IDENTIFYING RESISTANCE™
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no 2

This issue of IDENTIFYING RESISTANCE letter is an excellent illustration of how we can bridge the gap between phenotypic and genetic methods for the identification of Glycopeptide resistance in enterococci (GRE).

Who better to clearly explain this complex mechanism than the leader of the team who elucidated it?

We are pleased to see that all acquired resistance strains (van A, van B) are detected by VITEK 2. This is important as GRE infections and colonizations are spreading.

The bioMérieux group, with the recent acquisition of Organon Teknika completes its offer in microbiology (BacT/ALERT system) and expands its know-how in molecular biology (NASBA amplification platform, "BOOM" technology for extraction).

By combining phenotypic methods and molecular biology, we are clearly committed to being at the forefront of antimicrobial resistance.

Dr Christophe Mérieux,
Director of Research & Development

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STATE-OF-THE-ART

Glycopeptide resistance in enterococci

Patrice Courvalin

Patrice Courvalin, M.D., is a Professor at the Institut Pasteur (Paris, France), where he directs the French National Reference Center for Antibiotics and is Head of the Antibacterial Agents Unit. He is an expert in the genetics and biochemistry of antibiotic resistance. Among his many works he elucidated the resistance of enterococci to glycopeptides.

Glycopeptide antibiotics, vancomycin and teicoplanin, are used in the treatment of infections caused by Gram-positive bacteria in case of resistance or allergy to β-lactams. Due to their size, glycopeptides cannot penetrate the cytoplasmic membrane. They interact with the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) residue of peptidoglycan precursors and formation of these complexes blocks the transglycosylation and transpeptidation reactions and thus incorporation of the precursors into the bacterial cell wall [1].

Glycopeptide resistance in enterococci, first reported in 1986 [2, 3], has a broad geographical distribution and has become an increasing problem in clinical practice. Resistance to glycopeptides is phenotypically and genotypically heterogeneous. Five types of acquired resistance have been described in enterococci that can be distinguished on the basis of transferability, levels of resistance, and the spectrum of glycopeptides to which the

strains are resistant [table 1]. The VanA-type is characterized by high-level inducible resistance to both vancomycin and teicoplanin and is mediated by transposon Tn*1546* or closely related elements [4]. VanB-type strains have variable levels of inducible resistance to vancomycin only [5]. VanD-type enterococci are resistant to variable levels of vancomycin and teicoplanin [5, 6]. VanE and VanG, recently described in *Enterococcus faecalis* [8, 9], are characterized by a low-level of resistance to vancomycin and susceptibility to teicoplanin. VanC-type resistance is an intrinsic property of *Enterococcus gallinarum*, *E. casseliflavus* and *E. flavescens* [10, 11] and displays a low-level resistance to vancomycin. Glycopeptide resistance is due to the presence of an alternative pathway for peptidoglycan synthesis which allows (i) synthesis of low-affinity precursors in which the C-terminal D-Ala residue is replaced by a D-lactate (D-Lac) in VanA, VanB, and VanD phenotypes and by a D-serine (D-Ser) in the VanC, VanE, and VanG types and (ii) elimination of precursors normally produced by the host. Replacement of the D-Ala C-terminal residue by a D-Lac eliminates a hydrogen bond critical for antibiotic binding and considerably reduces the affinity for glycopeptides whereas substitution by a D-Ser does not alter the hydrogen bonds but is responsible for conformational changes which reduce slightly the affinity for vancomycin.

VanA-, VanB-, and VanD-type glycopeptide resistance

The VanA-type resistance was the first described [12]. Transposon Tn*1546* encodes proteins responsible for this acquired resistance [fig. 2]. The VanH dehydrogenase reduces pyruvate to D-Lac, the VanA ligase synthesizes the depsipeptide D-Ala-D-Lac which replaces the dipeptide D-Ala-D-Lac in the pathway of peptidoglycan synthesis, and the VanX D,D-dipeptidase hydrolyses the dipeptide D-Ala-D-Ala formed by the endogeneous chromosomal pathway. The distal portion of Tn*1546* encodes two proteins, VanY and VanZ, that are not necessary for glycopeptide resistance. The VanY D,D-carboxypeptidase hydrolyses the pentapeptide*Ala* synthesized from the dipeptide D-Ala-D-Ala which has escaped VanX hydrolysis. Thus, VanX and VanY act in series to prevent accumulation of the pentapeptide in the cytoplasm of glycopeptide-resistant enterococci favoring its replacement by pentadepsipeptide in cell wall assembly. VanZ confers low-level resistance to teicoplanin by an unknown mechanism.

The *van* alphabet

van A	*van* J	*van* $S_{A,B,C,D,E,G}$
B	K	$T_{C,E,G}$
$C_{1,2,3}$	L	U
D	M	V
E	N	$W_{B,G}$
F	O	$X_{A,B,D}$
G	P	$X_{YC,E,G}$
$H_{A,B,D}$	Q	$Y_{A,B,D,G}$
I	$R_{A,B,C,D,E,G}$	Z_A

Table 2

Glycopeptide resistance in enterococci

Resistance	Acquired					Intrinsic
Phenotype	VanA	VanB	VanD	VanG	VanE	VanC
MIC (mg/L)						
Vancomycin	64 - 1000	4 - 1000	64 - 128	8-16	16	2 - 32
Teicoplanin	16 - 512	0.5 - 1	4 - 64	0.5	0.5	0.5 - 1
Expression	Inducible		Constitutive	?	Inducible	Constitutive Inducible
Location	Plasmid Chromosome		Chromosome	?	Chromosome	Chromosome
Modified target	D-Ala-D-Lac			D-Ala-D-Ser		

Table 1

Glycopeptide resistance nomenclature

vanA: gene for the resistance ligase operon (gene cluster)
VanA: resistance ligase
VanA-type: harbours the *vanA* operon
VanA-phenotype: Vm^R Te^R
ddl: gene for the host ligase
Ddl: host ligase
D-Ala:D-Lac: ligase
D-Ala-D-Lac: depsipeptide

Table 3

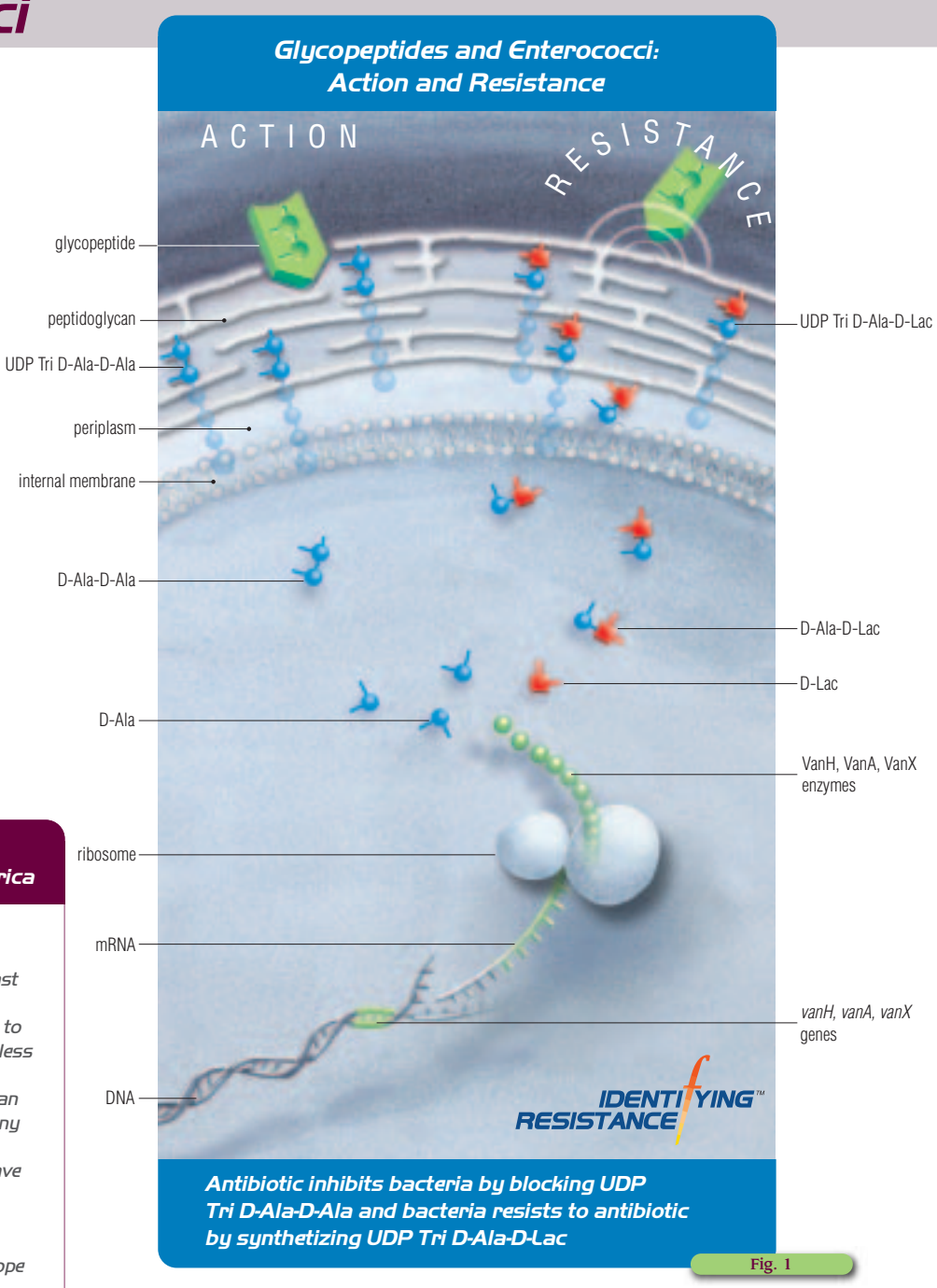


Fig. 1

An epidemiologic difference between Europe & North-America

There are major differences in the epidemiology of GRE between the United States and Europe. In contrast with Europe, GRE collected in the United States are multiple resistant to antibiotics and there appears to be less genetic variability among these isolates. The European GRE of human origin are usually susceptible to many other antibiotics and are highly polyclonal. These clinical isolates have the same susceptibility profiles as the GRE isolated from animals. The differences in epidemiology between the United States and Europe might be explained by the overconsumption of glycopeptides and other antibiotics in hospitals in the United States and the use of avoparcin (closely related in structure with vancomycin) as a growth promotor in food animals in Europe but not in the United States.

Relative occurrence of the main two Enterococcus species in clinical specimens

	North America	Europe
E. faecalis	80%	90 %
E. faecium	20 %	10 %
percentage of vanR strains		
E. faecalis	3 %	1 %
E. faecium	67 %	4 %

Data was derived from The Surveillance Network® (TSN®) Databases in North America and Europe for the year 2000, kindly provided by Focus Technologies Inc., Herndon, Virginia, USA.

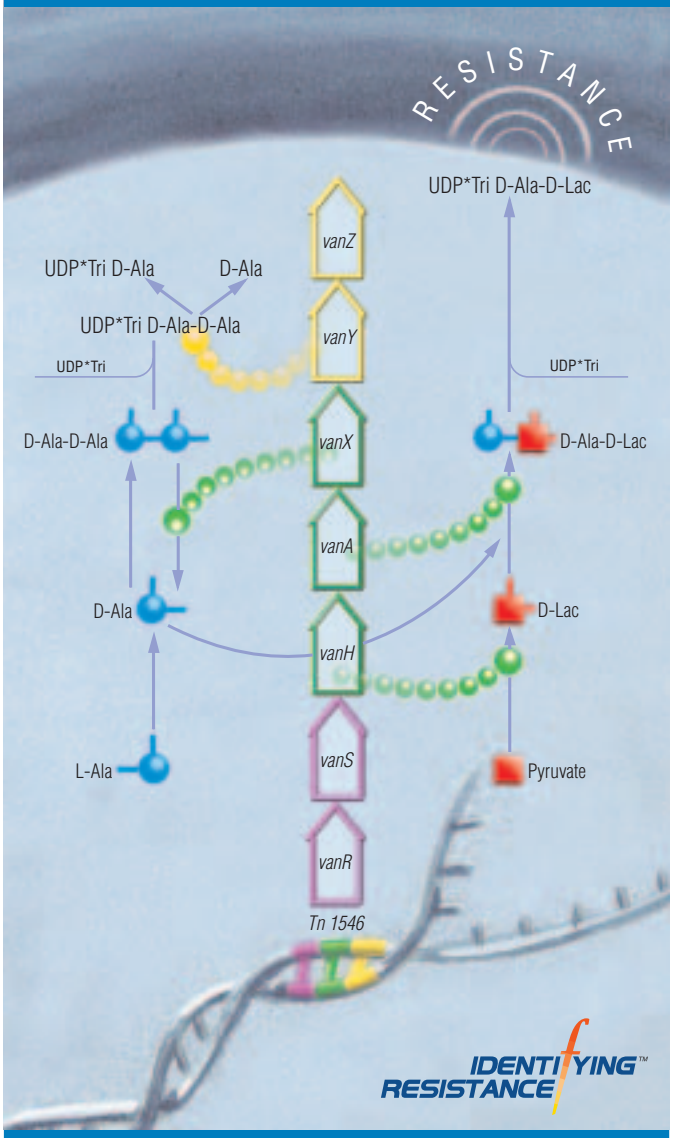
Regulation at the genetic level accounts for inducibility. Upstream from *vanH* are two genes encoding proteins structurally related to two-component regulatory systems. The two proteins control the level of expression of the resistance genes in response to the presence of glycopeptides in the culture medium.

The organisation of the *vanA*, *vanB*, and *vanD* operons is similar and it appears that the biochemical basis of glycopeptide resistance is identical in VanA and VanB.

As opposed to VanA-type enterococci, due to the fact that teicoplanin is not an inducer, the VanB strains remain susceptible to this antibiotic.

The VanD-type resistance exhibits certain peculiarities. In contrast to VanA and VanB, VanD resistance is constitutively expressed and is not transferable by conjugation to other enterococci **[6]**. The VanD isolates studied so far possess a host D-Ala:D-Ala ligase (Ddl) which is inactivated. Inactivation of the chromosomal Ddl results in the lack of precursors ending in D-Ala-D-Ala. However, the host metabolic pathway is replaced by the constitutively expressed resistance pathway allowing glycopeptide-independent growth of the two strains.

Glycopeptide resistance in Enterococci: genetics



Resistance is due to both the synthesis of UDP Tri D-Ala-D-Lac and the inhibition of the synthesis of UDP Tri D-Ala-D-Ala

Fig. 2

Glycopeptide-dependence

Clinical strains of enterococci that require the presence of vancomycin in the culture medium for growth have been isolated from patients treated for long periods with this antibiotic.
Mutants with a similar phenotype have also been obtained in vivo and in vitro.
Inactivation of the chromosomal ddl gene for the D-Ala:D-Ala ligase is responsible for vancomycin dependence.
The mutations lead to synthesis of a truncated protein or to the replacement of an essential amino acid. Due to lack of a functional Ddl, peptidoglycan precursors ending in D-Ala-D-Ala cannot be synthetised; therefore, presence of vancomycin is required for induction of the resistance pathway, resulting in synthesis of peptidoglycan precursors ending in D-Ala-D-Lac and growth of the bacteria.
It has been demonstrated that dependent strains can revert to a non-dependent, highly resistant phenotype.

VanC, VanE, and VanG glycopeptide resistance

VanC-type resistance is a characteristic of motile enterococci. Three *vanC* genes have been described : *vanC-1* in *E. gallinarum*, *vanC-2* in *E. casseliflavus*, and *vanC-3* in *E. flavescens* [10, 13]. The VanC-type is chromosomally encoded and generally constitutively expressed but may also be, in some strains, inducible. The organisation of the *vanC* operon is different from those of *vanA*, *vanB*, or *vanD*. Three genes, *vanT*, *vanC*, and *vanXY*$_C$, are required for VanC-type resistance [14, 15]. *vanT* encodes a membrane-bound serine racemase, VanT, which produces D-Ser [16]. The *vanC* gene product synthesizes D-Ala-D-serine (D-Ala-D-Ser) which is substituted for D-Ala-D-Ala in late peptidoglycan precursors [16]. In contrast to VanA-, VanB-, and VanD-type resistance, in which the VanX and VanY activities are encoded by two genes, VanXY$_C$ has both D,D-dipeptidase and D,D-carboxypeptidase activity and thus allows hydrolysis of the dipeptide D-Ala-D-Ala and removal of the ultimate D-Ala from pentapeptide[Ala] [15]. A two-component regulatory system, VanR$_C$-VanS$_C$, is also present. The *vanR-vanS* genes are located downstream from *vanT*, whereas in VanA, VanB, and VanD, they are located upstream from *vanH*.

The structural organisation of the *vanE* operon is similar to that of *vanC* whereas that of *vanG* appears more distantly related [9]. ■

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DID YOU KNOW?

NCCLS recommendations

NCCLS Guidelines provide MIC interpretive standards for Antibiotic Susceptibility Testing. Enterococci are treated in **Table 2D**, along with several comments.

Reference method: M7-A5

vancomycin	4	8	16	32
teicoplanin	4	8	16	32

S I R

Vancomycin is in Group B: agents that are "important clinically" with "primary testing".
Teicoplanin is Inv, "investigational", as it has not been approved by FDA.

Comment 8

- "When testing vancomycin, plates should be held a full 24 hours for accurate detection of resistance."
- "For isolates with MICs of 8-16 µg/ml, perform biochemical tests for identification, as listed under "Vancomycin Resistance" at the end of this table" ("test for motility and pigment production").

Comment 1

Do not report cephalosporins, aminoglycosides, clindamycin, trimethoprime-sulfamethoxazole. *They "may appear active in vitro, but are not effective clinically, and isolates should not be reported as susceptible."*

Comment 3

Because of limited alternatives, chloramphenicol, erythromycin, tetracycline (or doxycycline or minocycline), and rifampicin may be tested for vancomycin-resistant enterococci (VRE), and consultation with an infectious disease practitioner is recommended.

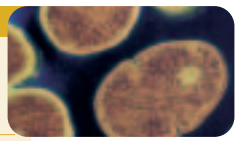
Screening test

Medium	BHI agar
Vancomycin	6 µg/mL
Inoculum	Growth or direct colony suspension to obtain 0.5 McFarland turbidity 1-10 µL of a 0.5 McFarland suspension spotted onto agar surface
Incubation	35°C; ambient air, 24 hours
Results	> 1 colony = presumptive resistance That means that every positive result should be confirmed by MIC determination

Adapted from NCCLS M100-S12, Jan. 2001.

Species Antibiotics Phenotypes

Species



Taxonomists have defined 17 species of enterococci. In clinical microbiology, the most common ones are *E. faecalis* and *E. faecium*. Other species are rare.

Enterococcus species	% *
Enterococcus faecalis	80
Enterococcus faecium	13
Enterococcus avium	3
Enterococcus gallinarum	2
Enterococcus casseliflavus	1
Enterococcus durans	1

* Relative proportion in a standard French clinical lab (Dr S.Tigaud, personal communication)

Glycopeptide antibiotics

This is a large family of agents with only a few used as antibiotics. The classification shows four groups, named after their main molecules: vancomycin, avoparcin, ristocetin, teicoplanin.

vancomycin Vancocin®, Lyphocin®	VAN	Eli Lilly, Shionogi, etc
teicoplanin Targocid®	TEC	Aventis

Others are under development such as daptomycin, ramoplanin, etc...

Phenotype

Phenotype	VAN	TEC
Wild S	S	S
VAN B	R	S
VAN A	R	R
VAN C	IR	S
~~Impossible~~	~~S~~	~~R~~

Enterococcus faecium	**VanA, VanB**
Enterococcus faecalis	**VanA, VanB**
Enterococcus gallinarum	VanC, **VanA**
Enterococcus casseliflavus	VanC, **VanA**
Enterococcus spp	rare or not described phenotypes

PRACTICAL ADVICE

How to diagnose VRE infections?

1 ■ Identify the strain

The goal is mainly to differentiate *E. faecium* and *E. faecalis* from *E. casseliflavus*, *E. gallinarum*, as the first two can acquire resistance while the latter two are naturally resistant.

2 ■ Detect resistance to glycopeptides

Test Vancomycin and Teicoplanin to obtain MIC values. Interpret according to NCCLS recommendations to report S, I, or R.

3 ■ Test alternative antibiotics

See Comment 3 of NCCLS recommendations, and the two drugs mentioned in Therapy section.

How to detect VRE carriers?

Detection of carriers is the only way to estimate the spread of resistance - as the infection/colonization ratio is relatively low.

The goal is to sort patients for isolation measures and to decide when to stop isolation.

Specimens are rectal swabs.

Perform a screening test: see NCCLS recommendations

What is the Reference Method?

The Gold Standard method consists of detecting resistance genes: *vanA*, *vanB*, *vanC*... using various molecular biology techniques.

What therapy is possible for VRE infections?

As Enterococci have many types of natural resistance (cephalosporins, clindamycin, imipenem...), treatment of severe infections with VRE is an issue.

"Old" antibiotics can be used (see comment no.3). The most promising alternatives are two recently marketed drugs: quinupristine/dalfopristine (Synercid®) for *E. faecium* only, and linezolid (Zyvox®,Zyvoxid®) for compassionate use.

(The Stanford Guide to Antimicrobial Therapy, 2000, p.21).

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Accuracy of the VITEK® 2 System To Detect Glycopeptide Resistance in Enterococci

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J. CLIN. MICROBIOL., VOL. 39, Jan. 2001, p 351-353, 2 tables, 14 references

Abstract

We evaluated the accuracy of the VITEK 2 fully automated system to detect and identify glycopeptide-resistant enterococci (GRE) compared to a reference agar dilution method. The sensitivity of vancomycin susceptibility testing with VITEK 2 for the detection of *vanA*, *vanB*, and *vanC1* strains was 100%. The sensitivity of vancomycin susceptibility testing of *vanC2* strains was 77%. The sensitivity of teicoplanin susceptibility testing of *vanA* strains was 90%. Of the 80 *vanC* enterococci, 78 (98%) were correctly identified by VITEK 2 as *Enterococcus gallinarum/Enterococcus casseliflavus*. Since the identification and susceptibility data are produced within 3 and 8 h, respectively, VITEK 2 appears a fast and reliable method for detection of GRE in microbiology laboratories.

Results

	VanA	VanB	VanC	GSE*
195 strains (no.)	50	15	80	50
Sensitivity on AST (%)	100	100	91	98
Sensitivity with ID (%)			97.5	

*GSE = glycopeptide susceptible enterococci

Methods

Reference methods were Rapid ID32® Strep for Identification and agar dilution for Antibiotic Susceptibility Testing.
All strains have been tested to be unique (PFGE).

Dr Nicole van den Braak
(with her recent thesis)
Erasmus Hospital Medical Center
Rotterdam (Pr.H.Verbrugh)

Quotes

"VITEK 2 is the first automated susceptibility method that tests both vancomycin and teicoplanin (1) for antimicrobial susceptibility, which is important for the description of the resistance phenotype."

"We have found that prolonging the opening hours of the microbiology laboratory and adapting the workflow allow the production of earlier reports is an achievable goal."

Key results

100 % detection for acquired resistant strains (*vanA*, *vanB*).
"Fast and reliable method for detection of GRE in microbiology laboratories." ■

(1) *Note : since teicoplanin is not FDA-approved, this molecule does not feature on the VITEK cards for the US market.*

Web sites

www.cdc.gov/ncidod/hip/Lab/FactSheet/vre.htm

Questions & Answers:
Importance of identification, differentiate colonization and infection, how to perform screening, how to type VRE strains?

www.phppo.cdc.gov/phtn/vrefacts.asp

VRE Facts with cost data: $18,000: hospitalization for a VRE Blood Stream infection

VRE control measures are cost-effective: one hospital can save $150,000/year

www.phppo.cdc.gov/dls/master/cs.asp

Case study of the month - september 2001

Another series of questions & answers

www.enterococcus.ouhsc.edu

from University of Oklahoma with substantial information and many links.

www.bacteriamuseum.org/species/enterococcus

with many links to other sites covering various aspects: fact sheets, lectures, scientific papers...

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