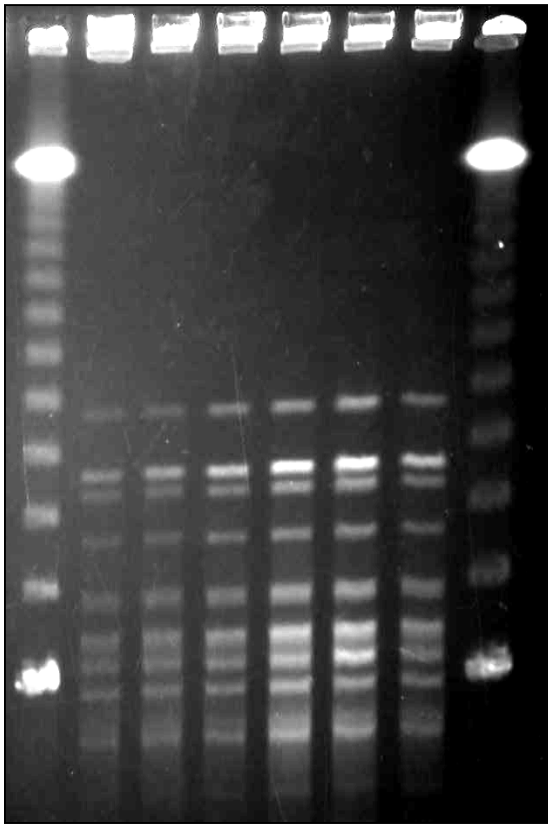
B. animalis ssp. *lactis* Strain Collection

Strain	Source
DSMZ 10140-Type Strain	Culture Collection
ATCC 27536	Culture Collection
Bl-04	Commercial
RB 0171	Commercial
RB 1280	Commercial
RB 1281	Commercial
RB 1573	Commercial
RB 1791	Commercial
RB 3046	Commercial
RB 4052	Commercial
RB 4536	Commercial

Strain	Source
RB 4753	Commercial
RB 4825	Commercial
RB 5251	Commercial
RB 5422	Commercial
RB 5733	Commercial
RB 5851	Commercial
RB 5859	Commercial
RB 7239	Commercial
RB 7339	Commercial
RB 8613	Commercial
RB 9321	Commercial
RB 9632	Commercial
HN019	Commercial

Possible Methods for Strain Differentiation

- **Phenotypic**
  - Carbohydrate Utilization
  - Enzyme Activity
  - Organic Acid Production
- **DNA-based**
  - PFGE
  - RAPD-PCR
  - Sequence of 16S-23S ITS
  - MLST



PFGE of *B. animalis* subsp. *lactis* performed with *Spe*I and *Xba*I yielded identical patterns for all strains except for ATCC 27536 digested with *Spe*I

(Briczinski 2007)

Differentiation of Strains[1] of *B. animalis* subsp. *lactis*

With a 24 isolate collection

Method	Difference
MLST	No differences
PFGE	ATCC 27536 (one extra band)
16S-23S ITS sequenced	No differences
RAPD-PCR	No differences
Lactic: Acetic Acid	No differences
Glucose utilization	12 “Fast” strains, 12 “Slow” strains

[1] Strains may simply be different “isolates” of a single strain

(Briczinski 2007)

When this project was initiated…

- No genome sequence was available for *B. animalis* subsp. *lactis*
- No reliable method was available to differentiate strains
- Diversity within the subspecies was not understood

Hypothesis

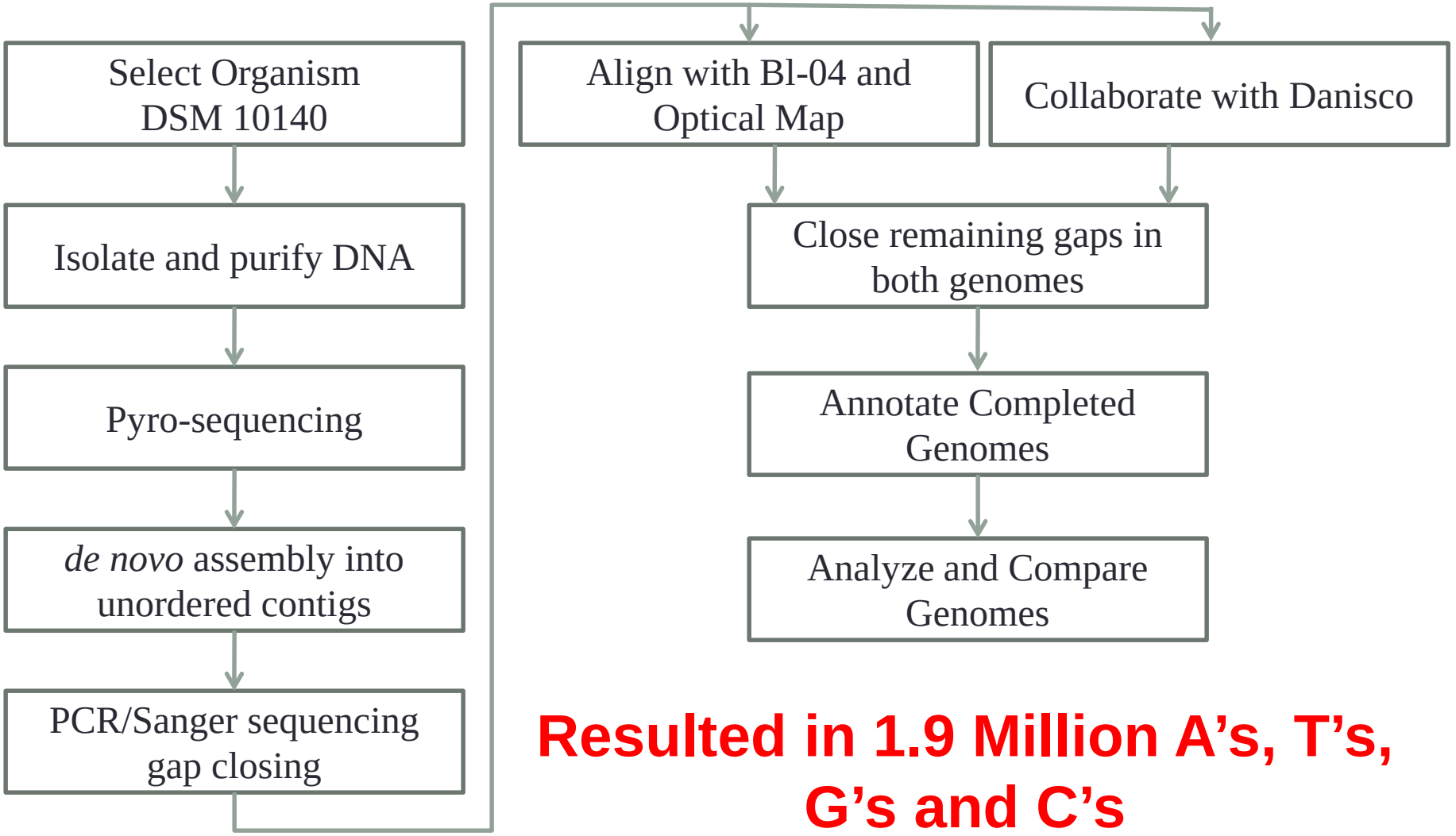
Sequencing the genomes of strains of *B. animalis* subsp. *lactis* will allow identification of targets for strain-level differentiation.

Objective 1

Sequence and compare the complete genomes of two strains of *B. animalis* subsp. *lactis*.

- DSM 10140 (the Type strain)
- Bl-04 (a commercial strain)

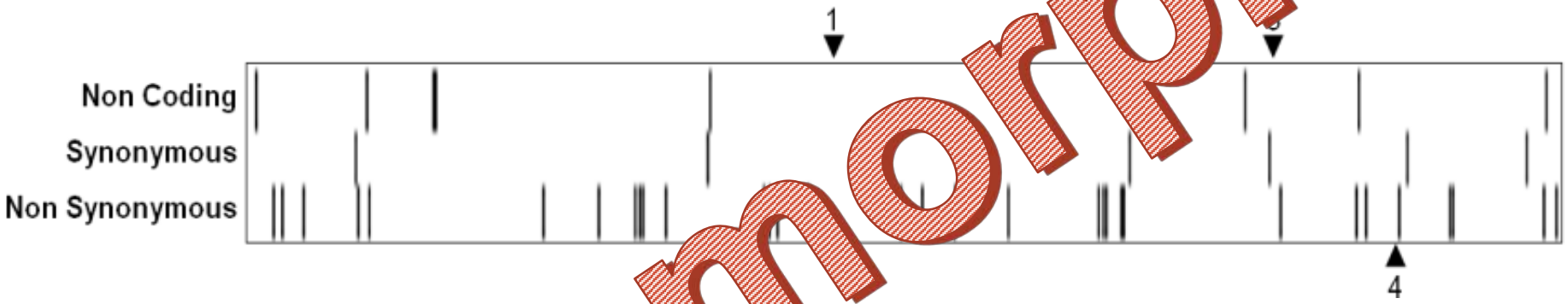
Project Flow



Resulted in 1.9 Million A's, T's, G's and C's

Comparison of the Genomes of *B. animalis* subsp. *lactis* DSM 10140 and Bl-04

- 47 SNPs and 4 INDELs were confirmed between t[illegible] tw[illegible] strains



Deletion #	Mis[illegible]g in…	Comment
1	DSMZ10140	tRNA-Ala-GGC
2	BL-04	Deletion in LCFA CoA-ligase
3	DSMZ10140	CRISPR Repeat
4	BL-04	Non-coding deletion

Monomorphic

Barrangou *et al*, 2009

Comparison of the Complete Genome Sequences of *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and Bl-04▽†

Rodolphe Barrangou,[1] Elizabeth P. Briczinski,[2,3] Lindsay L. Traeger,[1] Joseph R. Loquasto,[2] Melissa Richards,[1] Philippe Horvath,[4] Anne-Claire Coûté-Monvoisin,[4] Gregory Leyer,[1] Snjezana Rendulic,[5]‡ James L. Steele,[3] Jeffery R. Broadbent,[6] Taylor Oberg,[6] Edward G. Dudley,[2] Stephan Schuster,[5] Dennis A. Romero,[1] and Robert F. Roberts[2]*

Journal of Bacteriology, 2009. **191:**4144-4151.

- First complete and accurate sequence of *B. animalis* subsp. *lactis*

Objective 2

- Use the genome sequence information to develop a strain-level typing scheme *B. animalis* subsp. *lactis*.

Strain Collection (again)

Strain	Source
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Strain	Source
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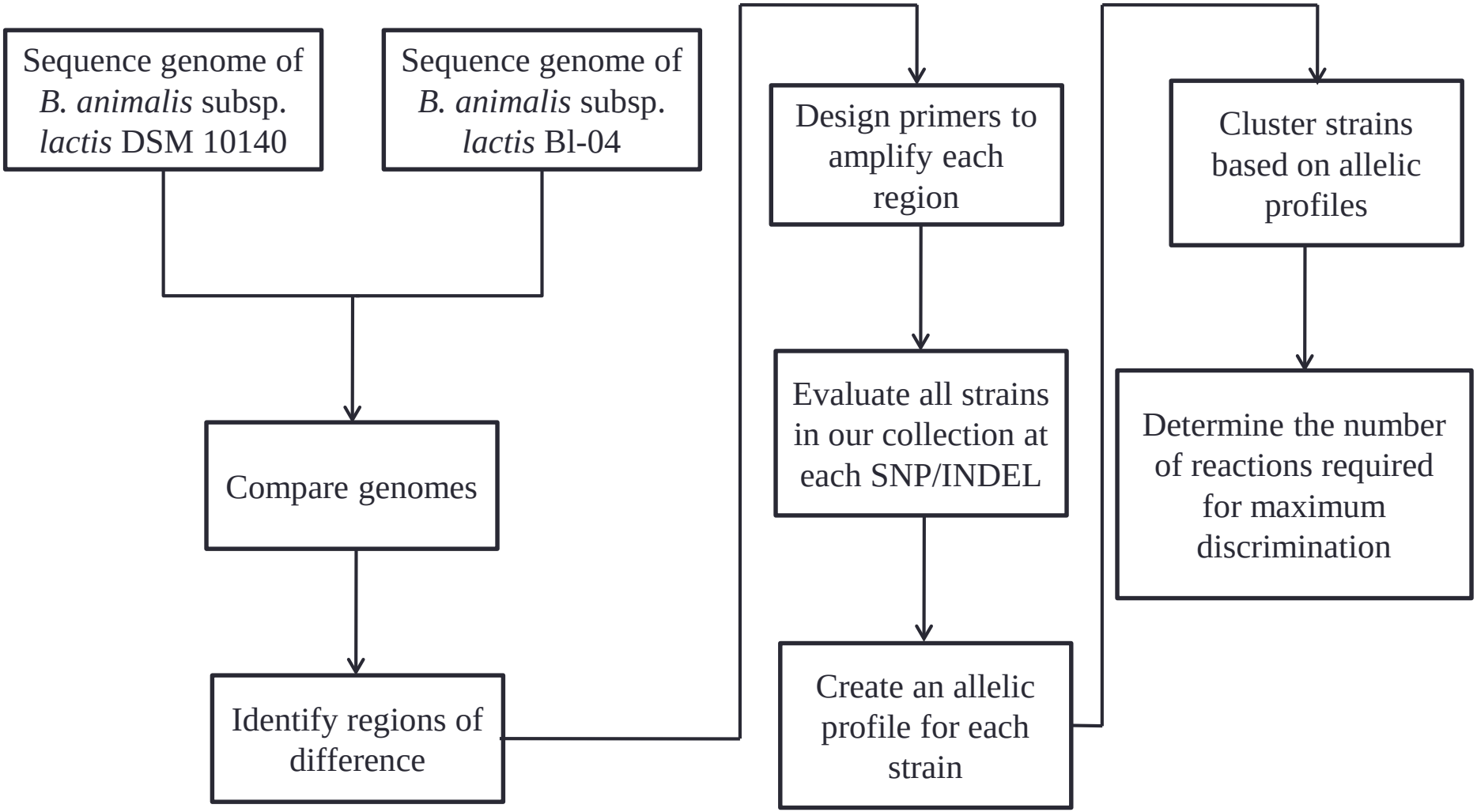
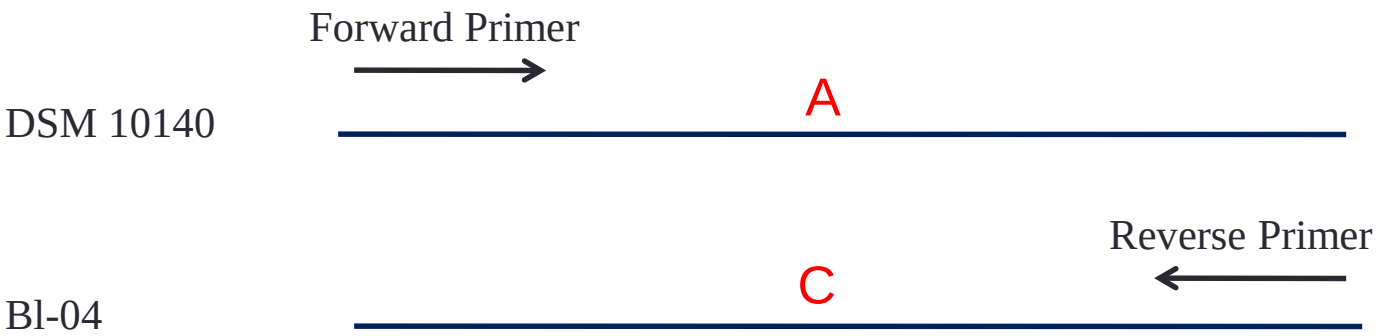
RB 5733	Commercial
RB 5851	Commercial
RB 5859	Commercial
RB 7239	Commercial
RB 7339	Commercial
RB 8613	Commercial
RB 9321	Commercial
RB 9632	Commercial
HN019	Commercial

Approach


Sequence genome of *B. animalis* subsp. *lactis* DSM 10140
Sequence genome of *B. animalis* subsp. *lactis* Bl-04
Compare genomes
Identify regions of difference
Design primers to amplify each region
Evaluate all strains in our collection at each SNP/INDEL
Create an allelic profile for each strain
Cluster strains based on allelic profiles
Determine the number of reactions required for maximum discrimination

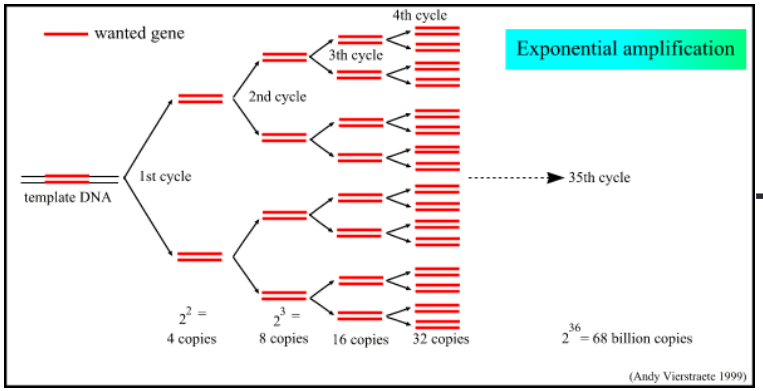
Design of PCR Primers

- Design one primer ~200bp upstream and ~200bp downstream of the putative SNP or INDEL

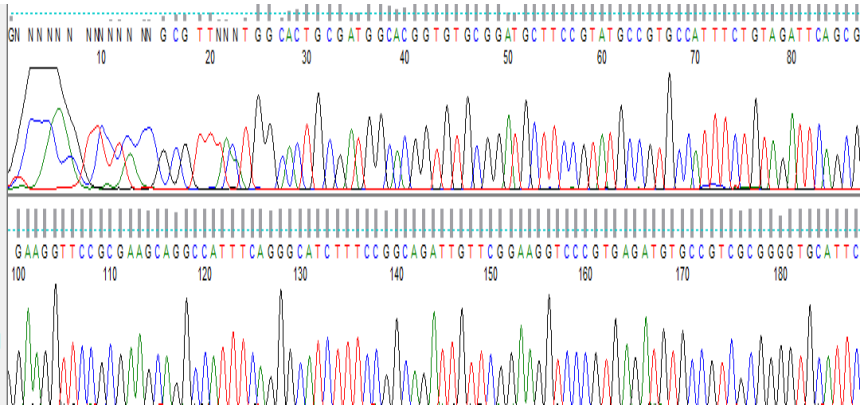


Amplification/Sequencing

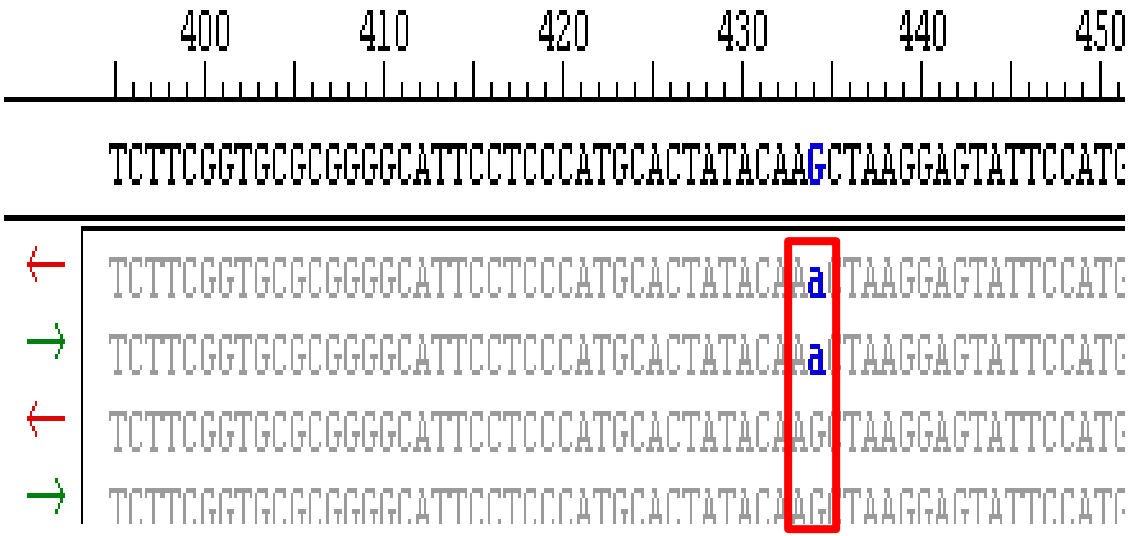
PCR



DNA Sequencing



Alignment



DSM 10140

Bl-04

Construct Allelic Profiles

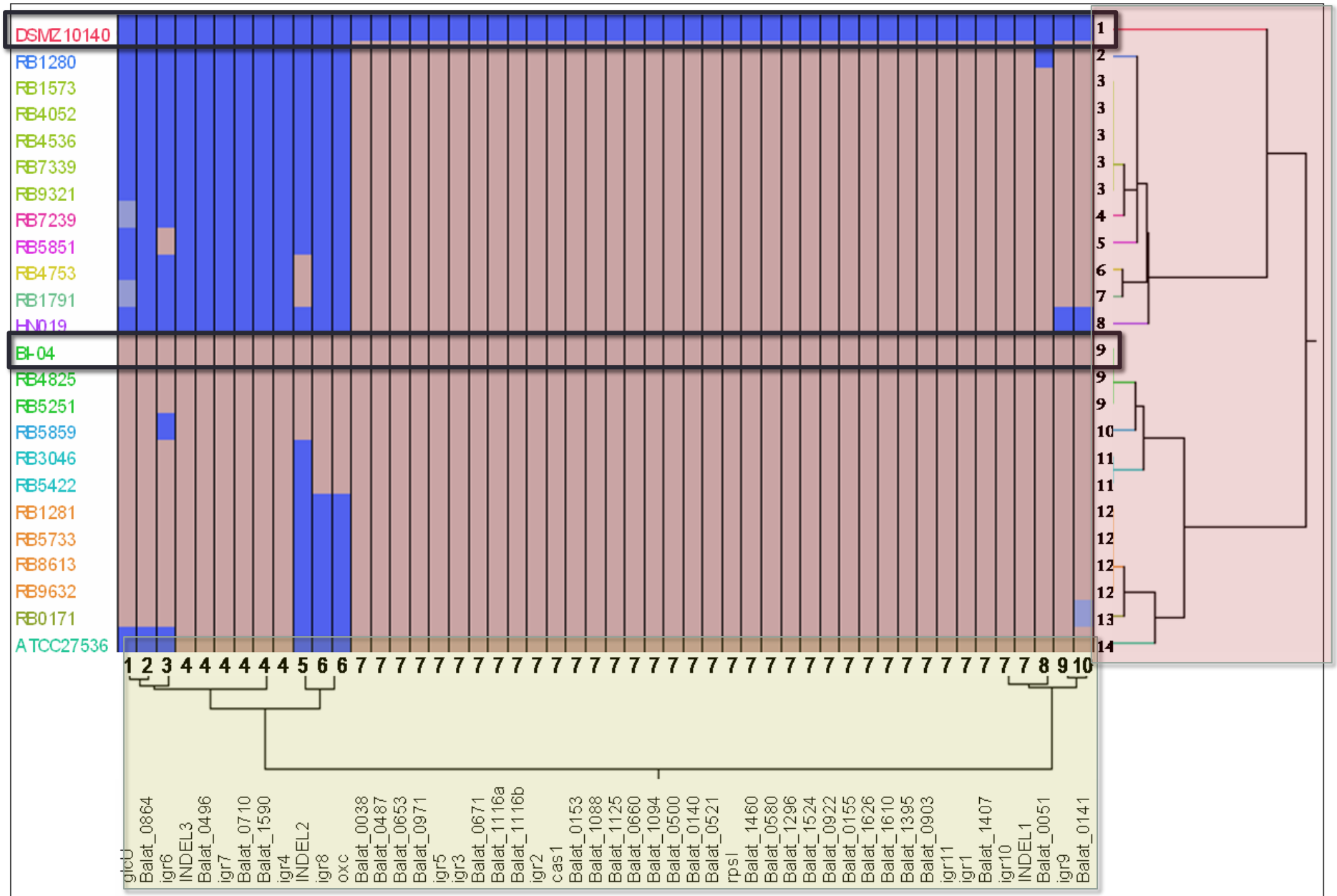
- Used DSM 10140 as reference strain
- Assigned all sequences that match DSMZ 10140 a value of "1"
- Assigned all sequences that match Bl-04 a value of "3"
- If the sequence didn't match either assign a value of "2"

DSMZ 10140	ATCGGGTCGTCAGCTAGCTCGATG	Allele 1
Bl-04	ATCGGGTCGTGAGCTAGCTCGATG	Allele 3
Strain A	ATCGGGTCGTCAGCTAGCTCGATG	Allele 1
Strain B	ATCGGGTCGTGAGCTAGCTCGATG	Allele 3
Strain C	ATCGGGTCGTTAGCTAGCTCGATG	Allele 2

Hierarchical Clustering

- JMP-Genomics (SAS-Cary, NC) was used to perform hierarchical clustering on allelic type data
- Hierarchical clustering allowed grouping of strains with the same allelic profile and differentiation of strains with different allelic profiles
- Hierarchical clustering also allowed determination of which loci and the minimum number of loci necessary to achieve maximum discriminatory power

Hierarchical Clustering of *B. animalis* subsp. *lactis*



Glucose Utilization

TABLE 1. Comparison of glucose utilization, glucose uptake, and SNPs in the glucose uptake gene among strains of *B. animalis* subsp. *lactis*

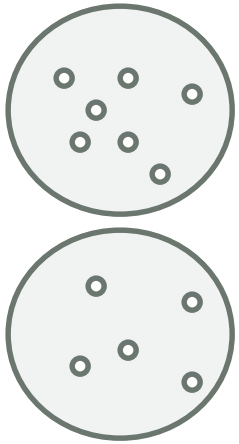
Strain[a]	Result of glucose fermentation with commercial kits[b]	Glucose uptake[c] (nmol/min/mg of cell protein)	SNP in *glcU* at position[d]:	
			1260073	1260380
DSMZ 10140	+	4.9	C	T
ATCC 27536	+	7.3	C	T
RB 1280	+	8.3	C	T
RB 1573	+	9.0	C	T
RB 1791	+	5.1	C	C
RB 4052	+	9.7	C	T
RB 4536	+	5.8	C	T
RB 4753	+	7.0	C	T
RB 5851	+	8.1	C	T
RB 7239	+	4.1	C	C
RB 7339	+	6.9	C	T
RB 9321	+	4.8	C	T
RB 0171	–	0.8	G	T
RB 1281	–	0.5	G	T
RB 3046	–	1.0	G	T
RB 4825	–	1.4	G	T
RB 5251	–	1.0	G	T
RB 5422	–	1.1	G	T
RB 5733	–	1.0	G	T
RB 5859	–	1.1	G	T
RB 8613	–	0.7	G	T
RB 9632	–	0.5	G	T

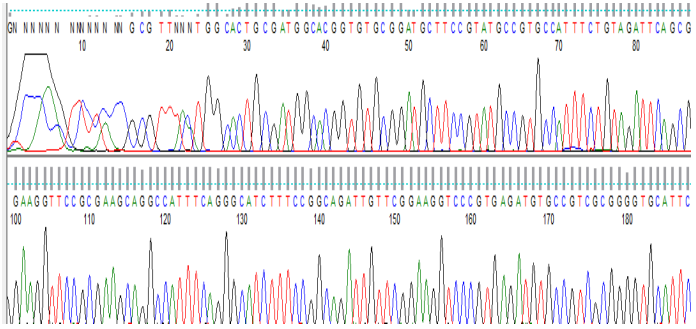
Practical Application of This Method

Strawberry yogurt drink supplemented with Bb-12

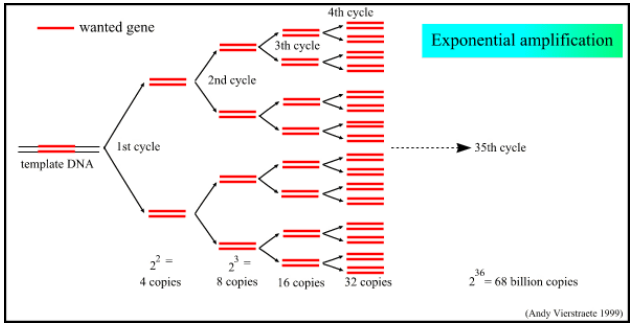
Plate on MRS + 0.05% cysteine + LiCl + Dicloxacillin

Pick colony counted as *B. animalis* subsp. *lactis* and isolate DNA



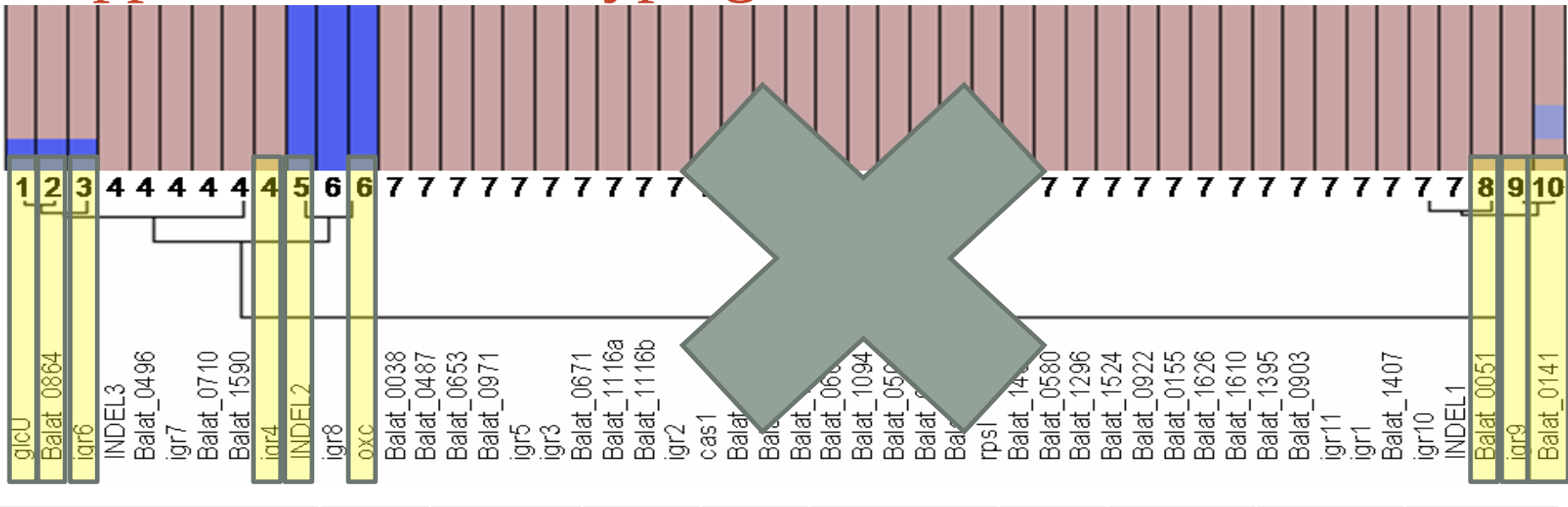


Sequence PCR product



PCR of differential loci

Application of SNP typing method



Loci Group	1	2	3	4	5	6	8	9	10
Loci	*glc*U	Balat_ 0864	Igr6	Igr4	INDEL 2	*oxc*	Balat_ 0051	Igr9	Balat_ 0141
Unknown	**C**	**T**	**G**	**T**	**1**	**C**	**A**	**G**	**-**
Allelic Profile (unknown)	1	1	1	1	1	1	3	3	3
Strain Cluster 3	C	T	G	T	1	C	A	G	-
Allelic Profile (3)	1	1	1	1	1	1	3	3	3

Whole Genome SNP/INDEL Typing

- Collection of 24 isolates was separated into 14 distinct genomic clusters.
- A minimum of nine loci need to be evaluated to obtain 14 genomic clusters.
- Fifteen of the 50 loci evaluated were highly informative loci (distinguished more than DSM 10140 and all other strains).

Potential Applications

- **Quality assurance** during manufacture of starter culture, dietary supplements and fermented dairy products

- **Clinical studies** to verify strains recovered from stool samples are the same group as those consumed (and not autochthonous strains)

- **Regulatory compliance** to assure the strain present is that claimed by the manufacturer

Advantages and Limitations

- **Advantages**
  - Sequence based data is highly portable
  - More discriminatory than previous phenotypic and DNA-based methods
  - A total of only 9 reactions are needed to achieve maximum discrimination
  - Appropriate method for differentiating monomorphic genomes with high degrees of similarity and synteny
- **Limitations**
  - DSM 10140 and Bl-04 define the outer limits of the method
  - Cannot detect genomic rearrangements
  - Cannot definitively identify a strain; only indicates it belongs to a certain group

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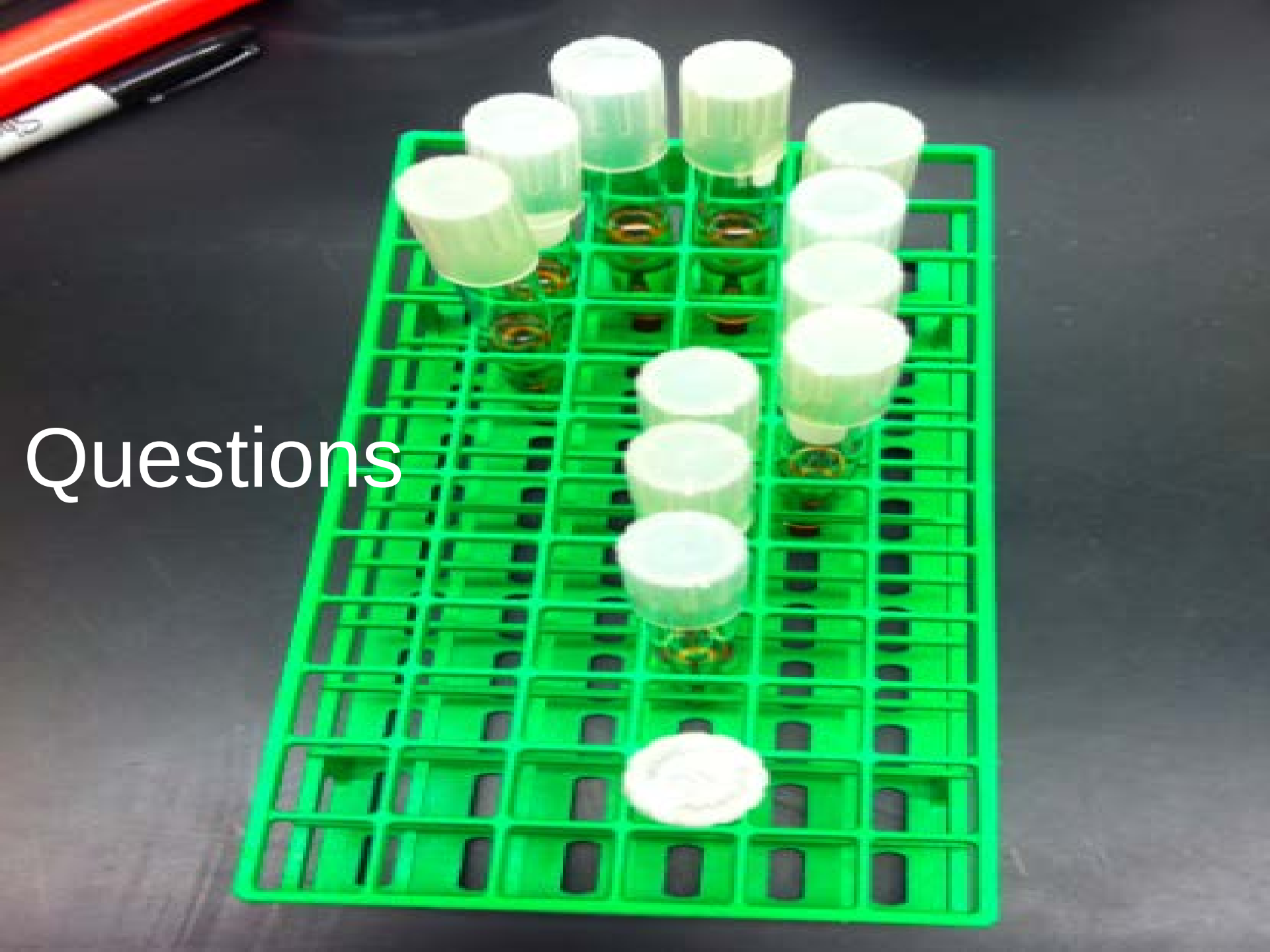
Questions