

ID of Environmentally Relevant Bacteria From Pharmaceutical Industries

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Agenda

- Environmentally relevant bacteria in Pharmaceutical Industries and ID
- Evaluation of the VITEK[®] 2 and the new cards for Gram-positive (GP), Gram-negative (GN) and *Bacillus* (BCL) identification, by using environmental isolates first identified by molecular method
- Conclusion

Introduction

- Identification (ID) of bacteria recovered from the environment of pharmaceutical industries (PI) is of concern.
 - It is a regulatory requirement.
 - It is part of process requirement.
 - It is standard practice in pharma. industries.
- Since isolates in these environments may be stressed, they are known to be sometimes difficult to identify using commercial kit databases (PDA TR #13).

Introduction

- The new VITEK 2 system and related identification cards (GP, GN and BCL) have been designed and released for improved automated identification of bacteria from the environment in the pharmaceutical industries (PI).
- We present an evaluation of this new system when using environmental isolates from PI, previously identified by molecular method (16S rRNA sequencing)

Introduction

- First, we will consider the « Bacterial Domain » to point out the actual diversity of the species we may theoretically face
- Second, we will present an overview of the main genera and species we did identify in 5 years, by using molecular method, as an expertise lab.
- Third, we will look at the performances of the VITEK 2 system with relevant environmental isolates from PI, previously identified by using nucleic acid based method of comparative sequencing (16S rRNA genes)
- Finally, the results will be discussed regarding the relevance of the species tested, and some comments will be done.

ID needs in Pharmaceutical industries

- In process control
 - Water
 - Raw material bioburden
 - **Environmental control and monitoring (EMP)**
 - Pre-sterilized bioburden
 - Sterility test

Environmental Monitoring

- Regulations and guidance documents
 - cGMP (USA, EU)
 - CFR (21CFR 211.42, 21 CFR 211.46, 21 CFR 211.22)
 - USP <1116> and EP
 - EMEA (annex I)
 - ISO 14644; ISO 13408; ISO14698-1
 - PDA Technical Report #13

ID requirements in Environmental Monitoring

- ID of environmental isolates is a requirement in many situations.
- Level of identification required (e.g. Group, Genus, Species, sub-Species) varies according to regulations and situations. Ability to set up a database to be able to reveal links in contaminations is a goal.
- It is stressed that environmental isolates are « particular » and that databases of automated systems are sometimes not adapted for them.
- It must be pointed out also that bacterial taxonomy is evolving with the improvement of knowledge, and identification is related to classification.
- Hence it is important to evaluate any automated system with environmental, well identified, isolates to validate how it performs.

« Bacterial Domain »



Bergey's Manual of Systematic Bacteriology
Volume 2: The Proteobacteria (Part B)
Volume package: Bergey's Manual of Systematic Bacteriology
Garrity, George (Ed.)
2nd ed., 2005, XXVIII, 1108 p. 222 illus., Hardcover
ISBN: 0-387-24144-2

Due: July 8, 2005

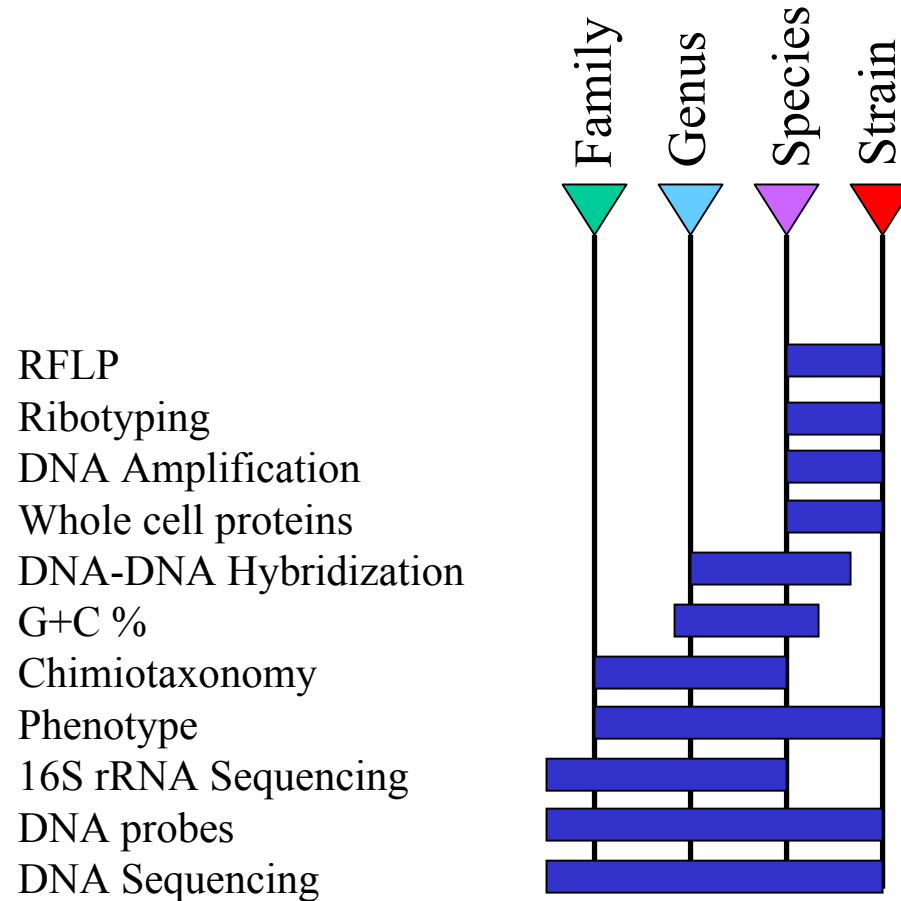
- New Classification : Bergey's 2005
 - Classification criteria
 - **Molecular** (Nucleic Acid based : Genomic and **phylogenetic**)
 - Specie definition
 - » $> 70\%$ DNA-DNA homology and $\Delta T_m < 5^\circ\text{C}$
 - » $\geq 97\text{-}98\%$ sequence homology in gene coding for 16S rRNA
 - » Multilocus Sequence Typing
 - Phenotypic
 - Morphology, biochemical, physiology (assimilations, fermentation, enzymatic reactions, susceptibilities, etc...)



(Stackebrandt et al., IJSEM 2002)

« Bacterial Domain »

- Taxonomic resolution of main ID methods



« Bacterial Domain »

- Species diversity :
 - Archaeobacteria :
 - > 259 species on the basis of 16S rRNA gene sequences
 - Eubacteria :
 - > 9021 species on the basis of 16S rRNA gene sequences
 - Gracillicutes (Gram-negative bacteria)
 - » Genera : > 538
 - » species : > 2000
 - Firmicutes (Gram-positive bacteria)
 - » Genera : > 235
 - » species : > 1346
 - Actinobacteria
 - » Genera : > 174
 - » species : > 1534
 - Others

« Bacterial Domain »

- Species diversity : exemples of the impact of molecular taxonomy
 - Eubacteria : new genera and species
 - Gracillicutes (Gram-negative bacteria)
 - *Pseudomonas* : > 8 genera; > 160 species
 - » *Acidovorax* (10), *Brevundimonas* (12), *Burkholderia* (35), *Comamonas* (10), *Delftia* (2), *Pseudomonas*(80), *Stenotrophomonas* (5), *Ralstonia* (5)
 - Firmicutes (Gram-positive bacteria)
 - *Bacillus* : > 20 genera; > 207 species
 - » *Alicyclobacillus* (5), *Amphibacillus* (3), *Aneurinibacillus* (6), *Anoxybacillus* (9), *Bacillus* (81), *Brevibacillus* (11), *Filobacillus* (2), *Geobacillus* (23), *Gracilibacillus* (2), *Halobacillus* (8), *Jeotgallibacillus* (2), *Lentibacillus* (5), *Marinibacillus* (2), *Oceanobacillus* (3), *Paenibacillus* (24), *Pontibacillus* (2), *Sporolactobacillus* (5), *Virgibacillus* (2), *Ureibacillus* (2), *Virgibacillus* (10).

« Bacterial Domain »

- Strain diversity :
 - Species definition : up to 30 % of DNA-DNA divergence between strains of the same species
 - *Genetic variability*
 - *Phenotypic variability*
 - *Representativity*
 - *Type strain of a species*
 - *Typical strains*
 - *Atypical strains*
 - *Environment effect :*
 - » *Selection pressure*
 - » *Stress*
 - » *Sanitizer effects*
 - » *Sterilization process effects (cheat, gaz, radio-sterilization, etc...)*

What do we really face ?

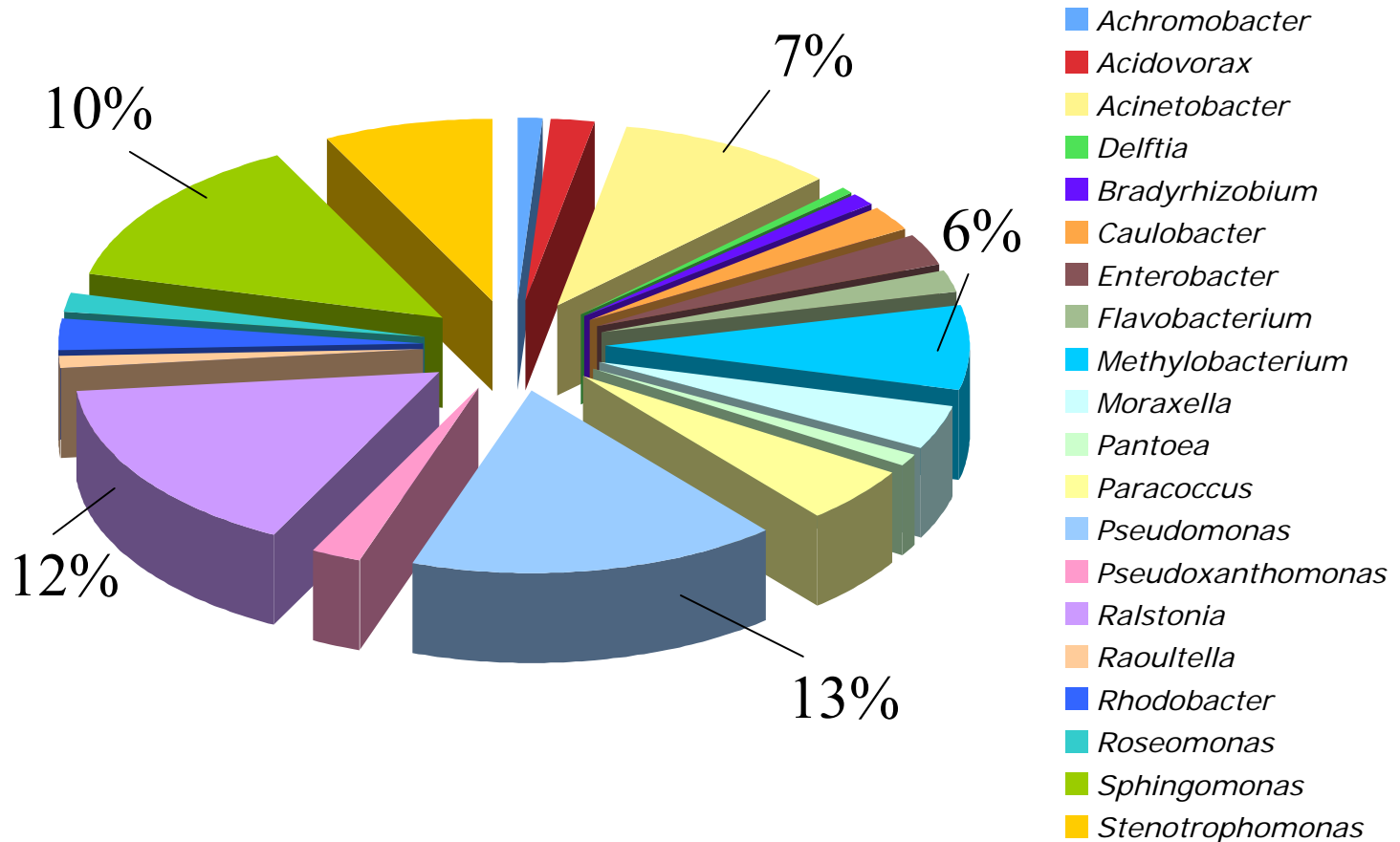
- Our experience after 5 years of molecular identification...
 - Quantity of bacteria identified : > 3000
 - Gracillicutes (gram negative bacteria) : **38 %**
 - Quantity of different species : < 200 ≠ species
 - Firmicutes (gram positive bacteria) : **62 %**
 - Quantity of different species : < 300 ≠ species
 - Sources :
 - Environmental monitoring (air-surfaces)
 - Water
 - Raw material, finished products,
 - process, personnel, quality controls
 - Sterility test positives, media-fill test positives, etc ...
 - Expert lab...
 - Often difficult to identify isolates...

What do we really face ?

- Global Analysis
 - Gram negative Bacteria
 - 70 genera
 - 160 species

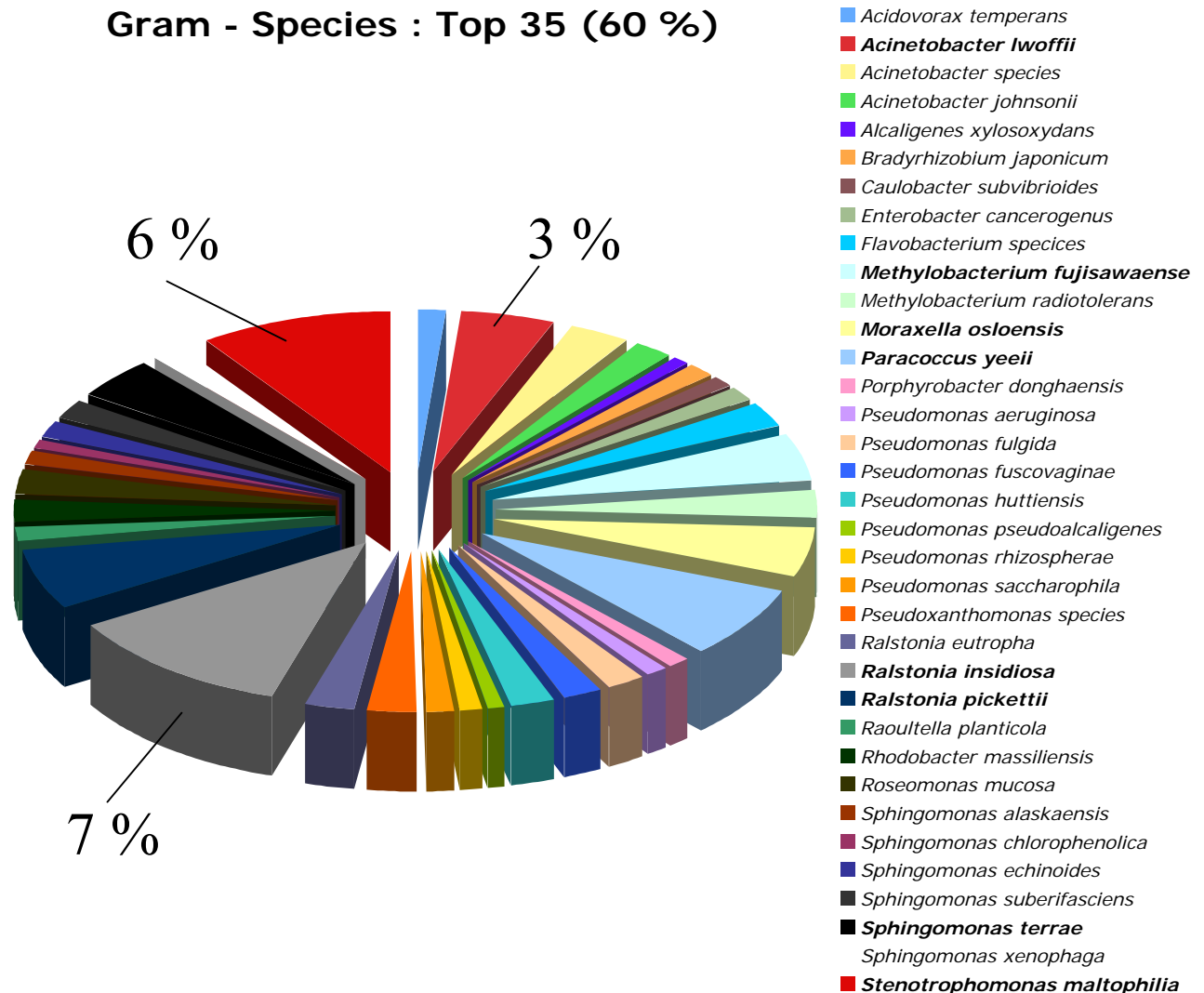
What do we really face ?

Gram - Genera : Top 20 (78 %)



What do we really face ?

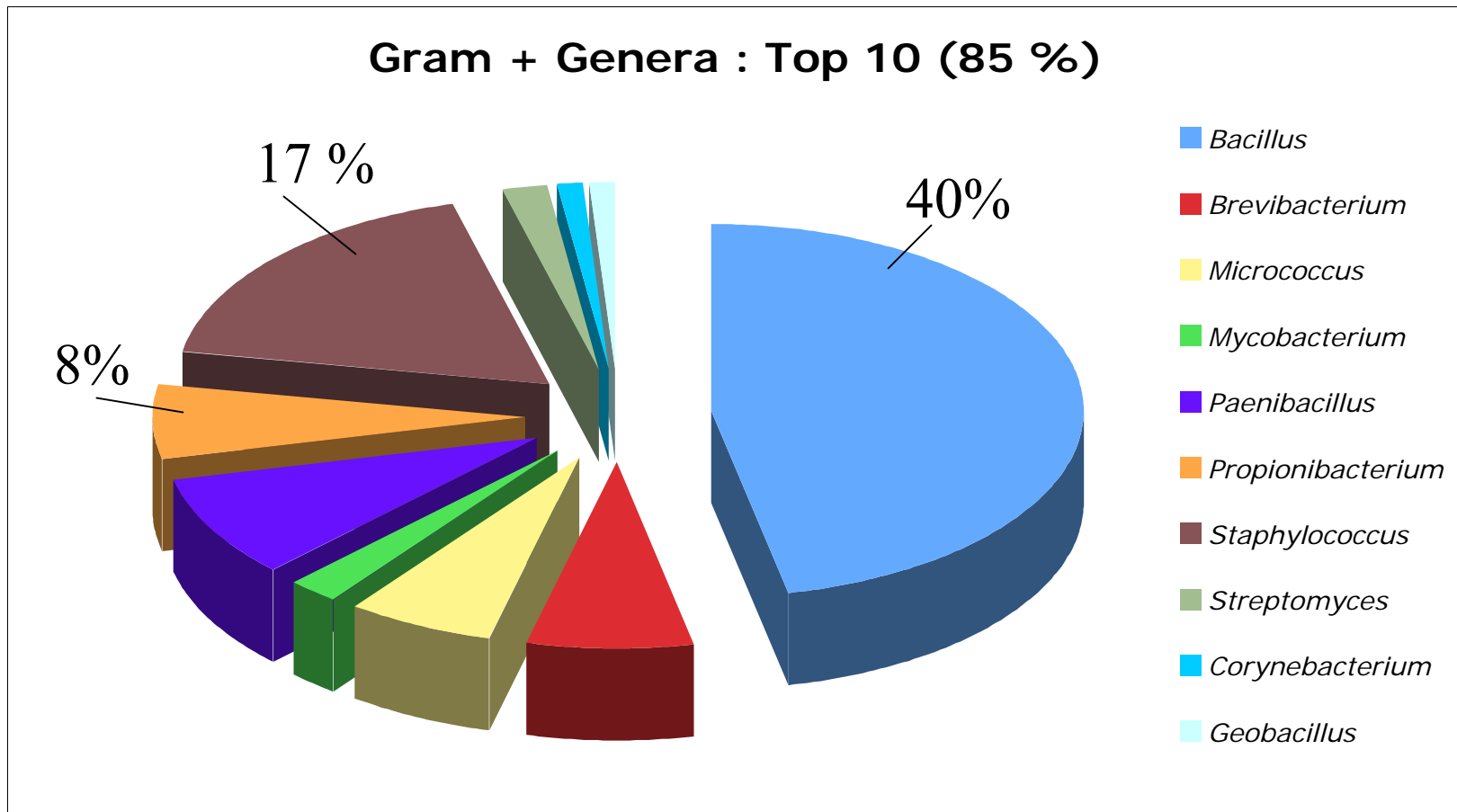
Gram - Species : Top 35 (60 %)



What do we really face ?

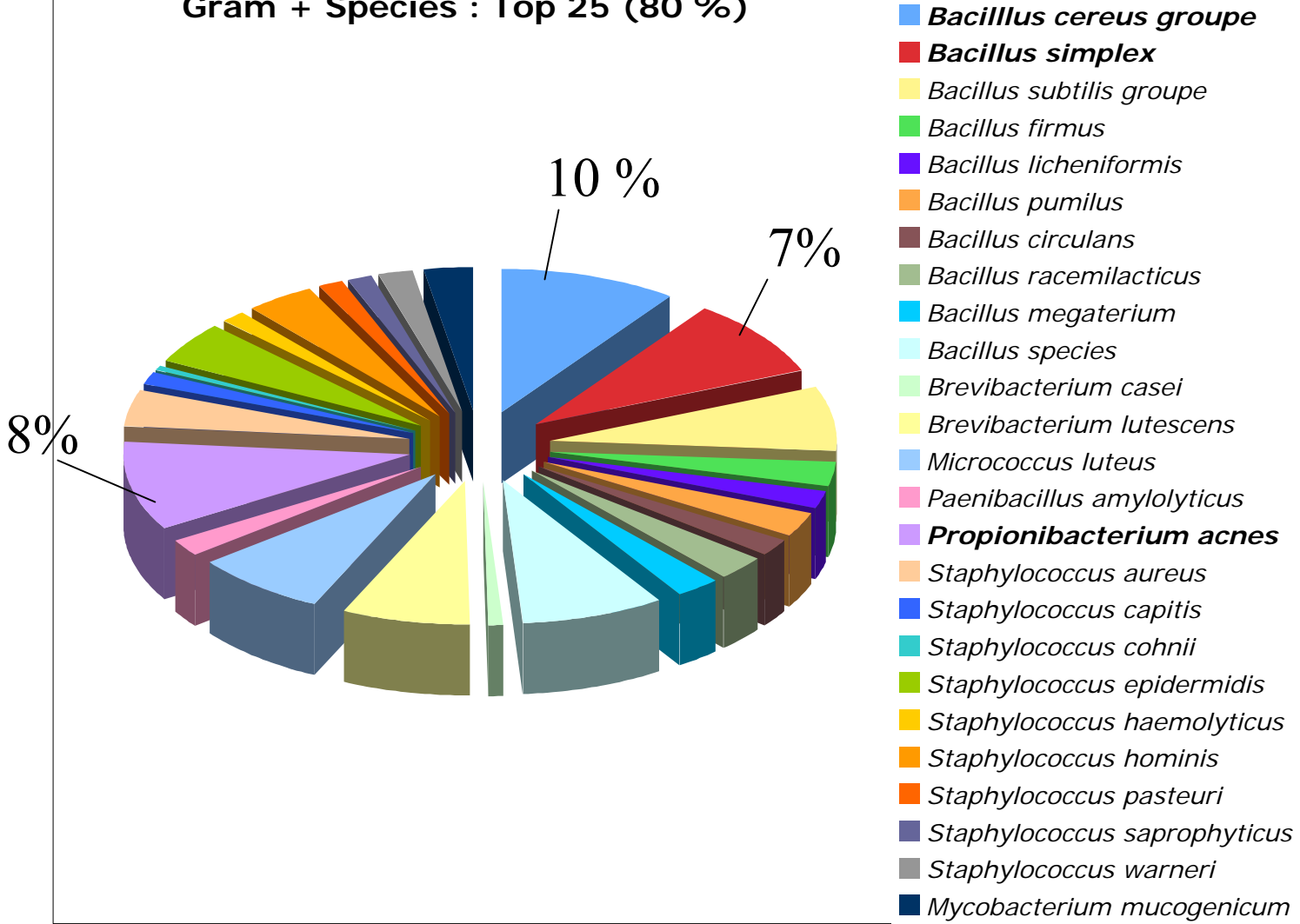
- Global Analysis
 - Gram Positive Bacteria
 - 80 genera
 - 260 species

What do we really face ?



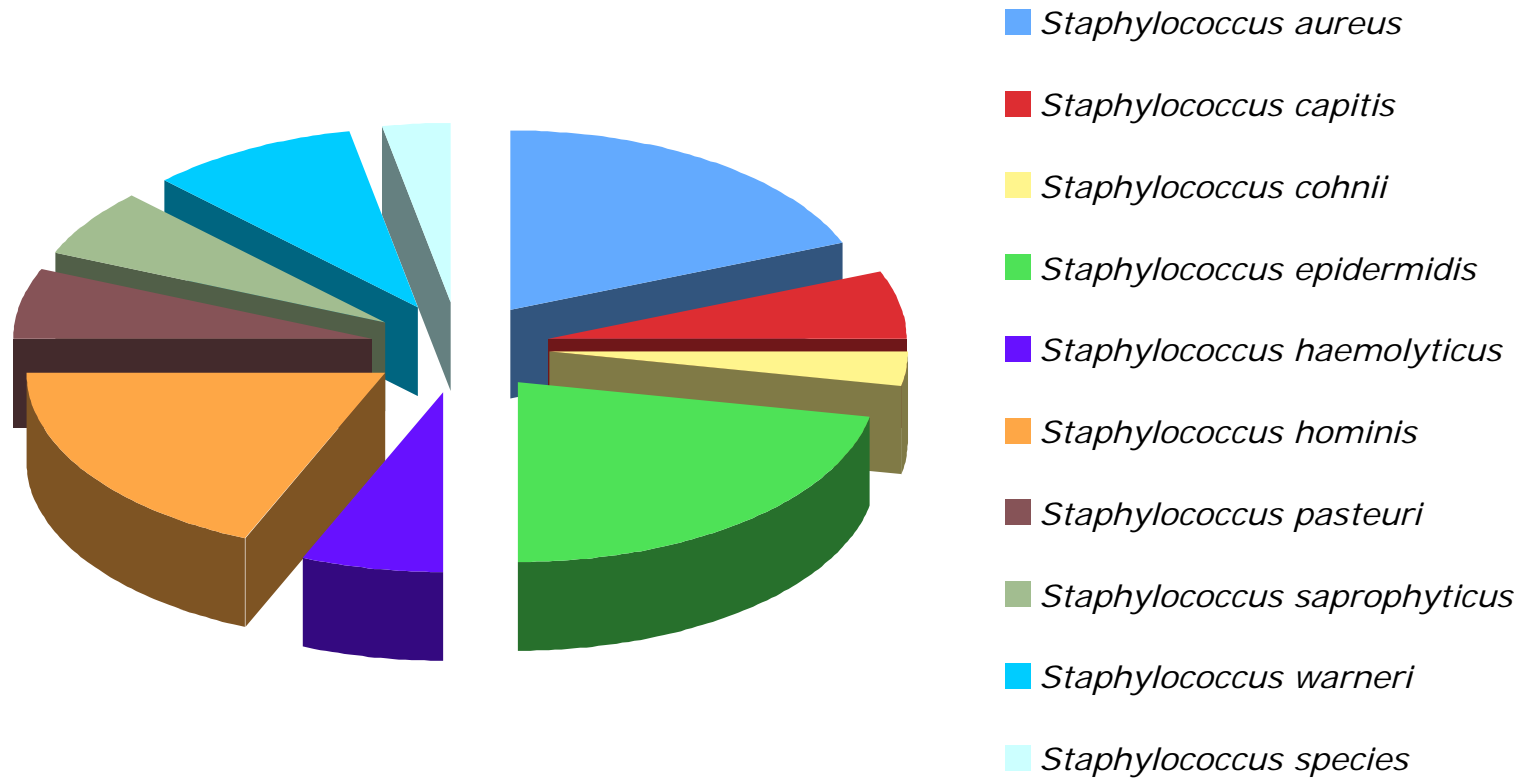
What do we really face ?

Gram + Species : Top 25 (80 %)



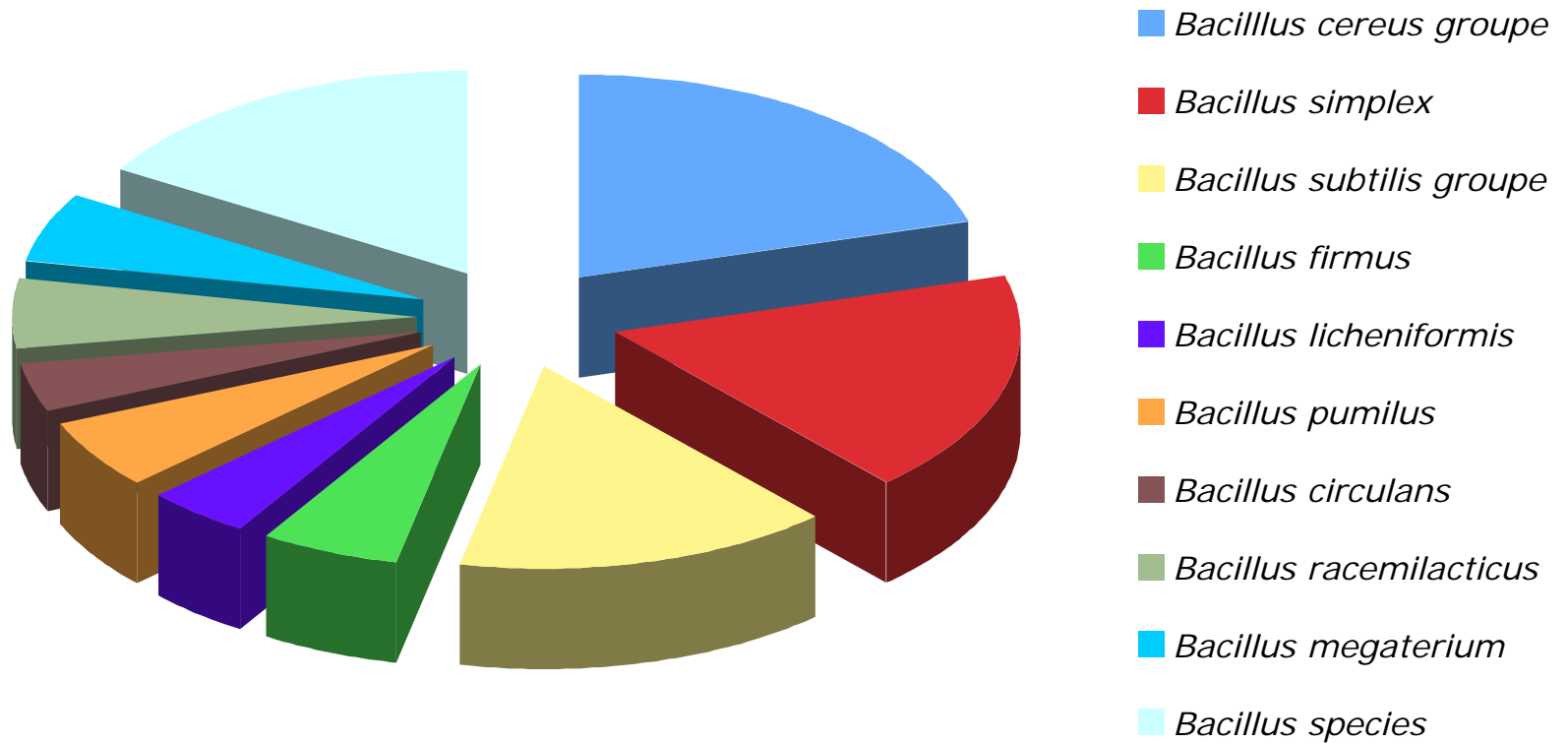
What do we really face ?

Gram + Species : Top 10



What do we really face ?

Gram + Species : Top 10



Environmentally relevant bacteria from P.I.

Theoretical variability is extremely high (> 9000 species)
New species are described almost every day...

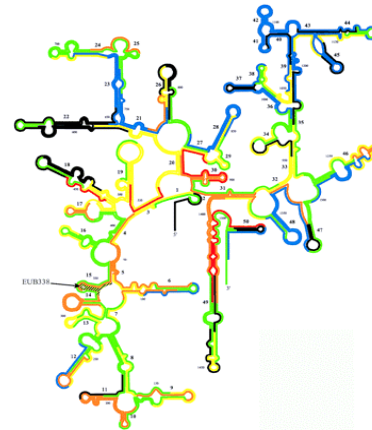
Practical variability is not that much high (< 500 species)
Even for expert lab...

In routine practice one can expect :

- 80 % of Gram-positive belong to roughly 25 species
- 60 % of Gram-negative belong to roughly 35 species

Evaluation of the VITEK[®] 2

- Objectives :
 - Evaluate the performances of the new VITEK[®]2 system with the GP, GN and BCL cards, for the identification of isolates from pharmaceutical industries.
 - Isolates have been identified by partial 16S rRNA gene sequencing method, considered the « Gold-Standard »



VITEK 2 Compact and the GP, GN and BCL cards

- The VITEK 2 Compact system



Evaluation of the VITEK 2 and *Gram-positive* Card (GP)

- The GP card

115 taxa claimed, representing
22 genera



Evaluation of the VITEK 2 and *Gram-negative* Card (GN)

- The GN card

135 taxa claimed, representing
55 genera

– *Enterobacteriaceae*

- 22 genera and 67 taxa

– *Non Enterobacteriaceae*

- 33 genera and 68 taxa



Evaluation of the VITEK 2 and *Bacillus* Card (BCL)

- The BCL card

42 taxa claimed, representing
6 genera

– *Aneurinibacillus* (1), *Bacillus** (18), *Brevibacillus* (8),
Geobacillus (3), *Paenibacillus* (10) et *Virgibacillus* (2)

* *B. anthracis* included and identified separately from
the other species in *B. cereus* group (*B. cereus*, *B.*
thuringiensis and *B. mesenteroides*)



Evaluation of the VITEK 2

- TEST ISOLATES :
- Environmentally relevant isolates from pharmaceutical industries
 - Originating from different pharmaceutical plants
 - Originating from different types of samples
 - Water analysis, environmental monitoring, sterility test positives, media-fill test positives, bioburden, pre-sterilized bioburden, personnel, etc...
 - In total 404 bacterial strains have been tested
 - 131 GP; 162 GN; 111 BCL
 - Identification of the isolates by using comparative sequencing of the 16S rRNA genes (partial)
 - Sequencing of the 5' end of the 16S rRNA genes (≥ 500 bp) and comparison with databases of sequences of reference strains
 - Microseq v 1.0 and public databases (NCBI)
 - Sequences homologies $\geq 97-98$ % with type strains of the species
 - « Gold-Standard »
 - Bacterial species claimed in knowledge databases of GN, GP and BCL cards

VITEK 2 system and GN card

- 162 isolates - 41 taxa
- Performances (Report 114-05NP rev. 1)
 - **92 % of correct identification**
 - **8 % of misidentification**
 - **0 % of unidentified**

Methode	Total testéd	CORRECT ID		One Choice		Low Discrim		Discordant		Unidentified	
		#	%	#	%	#	%	#	%	#	%
VITEK 2 (GN)	162	149	92	131	81	18	11	13	8	0	0

VITEK 2 system and GN card

- Relevance of the species claimed in database, regarding IDmyk's expert lab activities :
 - Roughly 60 % of the isolates from PI, we did identify in five years , belonged to species (taxa) claimed in the GN card database
 - *Improvement :*
 - *addition of 12 species most frequently encountered for many sites could increase to more than 75 % the percentage of isolates that can be identified by the VITEK 2 and GN card system.*

VITEK 2 system and GN card

- Comments :
 - *Escherichia coli* and *Escherichia coli* O157H7, *Shigella sp.*
 - *E. coli* and *Shigella dysenteriae* not distinguished by 16S rRNA sequences
 - *E. coli* and *Shigella group* distinguished by VITEK 2
 - *E. coli* and *E. coli* O157H7 distinguished by VITEK 2
 - *Brucella melitensis* and *Ochrobactrum anthropi*
 - Not distinguished by 16S rRNA sequences ($\geq 98\%$ homologies)
 - Distinguished by VITEK 2

VITEK 2 system and GP card

- 131 isolates - 33 taxa
- Performances (Report 114-05NP rev. 1)
 - **98.5 % of correct identification**
 - **1.5 % of misidentification**
 - **0 % of unidentified**

Methode	Total testéd	CORRECT ID		One Choice		Low Discrim		Discordant		Unidentified	
		#	%	#	%	#	%	#	%	#	%
VITEK 2 (GP)	131	129	98	120	92	9	6,9	2	1,5	0	0

VITEK 2 system and GP card

- Relevance of the species claimed in database, regarding IDmyk's expert lab activities :
 - > 80 % of the isolates from PI, we did identify in five years, belong to species (taxa) claimed in the GP card database
 - *Improvement : Microbacterium spp., Kocuria spp.*

VITEK 2 system and BCL card

- 111 isolates - 17 taxa
- Performances (Report 114-05NP rev. 1)
 - **88.3 % of correct identification**
 - **4.5 % of misidentification**
 - **7.2 % of unidentified**

Method	Total Tested	CORRECT ID		One Choice		Low Discrim.		Discordant		Unidentified	
		#	%	#	%	#	%	#	%	#	%
VITEK 2 (BCL)	111	98	88	89	80	9	8	5	4.5	8	7

VITEK 2 system and BCL card

• Results 1: Claimed Species

Genus	Species	Total Isolates	Correct ID		One Choice		Low. Disc.		Mis. ID		No ID	
		n=	n=	%	n=	%	n=	%	n=	%	n=	%
Bacillus	<i>amyloliquefaciens</i>	14	14	100%	14	100%	0	0%	0	0%	0	0,0%
Bacillus	<i>cereus group</i>	18	18	100%	15	83%	3	17%	0	0%	0	0,0%
Bacillus	<i>circulans</i>	5	2	40%	2	40%	0	0%	0	0%	3	60,0%
Bacillus	<i>firmus</i>	10	10	100%	9	90%	1	10%	0	0%	0	0,0%
<i>Bacillus</i>	<i>fusiformis</i>	1	1	100%	0	0%	1	100%	0	0%	0	0,0%
Bacillus	<i>licheniformis</i>	16	16	100%	16	100%	0	0%	0	0%	0	0,0%
Bacillus	<i>megaterium</i>	2	2	100%	2	100%	0	0%	0	0%	0	0,0%
Bacillus	<i>pumilus</i>	13	13	100%	13	100%	0	0%	0	0%	0	0,0%
<i>Bacillus</i>	<i>sporothermodurans</i>	2	0	0%	0	0%	0	0%	0	0%	2	100,0%
Bacillus	<i>subtilis group</i>	15	14	93%	13	87%	1	7%	0	0%	1	6,7%
<i>Brevibacillus</i>	<i>choshinensis</i>	1	1	100%	1	100%	0	0%	0	0%	0	0,0%
<i>Brevibacillus</i>	<i>parabrevis</i>	1	0	0%	0	0%	0	0%	1	100%	0	0,0%
<i>Geobacillus</i>	<i>stearothermophilus</i>	4	1	25%	1	25%	0	0%	1	25%	2	50,0%
<i>Paenibacillus</i>	<i>amylolyticus/pabuli</i>	2	1	50%	0	0%	1	50%	1	50%	0	0,0%
Paenibacillus	<i>glucanolyticus</i>	6	4	67%	2	33%	2	33%	2	33%	0	0,0%
<i>Paenibacillus</i>	<i>polymyxa</i>	1	1	100%	1	100%	0	0%	0	0%	0	0,0%
Total		111	98	88,3%	89	80,2%	9	8,1%	5	4,5%	8	7,2%

In **bold face**, species of the TOP 10 of *Bacillales* species found in routine, from PI, (> 80 % of total isolates identifiable)

VITEK 2 system and BCL card

- Relevance of the species claimed in database, regarding IDmyk's expert lab activities :
 - Roughly more than 15 % of *Bacillales* are not identifiable at the species level, even with molecular method of 16S rRNA gene comparative sequencing
 - No reference sequences : species still not described and/or accepted
 - Of the identifiable strains > 80 % of the isolates from PI, we did identify in five years, belong to species (taxa) claimed in the GP card database
 - *Improvement : adding Bacillus simplex (mix taxon with B. badius or single taxon) to BCL database add 7 % to the percentage of environmental isolates from PI covered by BCL card database.*

VITEK 2 system and BCL card

- Comments :

- *Bacillus cereus* group : *B. anthracis*, *B. cereus*, *B. thuringiensis* and *B. mesenteroides*
 - No distinction using 16S rRNA gene sequences ($\geq 98\%$ homologies)
 - Phenotype testing (haemolysis), etc...
 - Good distinction using VITEK 2 and BCL card system, especially for *B. anthracis*
- *Bacillus vallismortis* / *B. subtilis* / *B. atropheus*
 - Species are genotypically similar
 - (*B. vallismortis* sp. nov., a close relative of *B. subtilis*, isolated from soil in death valley california. Roberts et al., IJSB 1996)
- *Geobacillus stearothermophilus*
 - Take special care for sample preparation and incubation conditions with VITEK 2

Evaluation of the VITEK 2

- Claimed species global analysis
 - Gram-negative bacteria
 - **92 % of correct identification**
 - ID in 3 to 9 hours
 - Gram-positive bacteria (non-sporulating)
 - **98 % of correct identification**
 - ID in 3 to 7 hours
 - Bacillales
 - **88 % of correct identification**
 - After 14 hours (end point determination)

Excellent results with claimed species

VITEK 2

and relevant environmental isolates from PI

- Overview :
 - This is the first evaluation of an automated identification system using environmental strains from pharmaceutical industries, compared to the reference method of 16S rRNA gene sequencing
 - The evaluation provides validation of the performance of the VITEK 2 with GN, GP and BCL cards
 - The system is accurate, reliable and rapid, even with « tough » environmental isolates
 - Roughly, one can expect that more than 80 % of routine isolates can be exactly identified, rapidly, and in a cost effective maner
 - This evaluation demonstrates how the system is adapted for identification of bacteria found in environment of pharmaceutical industries
 - « Ease of Use » and rapidity of the system have been noted during evaluation

Conclusion

- The VITEK 2 system with the GN, GP and BCL cards identifies environmental isolates from pharmaceutical industries, with accuracies of 88% (BCL), 92% (GN) and 98% (GP) when they correspond to claimed taxa.
- Considering the difficulties routinely encountered to identify these organisms and considering the ease of use of the system, VITEK 2 is a significant advancement for the routine identification of environmental bacteria from pharmaceutical industries.

- Thank-you for your attention