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CHARACTERIZATION AND IDENTIFICATION OF AN ENTEROCOCCAL ISOLATE AND ITS BACTERIOPHAGE

By

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ABSTRACT

CHARACTERIZATION AND IDENTIFICATION OF AN ENTEROCOCCAL ISOLATE AND ITS BACTERIOPHAGE

By

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The purpose of this study was to characterize and identify the isolate BAW#1, and to characterize and classify a bacteriophage associated with that isolate. Identification and metabolic profile analysis using BIOLOG™, the API-STREP kit, and conventional testing identified the isolate as *Enterococcus casseliflavus*. Analysis of 16s rRNA sequence data supported the identification of the isolate BAW#1 as a member of the *E. casseliflavus-E. gallinarum* group., The major fermentation product of the isolate under anaerobic conditions was lactic acid.

The bacteriophage was examined with a transmission electron microscope after two different preparation methods. The phage has a noncontractile tail and an elongated head. Tail fibers and a base plate were visible. The size of the phage capsid measured 90 nm by 36 nm, the tail was 136 nm by 9 nm. Nucleic acid was extracted from the phage and a restriction digest carried out. Therefore, the phage has double stranded DNA. The phage belongs to the Siphoviridae family.

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ADM	anaerobic dilution media
AHDL	Animal Health Diagnostic Laboratory
Bp	base pair
C	degrees centigrade
dH20	distilled water
GCS-RF	Glucose-cellulose-starch-rumen fluid media
GCSX-RF	Glucose-cellulose-starch-xylose-rumen fluid media
HPLC	high pressure liquid chromatography
Hz	Hertz
Kb	kilobase 1000base pairs
ml	Milliliter
nm	Nanometer
OD_{600}	Optical density measured at a wavelength of 600nm
rpm	Revolutions per minute
str	Strains
TMV	Tobacco mosaic virus
TSA	Trypticase soy agar
TSB	Trypticase soy broth
UA	Uranyl acetate
ul	Microliter

INTRODUCTION

A bacterium and bacteriophage were recovered in a laboratory conducting research on rumen microbiology. The bacterium was thought to be a wild strain of *Ruminococcus albus* (Tadese 1993). The *Rumincoccus* are well known gram-positive anaerobic rumen microorganisms. They are significant for their role in the breakdown of cellulose in the rumen. Workers at the University of Illinois had also been working on the phage: bacterium pair, and had sequenced the 16s rRNA of the isolate. Based on this, using the Ribosomal Database Project sequence analysis programs, they had identified the isolate as *Enterococcus saccharolyticus* (Lawes 1995). The isolate was entered into the 16s rRNA databases as str.BAW1. It has been assigned the ascension U30931.

The species of the genus *Ruminococcus* are gram positive, anaerobic, cellulolytic, cocci. The genus *Enterococcus* is composed of gram positive, facultative anaerobic cocci. The first objective of this study was to test the previous identification of the isolate as a wild isolate of *R. albus* or as *E. saccharolyticus*. A preliminary battery of tests was devised to answer this question. These tests were oxygen sensitivity and fermentation product analysis.

Fermentation product analysis is an important tool in the identification of anaerobic bacteria. This analysis provides data indicative of the major metabolic pathways in bacteria. The members of the genus *Ruminococcus* produce acetate, ethanol and H_2 in pure culture (Iannotti et al 1973). Fermentation product analysis is not used in the identification of streptococcus, enterococcus and closely related species; in fact these species are all homofermentative. Anaerobically, the major fermentation product of the isolate was lactic acid. Aerobically, acetic acid, lactic acid and ethanol were produced. These are the fermentation products expected from members of the gram-positive facultatively anaerobic cocci. The isolate was tested for oxygen tolerance. It was found to be facultatively anaerobic. Based on these observations it became clear that the isolate was not a member of the genus *Ruminococcus*.

The identification of the isolate as *E. saccharolyticus* was tested. To determine if the isolate was actually *E. saccharolyticus*, or some other facultative cocci, a battery of phenotypic tests was used. The gram positive facultatively anaerobic cocci contains many genera, including *Abiotrophia, Aerococcus, Carnobacterium, Dolosigranumum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus* and *Vagaococcus.*

The isolate was tested with the microbial identification tools BIOLOG ™ and API-STREP. The identification of the isolate as a member of the genus *Enterococcus* was affirmed, however, these tests named the isolate *E. gallinarum* and *E. faecium* respectively. The proprietary characterization schemes of API- STREP and BIOLOG ™ were examined and compared with literature on the metabolic profiles of the various species. The above tests were supplemented with conventional tests suggested in Bergey's manual. A review of the characterization schemes found in the literature was undertaken to help resolve the identity of the isolate.

The 16s rRNA based identification was compared to a more complete compliment of enterococcal sequences using the Ribosomal Database Project program Similarity Rank. Sequences of type strains of the *E. gallinarum, E. saccharolyticus, E. faecium, E. mundtii* and *E. casseliflavus* were compared to the sequence of isolate BAW#1.

Preliminary research on the phage concluded that the phage was lysogenic to the bacterial isolate. The phage was thought to be infective of gram positive and gram negative bacteria. The preliminary research showed a tailed icosahedral phage, similar in appearance to the well known phage lambda (Tadese 1993).

Lawes found the phage lysate to contain two morphotypes, an icosahedral lambda like phage, and a phage with an elongated head and a noncontractile tail. Lawes tested the phage lysate on a battery of rumen microorganisms, no evidence of infection was found(Lawes 1995).

Because of the discrepancy in descriptions of the phage morphology, bacteriophage recovered from lytic infection of the isolate BAW#1 was examined using the transmission electron microscope. The lytic nature of the phage was tested.

CHAPTER ONE

Characterization and Identification of the Isolate BAW#1

Literature review:

The streptococci are a well known, if ill defined genera of the gram-positive cocci. Within the streptococci, the enterococci have historically been considered a sub generic grouping, well known members of this group are *Enterococcus* (*Streptococcus) faecalis* and *Enterococcus (Streptococcus) faecium.* 16s rRNA sequences have been determined for most of the enterococcus as well as many other gram-positive cocci. Based on this 16s rRNA sequence data as well as earlier DNA/DNA homology studies, the *Enterococci* have been established as a genus and placed in the clostridial subdivision of the gram-positive bacteria. This subdivision includes the genera *Aerococcus, Carnobacterium, Globicatella, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus, Vagaococcus, Dolosigranumum* and *Abiotrophia.* Within the clostridial subdivision, the *Enterococci* form a distinct cluster with the *Vagococcus, Tetragenococcus* and *Carnobacterium* (Hardie and Whiley 1997).

What 15 years ago was simply known as the genus *Streptococcus* has undergone significant phylogenetic changes. In 1984 *Enterococcus* and *Lactococcus* were split off as new genera from *Streptococcus.* S. *faecium* and S. *faecalis* of the group D *Streptococci* were redefined based on DNA-rDNA homology and oligonucleotide cataloging of 16s rRNA in 1984 (Schleifer and Klipper-Balz 1984).

The genus *Enterococcus* is defined as gram positive, spherical to ovoid facultative anaerobes occurring in chains or pairs. The major fermentation product from utilizable carbohydrates is lactate (Holt et al. 1993). Key distinguishing characteristics include ability to grow in 6.5 percent NaCl, at pH 9.6, at 10° C and 45 ° C, and to hydrolyze esculin in the presence of 40% bile. However, several species including E. *saccharolyticus* do not conform to these characteristics (Williams et al 1991).

Additionally, defining characteristics of the genus *Enterococcus* are:

> "Cells occur singly, in pairs, or in short chains, and are frequently elongated in the direction of the chain. Endospores are not formed. May be motile. Optimum growth temperature, ca. 35 ° C. Most strains survive heating at 60 ° C for 30 minutes. Hydrolyze pyrrolidonly-ß-napthylamide. Chemoorganotrophs. Metabolism fermentative. The predominant end product of glucose fermentation is L-lactic acid. Oxygen or other hydrogen acceptors may alter the end products of carbohydrate

metabolism. Hydrogen peroxide may or may not accumulate in the presence of oxygen. Do not contain heme compounds. Benzidine negative and usually catalase negative, but some strains contains pseudo catalase. Some strains synthesize cytochromes or catalase or both when they are provided with hemin. The minimal nutritional requirements are generally complex. React with Group D antisera; Some strains also react with group Q antisera. Some strains posses respiratory quinones (menaquinones or dimethylmenaquinones). Long chain fatty acids are predominantly of the straight chain saturated or monounsaturated types; some strains produce cyclopropane ring acids. Peptidoglycan type: Lys-D-Asp or Lys-Ala2-3 . G+C content of the DNA ranges from 37 to 45%" (Schleifer and Klipper-Balz 1984).

The type species for the newly defined genus was defined as *E. faecalis*.

The enterococci are best known for inhabiting the gastrointestinal tract, where they are usually not pathogenic. They are extremely versatile, mesophilic bacteria, and are capable of living in water and on plants. They are formidable opportunistic pathogens, and are implicated in disease of man, livestock and birds. Some species are used in cheese and yogurt production.

An important feature of the enterococci is their genomic plasticity. They are known to posses conjugative transposons, pheromone responsive plasmids and broad host range plasmids (Murray 1998).

Many members of the *Streptococcus* have been reassigned to the *Enterococcus* over the last fifteen years. Newly discovered species have also been assigned to the genus *Enterococcus* (Table 1).

Characterization of the species *E. Saccharolyticus.*

Data from researchers at the University of Illinois determined the 16s rRNA sequence of the isolate used in this research. Analysis of this sequence using the software of the Ribosomal Database Project led to the identification of the isolate as *E. saccharolyticus*. For this reason, the defining characteristics of *E. saccharolyticus* are presented here.

The species *E. saccharolyticus* was named in 1984. Analysis of DNA base composition and DNA/DNA homology studies were undertaken to determine the relationship of strains of *S. bovis, S. equinus* and related species. This work found many strains with low homology which were grouped as the same species, and others with high homology were being differentiated into separate species. Of 85 strains examined, six new groupings were distinguished. Two new species were defined, *S. alactolyticus* and *S. saccharolyticus*. Significantly the type strains of *S. bovis* and *S. equinus* were regrouped into the single species *S. equinus,* which was redefined. Strains isolated from straw bedding, cow belly swab, teat swab and rectal swab formed a tight genetic group with 80-100% DNA/DNA homology. Physiological and biochemical criteria also distinguish this group from others in the study (Farrow et al. 1984). The species name *S. saccharolyticus* was proposed for the group which also is characterized as:

> "Colonies on blood agar or nutrient agar are circular, smooth and entire. Non-pigmented. Non hemolytic. Cells are gram-positive mostly in pairs or short chains. Non-motile. Facultatively anaerobic. Catalase-negative. Growth at 10C and 45C; optimum ca.37C. No growth at 50C: does not survive heating at 60C for 30 min. Weak growth in 6.5% NaCl. Chemoorganotroph: metabolism fermentative. Acid and clot produced in litmus milk. Does not react with Lancefield group D antiserum. G+C content of DNA ranges from 37.6 to 38.3 Non-hemolytic. Does not react with Lancefield Group D antisera. G+C of DNA from 37.6 to 38.3 mol% as determined by T_M. Type strain is NCDO 2594" (Farrow et al 1984).

The species *S. saccharolyticus* phenotypically more closely resembles the new genus *Enterococcus* in growth at 10C and 45C and in 6.5% NaCl. It does not, however react with Lancefield Group D, lacks pyrrolidonylarylamidase (PYR) and is not hemolytic. 16s rRNA sequencing confirmed the phylogenetic position of *S. saccharolyticus* NCDO 2594 with the genus *Enterococcus*. The species was renamed *Enterococcus saccharolyticus*. (Rodrigues and Collins 1990).

E. saccharolyticus is phenotypically distinguished from other enterococcus groups in its negative VP reaction. Its lack of arginine hydrolysis is shared by members of the avium species group, the cecorum species group and *E. sulfureus* (Devriese et al. 1993).

Table 1. Transfers of species from the genus *Streptococcus* to the new genus *Enterococcus* 1984 to 1998.

New name	**Old name**	**Reference for change**
E. avium	*S. avium*	Collins et al 1984b
E. casseliflavus	*S. casseliflavus* and *S. faecium* subsp. *Mobilis*	Collins et al 1984b
E. cecorum	*S. cecorum*	Williams et al 1991
E. columbae	Unclassified	Devriese et al 1990
E. dispar	Unclassified	Collins et al 1991
E. durans	*S. durans*	Collins et al 1984b
E. faecalis	*S. faecalis*	Schleifer & Klipper-Balz 1984
E. faecium	*S. faecium*	Schleifer & Klipper-Balz 1984
E. flavescens	a variant of *E. casseliflavus*, status currently in question	Pompei et al 1992 and Descheemaeker et al 1997
E. gallinarum	*S. gallinarum*	Collins et al 1984b
E. hirae	atypical *E. faecium*	Farrow and Collins 1985
E. malodoratus	*S. malodoratus*	Collins et al 1984b
E. mundtii	Unclassified	Collins et al 1984a
E. pseudoavium	Unclassified	Collins et al 1989
E. raffinosus	Unclassified	Collins et al 1989
E. saccharolyticus	*S. saccharolyticus*	Rodrigues and Collins 1990
E. seriolicida	unclassified	Kusuda et al 1991
E. solitarius	Unclassified	Collins et al 1989
E. sulfureus	Unclassified	Martinez-Murcia and Collins 1991

Characterization of the species *E. casseliflavus*

The *species S. avium, S. casseliflavus, S. durans, S. gallinarum* and *S. faecalis* subsp *malodoratus* were transferred to the genus *Enterococcus* in 1984 based on biochemical, chemical and genetic data. The species *E. casseliflavus* was described as:

> 'Coccoid cells usually in chains or short pairs. Motile. Surface colonies on blood agar or nutrient agar are circular, smooth and entire. Yellow pigment produced... Isolated from plants, silage and soil. In many phenotypic characters *E. casseliflavus* resembles *E. faecium*, but strains of *E. casseliflavus* may be distinguished by some metabolic tests, by the production of yellow pigment and by the possession of respiratory quinones." (Collins et al 1984b).

The type strain is ATCC 25788 (=NCDO 2372). Metabolic tests results of *E. casseliflavus* are: hippurate production: (+), D-tagatose: (-), and production of β-glucuronidase: (-). *E. gallinarum* tested (-), (+), and (+) respectively in these tests (Collins et al 1984b).

The species *E. flavescens* has been described as very similar to *E. casseliflavus* (Pompei et al 1992). *E. flavescens* does not produce acid from ribose and does not produce alpha hemolysis on sheep's blood, beyond this it tests identically *to E. casseliflavus.* The species status of *E. flavescens* is being questioned at this time by several research groups (Quednau et al 1998, Carvalho et al 1998, and Descheemaeker et al 1997).

Phenotypic strategies to differentiate the Enterococcus, with a special emphasis on *E. Saccharolyticus*

The enumeration and identification of enterococci from environmental samples has been problematic for many years. The classification of the genus *Enterococcus* and related species is in a state of flux, and many identification schemes found in 1990's literature use the old nomenclature or leave the newer species out all together. Complicated by the presence of closely related organisms in the same environment, no particular medium ensures recovery of all enterococci For example, as many as 80 selective media have been used for the enumeration of enterococci from foods (Garg and Mital 1991.

A conventional test scheme for the identification of enterococcal species was proposed in 1989 by Facklam and Collins. The physiological identification scheme was constructed for group D Streptococcus species isolated from humans. ATCC strains, stored strains with atypical reactions and strains previously identifies as *S. avium, S. durans* or unidentified were retrieved from their culture collection and examined. Enterococcus was distinguished from Lactococcus and Leuconostoc species. Group D antigen was found unreliable as a differentiator. Lactococci were identified as Group N antigen. The Leuconostocs were identified by vancomycin resistance, gas from glucose, and PYR negative reaction. They noted that all enterococcus species are PYR positive (*E. saccharolyticus* is not), and that most enterococci are vancomycin resistant (see Clinical Section below). They found gas production from glucose to be a very reliable test, since only the Leuconostocs and about half of the Lactobacillus species (which may be confused as gram positive cocci) produce gas from glucose.

Of the enterococci studied, only the outlier *E. faecalis* produced sufficient gas to generate a positive test in this study. Differentiation of the enterococci into groups was based on fermentation of mannitol, sorbitol, sorbose and arginine. The paper did not include *E. saccharolyticus*. (Facklam and Collins 1989). The species *E. faecalis, E. solitarius, E. gallinarum, E. faecium, E. casseliflavus* and *E. mundtii* are placed together in a metabolic subgroup. These species were then differentiated based on production of acid from arabinose (+), sorbitol (+/-), lactose (+), motility (+) and pigment (+). The test results shown in parenthesis are those for *E. casseliflavus* . (Facklam and Collins 1989).

A 1991 review <u>Enterococcus in Milk and Milk Products</u> fails to mention *E. saccharolyticus;* though *E. gallinarum* and *E. casseliflavus* are mentioned. (Garg and Mital 1991).

Knudston and Hartman (1993) proposed flow charts of key tests which may be used without nucleic acid analysis in food and clinical labs. This study examined 13 of the 18 known ATCC strains of enterococcus along with *S. bovis* and *S. equinus*. However, *E. saccharolyticus, E. columbae, E. dispar, E. seriolocida* and *E. sulfureus* were not included in this study. A follow up study of enterococci in pork processing using the flow chart identified one percent of isolates as *E. casseliflavus* (Knudston and Hartman 1993).

More significantly, the four volume tome The Procaryotes, Second Edition, published in 1992 contains no reference to *E. saccharolyticus* either in the *Enterococcus* or *Streptococcus* chapters. *E. casseliflavus* is differentiated from *E. gallinarum* and *E. faecium* based on its yellow pigment production. *E. casseliflavus* is listed as motile, in contrast to the other yellow pigmented species, *E. mundtii.* (Devriese et al. 1992a).

E. sulfureus is another yellow pigmented species (Martinez-Murcia and Collins 1991), it is not listed in the review of the enterococcal genus by Devriese, Collins and Wirth (Devriese et al. 1992a). However, *E. sulfureus* is easily differentiated from *E. casseliflavus* and *E. mundtii* based on the lack of acid production from L- arabinose, inulin, mannitol, rhamnose and d-xylose (Martinez-Murcia and Collins 1991).

Testing for the motility of an isolate to identify *E. casseliflavus* is problematic. The type species of *E.* casseliflavus is not motile (Morrison et al 1997). Numerous other non-motile strains of E. casseliflavus have been cited (Teixeria et al 1996, Tyrell et al 1997).

The 1993 Cowan and Steele's Manual for the Identification of Medical Bacteria includes *E. casseliflavus* , *E. faecium* and *E. gallinarum* in its identification schemes (Cowan and Steele 1993). *E. casseliflavus* is differentiated from *E. gallinarum* based on a yellow pigment and a negative hippurate test by *E. casseliflavus* .

Devriese, Pot and Collins synthesized much of the data on the new genus in 1993. The discussion of the gallinarum species group was extended to include *E. flavescens.* In summary the authors stated that with the inclusion of new species, the simplified phenotypic tests of earlier years were inadequate to isolate and identify the enterococci. Many of the new enterococcal species do not grow on enterococcal selective media. Isolation and identification methods adapted to the "classical" enterococci are still valuable for monitoring drinking water and identification of human pathogens (Devriese et al 1993). Identification schemes of gram positive cocci found in clinical and veterinary literature are frequently based on Lancefield and hemolysis test criteria, however, this criteria is insufficient for differentiating the newer species.

Studies in the last year have focused on differentiating *E. casseliflavus* and *E. gallinarum* from *E faecium* and *E. faecalis.* Atypical strains of these species are difficult to distinguish using standard techniques. Carvalho, Teixeira and Facklam (1998) suggest adding tests for susceptibility to efrotomycin and acidification of methyl-α-D-glucopyranoside. Use of these tests on 107 typical and atypical strains of enterococci differentiated the strains correctly as determined by SDS-PAGE and DNA:DNA re-association experiments.

To summarize the phenotypic identification and characterization of the *Enterococcus*, the following points should be considered:

- Gram Positive facultative anaerobic cocci or ovoid cells growing in chains-could be *Streptococci, , Lactobacillus, Aerococcus, Leuconostoc, Pediococcus, Gemella* or *Enterococcus* (Cowan and Steele's 1993*).*
- Isolates tolerant of azide (0.04%) and salt (6.5%) would most likely be an enterococci. However, *E. cecorum* and *E. pseudoavium* are not salt tolerant (Holt et al 1993).

The *E. faecium* species group members include *E. faecium, E. casseliflavus, E. mundtii, E. gallinarum* and *E. faecalis.* Members of this species group are differentiated from other enterococci by their ability to produce acid from mannitol and arginine, and inability to produce acid from sorbitol (Carvalho et al 1998). Pigmentation is unique to *E. casseliflavus, E. mundtii* and *E. sulfureus,* however *E. casseliflavus* is variable in pigment production.

The inability to hydrolyze hippurate separates *E. casseliflavus* from *E. gallinarum* and *E. sulfureus.* The ability to utilize raffinose and inulin also separates *E. casseliflavus* from *E. mundtii* and *E. sulfureus.* A summary of phenotypic characteristics which may be used to identify the Enterococci is presented in the Results section of this thesis (Table 4)

Considerable progress has been made in the development of DNA and 16s rRNA based identification methods. Small subunit rRNA is currently the most widely used molecule for determining phylogenetic relationships of microbial species.

16S rRNA is a good tool for assessing relationships among species. It has universality, it is present in all organisms (not phage and virus). The molecule has experienced little selective pressure and lateral gene transfer. 16s rRNA has invariable sites with highly conserved structure which allows the phylogenetic separation of distantly related organisms. 16s rRNA also has hypervariable sites. The variability allows differentiation down to the species and subspecies level.

The phylogenetic theory is based on comparison of homology of 16s rRNA of different species, on a nucleotide by nucleotide basis. The greater the number of differences between two sequences, the greater the evolutionary distance between the two species (VanDamme et al 1996a).

The new system provides a greater ability to predict success in genetic engineering. Aside from energy source utilization, which formed the backbone of the old classification system, a close phylogenetic relationship predicts that two species are highly similar in fundamental biochemistry, nature of gene expression systems, main regulatory properties, main biosynthetic and degradative pathways, and that individual genes have closer homology to close relatives. Greater knowledge of species provides a greater potential for genetic engineering.

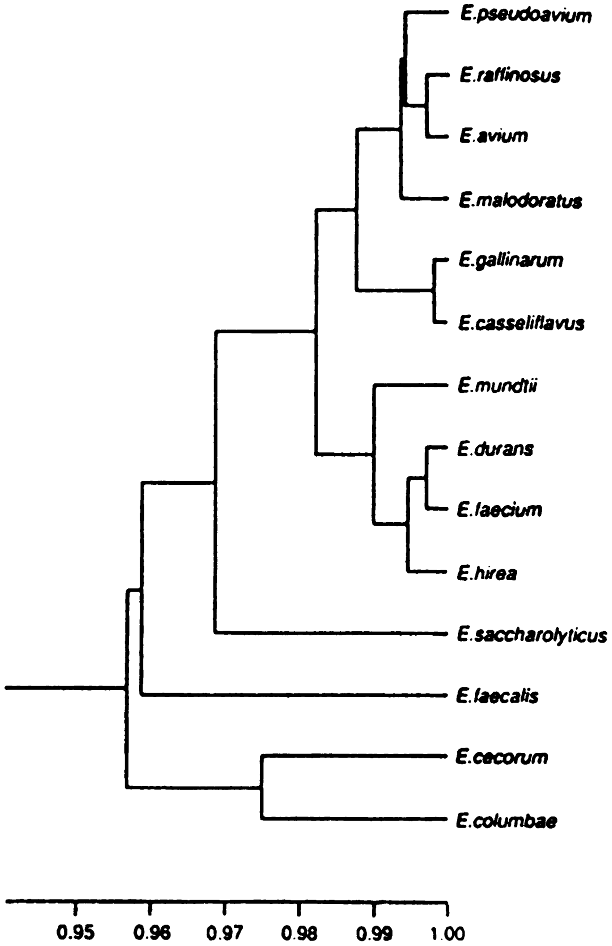
The entire 16s rRNA sequence has been determined for most enterococcus species (Table 2). Four species groups have been identified based on this 16s rRNA data. (See Figure 1). *E. casseliflavus* is grouped with *E. gallinarum,* and this group is adjacent to one containing *E. mundtii, E. hirae, E. durans* and *E. faecium*; *E. saccharolyticus* is quite distant (Williams et al 1991). These groupings are quite similar to the species groups derived from metabolic data.

rRNA targeted oligonucleotide probes were synthesized and flourescently labeled for six species of gram positive facultative anaerobic cocci. Lysozyme treatment of the enterococci, lactococci, and streptococci allowed strong and consistent whole cell hybridizations. Identification of these species in a milk sample was completed within about 8 hours using this method. *E. saccharolyticus* was not included nor were any members of Devriese' gallinarum species group. The methodology is presented as a potential solution to difficulties identifying cocci with traditional metabolic test methods (Beimfohr et al 1993).

Table 2. Reference source for 16s rRNA sequences of species in the genus *Enterococcus*.

Species	***Data source for rRNA sequence***	***Publication***
E. avium, E. casseliflavus, E. cecorum, E. columbae, E. durans, E. faecium,. E. gallinarum, E. hirae, E. malodoratus, E. mundtii, E. psuedoavium, E. raffinosa	*SSU rRNA*	*Williams, A.M., Rodrigues, U.M. and Collins, M.D. 1991*
E. dispar	*SSU rRNA*	*Collins, M.D., Rodrigues, U.M., Pigott, N.E. and Facklam, R.R. 1991*
E. faecalis	*RDP*	*** CR Woese Lab, U. of Illinois*
E. hirae	*RDP*	*Sechi, L.A., Fazari, S. and Daneo-Moore, L. 1993*
E. saccharolyticus	*RDP*	*Rodrigues, U.M. and M.D. Collins 1990*
E. seriolicida	*SSU rRNA*	Miller J.M.; Unpublished *"16S rRNA gene sequence of* Enterococcus seriolicida"
E.sp	*SSU rRNA*	Cai J. and Collins M.D.; *"Unpublished"*
E. sulfureus	*SSU rRNA & RDP*	*Martinez-Murcia, A.J. and Collins, M.D. 1991*
E.BAW#1	*RDP*	*A.Lawes 1995*

Figure 1. Dendrogram showing the similarities between strains based on sequence homologies and clustering by the unweighted pair group method. A 16s rRNA derived dendrogram of 14 species of the genus *Enterococcus* by Williams et al 1991.



Restriction Fragment Length Polymorphism (RFLP) analysis of 16S rDNA of 12 ATCC type species was used to create a data base to identify streptococcus and enterococcus species of bovine origin. Using this database, eleven biochemically atypical isolates from bovine mammary secretions including *E. saccharolyticus* were correctly identified. This use of 16S rDNA RFLP demonstrated the potential for the technique. Typing of the same *E. saccharolyticus* isolate by API STREP resulted in low discrimination and the Vitek system did not identify the organism. The species tested did not include any of Devriese et al's gallinarum species groups, it did include *E. faecium* (Jayarao et al 1992).

In a related study, Polymerase Chain Reaction (PCR) amplification using arbitrary primer 8.6d resulted in a characteristic pattern for each of 12 ATCC type strains including *E. saccharolyticus*. PCR based DNA fingerprinting was then used to successfully ID streptococcal and enterococcal species from bovine milk. The database, developed to identify important mastitis pathogens, did not contain information to identify lactococcal and aerococal isolates (Jayaroa & Oliver 1994). Species identification schemes have also been developed based on intergenic ribosomal PCR, this methods clearly differentiates many of the important nosocomial enterococci including *E. mundtii* and *E. casseliflavus* (Tyrell et al 1997).

A more recent PCR based study focused on medically important strains, the E. faecium species group in particular. The primer D11344 proved to be useful for species specific identification. The band patterns are identifiable visually (Descheemaeker et al 1997).

Clinical significance of enterococci.

Nosocomial infections are hospital acquired infections. The source of the infectious agent may be endogenous, commonly the patients gastrointestinal tract, female genital tract or skin. Exogenous sources include health care personnel, biomedical devices and the hospital environment (Hindler et al. 1994). The enterococci are frequently non pathogenic commensals when found in intestinal, vaginal, and oral tracts, however enterococci posses properties that allow them to take on a pathogenic role. Their natural ability to acquire, transfer and accumulate extrachromasomal elements confers a plasticity to their genome. Survival under stresses including antibiotic therapy is facilitated by this plasticity (Jett et al. 1994).

Hospital wide infection data covers all sites of nosocomial infection for all patients. From January 1990 to March 1996 the enterococci were found in ten percent of 101,821 nosocomial infections. The enterococci were most frequently found in urinary tract infections (UTI: 5613 cases) and surgical site infections (SSI: 2120 cases). Lower incidence was found in blood stream infections (1298 cases), pneumonia (267 cases) and other sites (1061) (CDC 1996). Enterococci are second only to *Escherichia coli* in Intensive Care Unit (ICU) nosocomial isolates (De Vera et al. 1996). *Enterococcus faecalis* is the most frequently found enterococcal pathogen (Tailor et al. 1993). It has been implicated in endocarditis, bacteremia, UTI and intraabdominal infections.

In addition, enterococci have been cultured from other infections.

- Enterococci have been isolated as the disease causing organism in 5.2% of clinical cases of endocarditis (Watanakunakorn and Burkert 1993).
- In necrotizing fascitis, a fast spreading necrosis of the fascia and subcutaneous tissue, enterococci are one of several pathogens found. The infections are usually polymicrobial (Ou et al.1993). Fourniers gangrene (gangrenous ulcers of the scrotum and or penis) is probably the same disease and a similar complement of pathogens which includes enterococci is found (Efem 1994).
- Septic arthritis due to enterococcal infection occurs with prosthetic and natural joints. In cases of prosthetic joint infection, antimicrobial therapy is successful. In two of 8 cases reported of native joint infections, amputation was necessary to cure the infection (Raymond et al. 1995).

- 29 percent of patients with pyogenic liver abscess had enterococci as the causal organism. This was the second most common organism cultured, after Klebsiella (Hansen and Vargish 1993).

Patient risk factors for enterococcal infections are immuno-compromised state, significant medical problems, recent surgery, and hospitalization for extended periods (Boullanger et al 1991).

Strains implicated in nosocomial infections are *E. faecalis, E. faecium, E. gallinarum, E. avium, E casseliflavus, E. raffinosus, E. hirae, E. durans* and *E. mundtii* (Vandamme et al 1996b, Pompei et al 1992, Clark et al 1993).

Antibiotic therapy and resistance of enterococcal pathogens.

Several classes of antibiotics have been utilized successfully against enterococcal infections. These are the β-lactams, aminoglycosides, glycopeptides and flouroquinones. Enterococci which are resistant to antibiotics are becoming an increasing problem in nosocomial infections. High level antibiotic resistance has been found to vancomycin (Ortega et al 1991), teicoplanin (Schmit 1992), gentamicin, kanamycin and streptomycin (Ismaeel 1992).

Enterococci resistant to the β-lactams have been isolated from clinical specimens. There are two basic mechanisms of resistance to the β-lactams, which inhibit bacterial cell wall synthesis: These are the production of β-lactamase and a change in the penicillin binding protein

(PBB) structures of the cell wall. Of special significance to the future of antibiotic use is a recent publication on antibiotic resistance gene swapping. Restriction mapping of the structural part of β-lactamase genes isolated from enterococcal and staphylococcal were found identical, however, the surrounding areas were not identical (Zsckech et al 1998).

The fluoroquinones are an important class of antimicrobials in the treatment of nosocomial infections by inhibiting bacterial DNA gyrase. Ciprofloxacin is effective against most enterococcal strains and is useful for treatment of the elderly with urinary tract infections (UTI) (Wiseman and Balfour 1994).

The aminoglycosides have been effective in treatment of enterococcal infections. This class, which includes gentamicin and streptomycin, are bacteriocidal and disrupt microbial protein synthesis. Enterococcal resistance to the aminoglycosides is a growing problem in clinical microbiology. High level resistance to the aminoglycoside gentamicin was found in 61% of isolates in 1989 and 1990 but in none of the isolates from 1969 to 1988 (Grayson et al 1991). *E. faecalis* is the most frequently found enterococcal pathogen in bacteremia (Tailor 1993). An examination of *E. faecalis* bacteremia in a hospital found 48% of 199 isolates over a 32 month period were highly resistant to gentamicin. A mortality rate of 65% was found for patients with resistant and non resistant infections. RFLP analysis of plasmid patterns found one plasmid pattern in 15 isolates. There was no evidence for direct patient to patient transfer. The authors suggested that *E. faecalis* is a marker for severe illness and the investigation did not find clinical factors associated with high level aminoglycoside resistance (Antalek et al 1995).

The glycopeptides vancomycin and teicoplanin have been important weapons against gram positive bacterial infections. Combination therapy of vancomycin and aminoglycosides have been effective in serious enterococcal infections where β-lactam therapy was not an option. (Hindler et al 1994). Because aminoglycosides alone are unable to penetrate the cell wall at clinically acceptable levels, their use is supplemented with a cell wall active agent, such as vancomycin which inhibits peptidoglycan synthesis (Hindler et al. 1994).

National data from the US Center for Disease Control (CDC) and Belgium indicate incidence of vancomycin resistant enterococci (VRE) as an emerging threat. From 1989 through 1993, the proportion of enterococcal isolates resistant to vancomycin (VRE) reported to CDC's National Nosocomial Infections Surveillance (NNIS) system increased from 0.3% to 7.9%. (CDC 1993).

In one year the percentage of nosocomial enterococci reported as resistant to vancomycin increased from 11.5% in 1993 to 13.6% in 1994 among Intensive Care Unit (ICU) isolates. The resistance level rose from

4.9% to 9.1% among noncritical care unit isolates. The increase was more dramatic among isolates from noncritical care units, suggesting that vancomycin-resistant enterococci are spreading from their focus in ICUs. (CDC 1995).

A nationwide study of enterococcal pathogens in Belgium examined nearly 500 strains. Of these, over 50 percent were resistant to the aminoglycoside streptomycin, while only 8.7 percent were resistant to gentamicin. Vancomycin resistance was found in 16.3 percent of the *E. faecium* strains and 1.5 percent of all enterococcal strains (VanDamme et al 1996b).

Single hospital based studies draw a picture of VRE as an opportunistic pathogen found in the most seriously ill patients, who have already undergone antibiotic therapy, and been hospitalized repeatedly or for long periods.

An analysis of 71 patients hospitalized between 1991 and 1994 was conducted to describe the population afflicted with bacteremial VRE. 73% were hospitalized in an intensive care unit, adult oncology or AIDS unit. These severely ill patients had received extensive antibiotic treatment during prolonged hospital stays (Montecalvo et al 1996). A review of patients in cardiothoracic ICU with vancomycin resistant *E. faecium* found prior nosocomial infection and a prior exposure to vancomycin as important variables. The antimicrobial susceptibility of the isolates was identical from the six patients (Karanfil et al 1992). Evidence of a similar outbreak was found in an ICU which affected nine patients. Isolates were resistant to glycopeptides, penicillins and aminoglycosides. Restriction digests data of genomic DNA were consistent with the spread of a single isolate. Prior extensive antibiotic exposure, particularly vancomycin, as well as renal insufficiency and length of hospital stay were risk factors for infection with the isolate (Handwerger et al 1993).

A great deal of research is currently underway examining the biochemistry and genetics of vancomycin resistance. The vanA gene has been implicated in high level resistance to vancomycin and is thought to be transferred by plasmid (Clark 1993 et al, Leclerq et al 1988, Barbier et al 1996).

The vancomycin resistance phenotype VanC is constitutive *to E. gallinarum* and *E. casseliflavus.* It confers low level vancomycin resistance. The majority of isolates reported to the CDC are *E. faecium* with the VanA phenotype (CDC 1993). The VanA and VanB genes may be transferred between strains or species of enterococci (Morrison et al 1997). Strains of *E. casseliflavus* have been isolated which harbor the VanA gene (Morrison et al 1997, Tyrell et al 1997). Concerns have been raised that enterococcus may provide a reservoir for genetic elements conferring resistance, and that these elements may be transferred to other genera.

Incidence of Enterococci Associated with Farm Animals

Enterococci have been found in and on many animals. As with the human animal they may be pathogenic or commensal. The enterococci have been isolated from healthy cats, dogs, cows, bird of various species, and pigs (Devriese et al 1987, Devriese et al 1991, Devriese et al 1992a, Devriese et al 1992b, and Saika et al 1994).

E. saccharolyticus has been associated directly with bovine mastitis. 317 gram positive catalase negative cocci isolated from bovine mammary glands were characterized. The most frequently isolated organisms from teat canal swabs were *S. dysgalctiae* and *S. saccharolyticus*. One cow with a teat canal colonized by *S. saccharolyticus* developed clinical mastitis 1 week later, demonstrating the pathogenic potential of this organism (Watts 1988). In a later study of 377 strains isolated from bovine IMI, 41 were identified as E. *saccharolyticus* (Watts 1993).

In a study evaluating the popular Rapid STREP, Rapid STREP identification of 199 strains of streptococci and enterococci associated with bovine intramammary infections (IMI) was tested. Enterococcal species were correctly identified 83.3% of the time. 14 of 25 *E. faecalis* isolates were incorrectly identified as *E. faecium*. No discussion was made of *E. casseliflavus.* API 20 STREP is a commercial system in frequent use, identifying streptococci in 4 to 24 hours. One of four *E. saccharolyticus* strains was misidentified as *S. bovis* using the test system in combination with hemolysis on bovine blood agar plates. *S. bovis* and *E. saccharolyticus* are both frequently isolated from bovine mammary glands. (Watts 1989).

Vancomycin resistant enterococci have been found in farm animals and pets (Devriese et al. 1996). Avoparcin is a glycopeptide antibiotic which is sometimes used in farm animals for growth promotion. VRE strains are cross resistant to avoparicin. Investigators surveyed farm animals in Europe and found six to eight percent positive for VRE. (Devriese et al 1996). In the US research is examining possible links between the spread of VRE and antibiotic usage. A study of hospitalized patients and over 50 farm animals failed to find any VRE with high level resistance to vancomycin (carrying VanA or VanB genes) except among hospital patients with risk factors associated with multiple hospitalizations (Coque et al. 1996).

Objectives:

1) To test the isolate BAW #1 and *S. bovis* ATCC strain 33317 91-08 for gram stain and ability to grow on aerobic and anaerobic media.
2) To determine fermentation products of the isolate BAW #1 using HPLC. To test culture supernatants of the isolate grown in Trypticase Soy Broth (TSB) and Glucose-Cellulose-Starch-Xylose- Rumen Fluid (GCSX-RF). To determine the fermentation products of *S. bovis* ATCC strain 33317 91-08 grown in TSB.
3) To determine the metabolic profile of the isolate BAW #1 . To identify the isolate BAW #1 using BIOLOG™, API STREP™ and conventional testing.
4) To identify the isolate BAW#1 using 16s rRNA sequence data.

Materials and Methods

1. Purification of the isolate BAW #1

The anaerobic method was used in purification of the isolate BAW #1. Standard anaerobic technique as described by Bryant (1972) was used to cultivate the isolate BAW #1 prior to its identification as an enterococcus.

Host culture purification: This procedure was carried out using anaerobic technique. Media used were GCS-RF broth, slants, plates, roll tubes, soft agar and anaerobic dilution medium (ADM). Please see Appendix I for complete media formulations. Oxidized media was discarded. All transfers were made under CO_2 gas, plates were incubated in an anaerobic jar that was sealed and gassed for 30 minutes with CO_2. All incubations were at 39°C.Two tubes of GCS-RF broth were inoculated with the isolate BAW #1 and incubated overnight at 39 °C under anaerobic conditions. The broth was transferred by sterile loop under CO_2 to a slant of GCS-RF. Culture was transferred to broth grown overnight and the following day a dilution series was prepared in ADM. Roll tubes were inoculated under CO_2 at 39°C overnight. A well isolated colony was picked and used to inoculate a fresh broth. The broth culture was gram stained and examined microscopically. The isolation procedure was repeated three times to insure purity of isolate.

2. Conventional Microbiological tests.

The isolate BAW #1 was tested on anaerobic and aerobic medium, including several types of selective media.

The isolate BAW #1 was tested for its ability to grow on aerobic media. Trypticase Soy Agar (TSA), TSA with 5% defibrinated sheep's blood and BIOLOG ™ BUGM were inoculated by loop from a log phase culture of the isolate.

A growth curve study of the isolate BAW #1 in GCS-RF broth under anaerobic conditions was conducted to facilitate work with the phage. Duplicate tubes were inoculated with 0.1 ml from an overnight culture. Samples were withdrawn at regular time points and diluted into ADM. A dilution series was set up and plated. The study was continued until OD_{600} readings indicated the end of the log phase had been reached. The growth curve was determined, and correlation's of population size, OD_{600} and time were made. (Meynell and Meynell 1965). The relationship between the number of cells per chain and nutritional conditions was not determined, therefore the colony forming units may not correlate exactly to numbers of cells. The OD_{600} was determined on a Spectronic 21D Spectrophotometer.

The isolate BAW #1 was inoculated from a log phase TSA culture into the following selective media with a sterile loop. Selective media tests were run in duplicate. Complete media formulations are in Appendix I.

The isolate BAW #1 was tested on m-enterococcus agar which contains 0.04% sodium azide and 2,3,5, triphenyl tetrazolium chloride This is a standard medium for the isolation, culture and enumeration of enterococci from water, sewage and feces. The isolate BAW #1 was tested for growth in TSB broth with 6.5% NaCl. The isolate BAW #1 was tested for growth on TSA slants with 15% glycerol.

The isolate BAW #1 was tested on motility sulfide plates and slants which contain 0.2% ferric citrate and 0.2% L-cystine. At 48 hours plates were examined for presence, pigmentation and spreading of colonies. A darkened pigment is indicative of production of H_2S from L-cystine. Colony spreading is a positive test for motility.

The isolate BAW #1 was tested for pigment production. Sterile cotton swabs were used to pick up colonies off of TSA and BIOLOG ™ BUGM. Control swabs were rolled across TSA and BIOLOG ™ BUGM with no inoculate.

3. High-pressure liquid chromatography (HPLC) analysis of fermentation products of the isolate BAW #1 .

Fermentation products were determined using HPLC. To prepare samples for HPLC, cultures of the isolate BAW #1 were grown in triplicate in anaerobic (GSCX-RF) and aerobic (TSB) liquid media. *S. bovis* was grown in triplicate in aerobic (TSB) liquid media

After overnight incubation, cultures were centrifuged at 13000g for 30 minutes, the supernatants were transferred to a clean centrifuge tubes and respun as before. Supernatants were transferred to HPLC tubes and held at -20 ° C until analysis. Supernatants were analyzed for fermentation end products by ion exchange exclusion HPLC. The column temperature was 65° C. Mobile phase consisted of 0.005 N H_2SO_4 at a flow rate of 0.9ml/min. Twenty ul of the filtered samples were autoinjected and analytes detected by refractive index. Integration areas were quantified by commercial HPLC software and compared to standards of acetic, butyric, citric, isobutyric, lactic and propionic acid, and ethanol. Controls of uninocculated media were used to determine baseline values.

4. BIOLOG ™ Identification

BIOLOG ™ Identification followed the protocol for gram positive organisms in the BIOLOG ™ manual. (Bochner 1996) The BIOLOG ™ identification system tests the organisms carbon source utilization of 95 substrates, with water as a control in the 96th well. The isolate BAW #1 was tested on BIOLOG ™ gram positive plates, and the BIOLOG ™ gram positive database was selected for data analysis. The redox based dye tetrazolium violet is used to detect metabolic activity colorimetrically. Positive utilization is observed as the dye is irreversibly reduced to a purple formazon. Any chemical substrate oxidized will result in the production of NADH. The tetrazolium is reduced by electrons from the electron transport chain.

The isolate BAW #1 was tested anaerobically and aerobically. The first test was anaerobic. The isolate was grown on GCS-RF plates and transferred by sterile swab into ADM to an OD of 0.25 using anaerobic technique. Two BIOLOG ™ plates were inoculated with this solution and the plates were incubated under CO_2 at 39°C and read at 4 hours and 24 hours. After the isolate tested positive for growth under aerobic conditions, it was tested again on two BIOLOG ™ plates using standard BIOLOG ™ BUGM media and saline to prepare the inoculate. The aerobically incubated plates were incubated at 39°C for 24 hours. In both cases metabolic activity on 95 substrates was measured by the BIOLOG ™ Plate Reader and the BIOLOG ™ Gram Positive Database used to identify the organism BIOLOG ™ calculations and interpretations of BIOLOG ™ data are as follows:

The data produced by BIOLOG ™ is presented on printed data sheets as:

BIOLOG ™ DATA FORM	SUBSTRATE UTILIZATION
<xxx>	positive
{xxx}	borderline
{xxx+	borderline
{xxx-	borderline
xxx	negative

Numerical values were assigned for the BIOLOG ™ dataforms, 10 for positive, 5 for borderline and 0 for negative.

The average value for each substrate was calculated and variability noted. This data manipulation serves to produce a set of numbers comparable to other data in the microbiological literature, including Bergey's Manual for Determinative Bacteriology (9th) (Holt et al 1993) . Cluster Analysis was generated using the BIOLOG ™ software by entering the BIOLOG ™ Bionumber generated at the time of the plate reading.

5. API STREP Test

The isolate BAW #1 was brought to the Animal Health Diagnostic Laboratory (AHDL) of the College of Veterinary Medicine at MSU for testing on API Rapid STREP system. The API STREP strip tests the isolate for acetoin production, β-glucosidase, pyrrolidonylaryl-amidase, β-galactosidase, leucine arylamidase, arginine hydrolysis, ribose, L-arabinose, mannitol, lactose, trehalose, raffinose, starch, hippurate hydrolysis, α-galactosidase, β-glucuronidase, alkaline phospatase, sorbitol, inulin, glycogen and β-hemolysis. Test results are presented as positive or negative for each test. From this a seven digit profile is produced which is used to determine species identification. This profile was called in on two separate occasions to the API STREP/ bioMerieux phone line. Operators used the most recent version 3 for data analysis.

The database includes *E. gallinarum*, but does not include *E. saccharolyticus*. An identification which is "good to the species level" indicates that the isolate BAW #1 matches at least 90% of what would be expected for the designated species.

6. Antibiotic resistance testing

The isolate BAW #1 was sent to the Animal Health Diagnostic Laboratory (AHDL) of the College of Veterinary Medicine at MSU for analysis of antibiotic susceptibility. The isolate BAW #1 was tested for sensitivity to: ampicillin, cephalothin, cefotoxin, ciprofloxacin, clinamycin, erythromycin, gentamicin, nitrofurantoin, oxacillin, penicillin, tetracycline and trimethoprim -sulfa.

7. Analysis of 16s rRNA sequence of the isolate BAW#1

The 16S rRNA sequence of the isolate BAW #1 has been determined (Lawes 1995) and was entered into the Ribosomal Database Project (RDP) in 1995 by Lawes . The RDP programs identified the isolate BAW #1 as a strain of *E. saccharolyticus*. In 1995, The RDP database only included six enterococcal species*: E. cecorum, E. faecalis, E. hirae, E. sulfureus, E. columbae* and *E. saccharolyticus*.

Phenotypic data in this study did not support that identification, in fact the isolate was identified by API STREP and BIOLOG as *E. casseliflavus*. The sequences in the RDP database at the time of the

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Lawes analysis did not include members of the *E. faecium* species group. To test the identification of the isolate, a full species complement of the enterococcus was needed. Additional 16s rRNA enterococcal sequences were included in the analysis. These were E. *casseliflavus* NCDO2376, *E. gallinarum* NCDO 2313 (T), *E. faecium* NCDO 942 (T), *E. Mundtii* NCDO 2375, and *E. durans*, NCDO 596 (T). Partial sequences for two strains of *E. casseliflavus*, strain AFO 39903 and strain AFO39989, and two strains of *E. gallinarum,* strains AFO39900 and AFO 39898 were also included in the analysis (Patel et al 1998). The type strain of *E. saccharolyticus* was also included in the analysis. The additional sequence data was entered into the ARB program Fast DNA ml.

These sequences were examined for phylogenetic relationship using the least squares distance matrix program of ARB. An alignment and a phylogenetic tree were generated.

Results

1. Purification of the isolate BAW #1

The isolate BAW #1 readily grew overnight at 39 ° C under anaerobic conditions on the complex medium GCS-RF. The isolate is gram positive and occurs in chains of two up to 20 cocci in length. The cocci are slightly ovoid in shape.

2. Conventional Microbiological tests.

Conventional test results include data generated when the isolate BAW #1 was tested on anaerobic and aerobic media, including several types of selective media. Aerobic media supportive of overnight growth at 39 ° C included TSA, TSA with 5% defibrinated sheep's blood and BIOLOG ™ BUGM. The isolate BAW #1 grew rapidly in TSB, reaching an OD_{600} of 0.5 in 4-6 hours. The results of the growth curve study of the isolate BAW #1 in GCS-RF broth are presented in Table 3 and Figure 3. The isolate BAW #1 attained rapid growth rates in anaerobic media, with a peak doubling time of less than one hour. The isolate entered log phase growth approximately three hours after inoculation. Optical densities indicative of log phase growth are in the 0.2 to 0.6 range for this media (Figure 2). The stationary phase of growth was entered when the colony forming units per milliliter reached 8×10^7 in GCS-RF broth. The isolate

BAW#1 grows rapidly, even under anaerobic conditions. The growth rate of the isolate was determined by the formula (Meynell and Meynell 1965):

$\mu=(\log N_{(t)}-\log N)/t$

where

$\mu=$ Specific growth rate

$N=$ Population at a given time

$N_{(t)}=$ Population after an elapsed time

$T=$ Time elapsed since last measurement

The facultative nature of the isolate provided evidence that it could not be a member of the genus *Ruminococcus.*

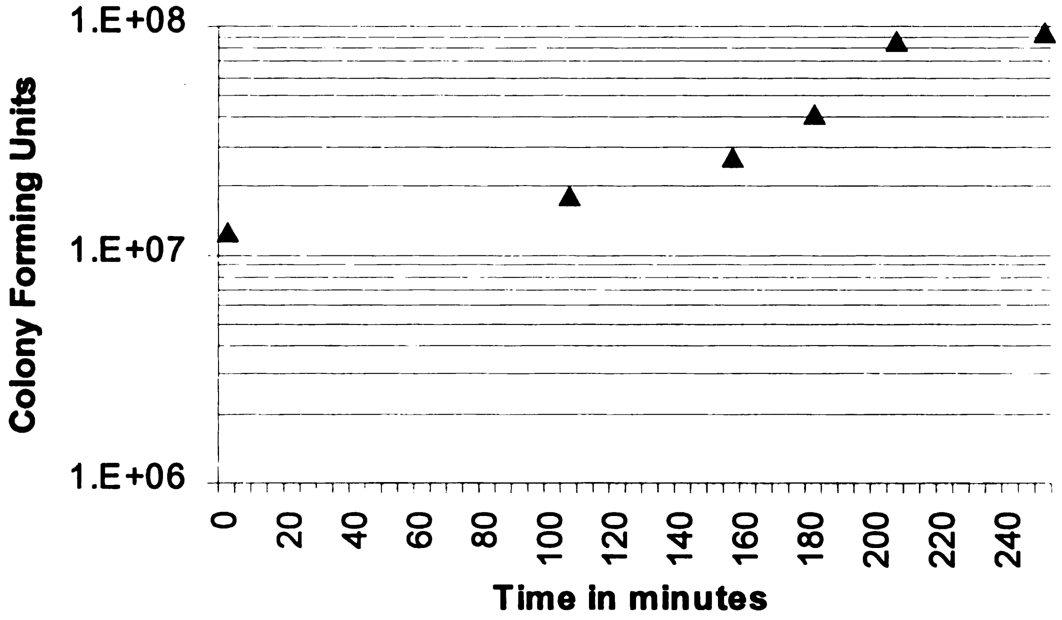
The isolate BAW #1 was tested on m-enterococcus agar, which contains 0.04% sodium azide and 2,3,5, triphenyl tetrazolium chloride. Colonies were tiny, pink and visible after 24 hours. The isolate was tested on motility sulfide media which contains 0.2% ferric citrate and 0.2% L-cystine. After 48 hours, growth was positive, but not pigmented, indicating the isolate does not produce H_2S from L-cystine. The colonies did not spread, indicating a lack of motility.

The isolate BAW #1 tested positive for growth in TSA and Antibiotic Media with growth evident after 24 hours. The isolate BAW #1 was tested in TSB broth with 6.5% NaCl with growth evident after 24 hours. The isolate BAW #1 grew overnight on TSA slants with 15% glycerol.

Table 3. Growth characteristics of the isolate BAW #1 in GCS-RF broth: Optical Density and Colony Forming Units charted against time

		Optical Density		**Colony Forming Units**	**Specific Growth Rate**
TIME	**Minutes**	**Tube A**	**Tube B**	**average**	**$\mu=(\log(N_t)-\log N)/t$**
9:30 AM	0	0.04	0.05	12540000	
11:15 AM	105	0.09	0.08	17995000	0.0015
11:55 AM	155	0.11	0.12	26962500	0.0035
12:30 PM	180	0.17	0.18	41050000	0.0073
12:55 PM	205	0.25	0.25	86000000	0.0128
1:40 AM	250	0.43	0.42	94075000	0.0009

Figure 2. Growth data for isolate BAW #1 grown in GCS-RF anaerobically: Colony Forming Units and Time.



The ability of the isolate to tolerate high salt and azide concentrations confirmed its identification as a member of the genus *Enterococcus.* Sterile cotton swabs used to pick up colonies off of TSA and BIOLOG ™ BUGM turned yellow. Control swabs rolled across TSA and BIOLOG ™ BUGM with no inoculate picked up no pigmentation. Control swabs rolled across plates of *S. bovis* grown on TSA and BIOLOG ™ BUGM also picked up no pigmentation. The pigment production of the isolate contradicted its identification as *E. saccharolyticus,* since this species is not pigmented. Based on pigmentation, the isolate BAW#1 could be *E. mundtii, E. casseliflavus* or *E. sulfureus,* however, the lack of motility would exclude most strains of *E. casseliflavus.*

3. High-pressure liquid chromatography (HPLC) analysis of fermentation products of the isolate BAW #1 .

The end point fermentation products of the isolate BAW #1 grown in TSB are 51% acetic acid, 34% lactic acid and 15% ethanol (Table 4). The end point fermentation products of the isolate BAW #1 grown anaerobically in GCSX-RF are 95% lactic acid and 5% ethanol. The endpoint fermentation products of S. bovis are 89% lactic acid, 2% acetic acid and 9 % citric acid. (Table 4).The fermentation product analysis provided further evidence that isolate is not a strain of Ruminococcus, and supports the thesis that the isolate is an enterococci.

Table 4. Fermentation Products of the isolate BAW#1 and *Streptococcus bovis* as determined by High Pressure Liquid Chromatography.

I. Fermentation Products of the isolate BAW#1 Cultured aerobically in Trypticase Soy Broth.

Fermentation Product	Percent of Total
Lactic acid	0.34
Acetic acid	0.51
Ethanol	0.15

II. Fermentation Products of the isolate BAW#1 Cultured Anaerobically in GCSX-RF

Fermentation Product	Percent of Total
Lactic acid	0.95
Ethanol	0.05

III. Fermentation Products of *Streptococcus bovis* Cultured Aerobically in Trypticase Soy Broth

Fermentation Product	Percent of Total
Citric acid	0.09
Lactic acid	0.89
Acetic acid	0.02

4. BIOLOG ™ Identification

The BIOLOG ™ identification system tests the organisms carbon source utilization of 95 substrates, with water as a control in the 96th well. Thirty-nine of the substrates tested positive, fifteen showed an intermediate reaction and forty-one tested negative. A complete listing of substrate reactions is provided in Tables 5, 6, and 7. Some variability was found in substrate utilization on the four plates. Some substrates tested positive on one plate and borderline on another, as well as negative on one plate and borderline on another. None of the substrates tested positive on one plate and negative on another. An anaerobic environment was more conducive to the utilization of α-ketoglutaric acid, α -ketovaleric acid, L-rhamnose, L-lactic acid and pyruvic acid. The isolate demonstrated greater utilization of D-ribose and uridine 5-monophosphate under aerobic conditions.

The BIOLOG ™ software assigns a Bionumber to a reading which is a condensed version of the test results. The BIOLOG ™ numbers for the four plates were:

A: 3627-5656-7764-5623-3000-1300-0001-7600
B: 3607-5656-7764-5623-3000-1310-0001-7600
C: 3627-5656-7764-5663-3000-0300-0001-7600
D: 3607-5656-7764-5663-3200-1310-0001-7600

Based on this profile, the BIOLOG ™ identification system identified the isolate BAW #1 as *E. gallinarum* on each plate using BIOLOG ™ Gram Positive Release 3.5. The BIOLOG ™ program rated the similarity and distance of the isolate BAW #1 to *E. gallinarum* for each plate read (Table 8.)

BIOLOG ™ Similarity is a calling criteria, not a probability, and is based on empirical results. It is considered a judge of the reliability and confidence of the identification. The value of distance in the BIOLOG ™ system roughly approximates the number of mismatches, with allowances for substrates known to be variable for a given species. A similarity index of greater than 0.5 is considered an acceptable identification for bacterial species measured with the BIOLOG ™ system. Therefor, the identification of the isolate BAW #1 as *E. gallinarum* was considered greater than acceptable using the BIOLOG ™ Gram Positive Release 3.5.

This identification conflicted with the API STREP identification of the isolate BAW #1 as *E. casseliflavus* and with the RDP identification of the isolate BAW #1 as *E. saccharolyticus*. The BIOLOG ™ Software Release 3.5 includes *E. casseliflavus* , *E. gallinarum and E. saccharolyticus* in its database.

Table 5. BIOLOG ™ SYSTEM Substrate utilization profile of the isolate BAW #1. PART 1. Substrates the isolate was unable to utilize. (mean utilization value less than 2).

	BIOLOG WELL	**MEAN**	**PLATE**			
			A	**B**	**C**	**D**
3-methyl glucose	c11	1.25	0	0	0	5
α-hydroxybutyric acid	e7	0	0	0	0	0
α-methyl D mannoside	d2	0	0	0	0	0
α-methyl D-glucoside	c12	0	0	0	0	0
acetic acid	e6	0	0	0	0	0
adenosine-5'monophosphate	h6	1.25	0	0	0	5
alaninamide	g1	0	0	0	0	0
asparagine	g5	0	0	0	0	0
ß-hydroxybutyric acid	e8	0	0	0	0	0
D- arabitol	b2	0	0	0	0	0
D-alanine	g2	1.25	5	0	0	0
D-galacturonic acid	b8	0	0	0	0	0
D-lactic acid methyl ester	f2	0	0	0	0	0
D-malic acid	f4	1.25	5	0	0	0
D-tagatose	e1	1.25	5	0	0	0
fructose-6-phosphate	h9	0	0	0	0	0
γ-hydroxybutyric acid	e9	1.25	5	0	0	0
glycyl-L glutamic acid	g7	0	0	0	0	0
glucose-1-phosphate	h10	0	0	0	0	0
glucose-6-phosphate	h11	0	0	0	0	0
inulin	a6	0	0	0	0	0
L-alanine	g3	1.25	5	0	0	0
L-alanyl-glycine	g4	0	0	0	0	0
L-fucose	b6	0	0	0	0	0
L-glutamic acid	g6	0	0	0	0	0
L-pyrglutamic acid	g8	0	0	0	0	0
L-serine	g9	0	0	0	0	0
lactamide	f1	0	0	0	0	0
m-inositol	b12	1.25	0	5	0	0
mannan	a7	0	0	0	0	0
methyl succinate	f7	0	0	0	0	0
N-acetyl L-glutamic acid	f12	1.25	0	5	0	0
ρ-hydroxybutyric acid	e10	0	0	0	0	0
propionic acid	f8	1.25	5	0	0	0
putrescine	g10	1.25	5	0	0	0
sedoheptulose	d9	1.25	0	0	0	5
succinamic acid	f10	0	0	0	0	0
succinic acid	f11	0	0	0	0	0
thymidine-5'monophosphate	h7	0	0	0	0	0
tween 80	a9	0	0	0	0	0
xylitol	e4	0	0	0	0	0

Table 6. BIOLOG ™ SYSTEM Substrate utilization profile of the isolate BAW#1. PART 2. Substrates the isolate was able to utilize weakly. (mean utilization value between 2 and 8).

SUBSTRATE	**BIOLOG WELL**	**MEAN**	**PLATE**			
			A	**B**	**C**	**D**
α–methyl D galactoside	c9	2.5	0	5	0	5
2,3-butanediol	g11	2.5	5	0	0	5
α–ketoglutaric acid	e11	2.5	5	5	0	0
α–ketovaleric acid	e12	2.5	5	5	0	0
β–cyclodextrin	a3	7.5	5	10	5	10
D-L-α glycerol phosphate	h12	2.5	0	5	0	5
D-melezitose	c7	6.25	5	5	5	10
D-ribose	d7	7.5	5	5	10	10
D-sorbitol	d10	5	5	5	5	5
L-lactic acid	f3	7.5	10	10	5	5
L-rhamnose	d6	2.5	5	5	0	0
Pyruvic acid	f9	5	10	5	0	5
Tween 40	a8	7.5	5	10	10	5
Uridine-5'monophosphate	h8	2.5	0	0	5	5
Xylose	e5	3.75	5	0	5	5

Table 7. BIOLOG ™ SYSTEM Substrate Utilization by the isolate BAW #1 as tested in the BIOLOG™ system. Part 3. Substrates the isolate was able to utilize in the BIOLOG™ system (mean utilization value greater than 8).

SUBSTRATE	**BIOLOG WELL**	**MEAN**	**PLATE**			
			A	**B**	**C**	**D**
Arbutin	b3	10	10	10	10	10
2'-deoxyadenosine	h2	10	10	10	10	10
α–cyclodextrin	a2	10	10	10	10	10
α–D glucose	b11	10	10	10	10	10
α–D- lactose	c1	10	10	10	10	10
Adenosine	h1	10	10	10	10	10
Amygdalin	a12	10	10	10	10	10
β–methyl D galactoside	c10	10	10	10	10	10
β–methyl D-glucoside	d1	10	10	10	10	10
Cellobiose	b4	10	10	10	10	10
D- glucuronic acid	b10	10	10	10	10	10
D- mannose	c6	10	10	10	10	10
D-fructose	b5	10	10	10	10	10
D-galactose	b7	10	10	10	10	10
D-mannitol	c5	10	10	10	10	10
D-melibiose	c8	10	10	10	10	10
D-piscose	d4	10	10	10	10	10
D-raffinose	d5	10	10	10	10	10
D-trehalose	e2	10	10	10	10	10
Dextrin	a4	10	10	10	10	10
Gentibiose	b9	10	10	10	10	10
Glycerol	g12	10	10	10	10	10
Glycogen	a5	10	10	10	10	10
Inosine	h3	10	10	10	10	10
L- arabinose	b1	10	10	10	10	10
L-malic acid	f5	10	10	10	10	10
Lactulose	c2	10	10	10	10	10
Maltose	c3	10	10	10	10	10
Maltotriose	c4	10	10	10	10	10
Methyl pyruvate	f6	10	10	10	10	10
N- acetyl glucoseamine	a10	10	10	10	10	10
N- acetyl mannoseamine	a11	10	10	10	10	10
Palitinose	d3	10	10	10	10	10
Salacin	d8	10	10	10	10	10
Stachyose	d11	10	10	10	10	10
Sucrose	d12	10	10	10	10	10
Thymidine	h4	8.75	10	5	10	10
Turanose	e3	10	10	10	10	10
Uridine	h5	10	10	10	10	10

Table 8. Similarity and Distance as determined by BIOLOG™ software for the isolate. The first data set is based on BIOLOG ™ software release 3.5, the second is based on BIOLOG ™ software release 3.7.

Plate	BIOLOG ™ RELEASE	Similarity	Distance
A	3.5	0.813	2.807
B	3.5	0.809	2.871
C	3.5	0.821	2.683
D	3.5	0.821	2.683
A	3.7	0.922	1.160
B	3.7	0.915	1.258
C	3.7	0.828	2.571
D	3.7	0.868	1.956

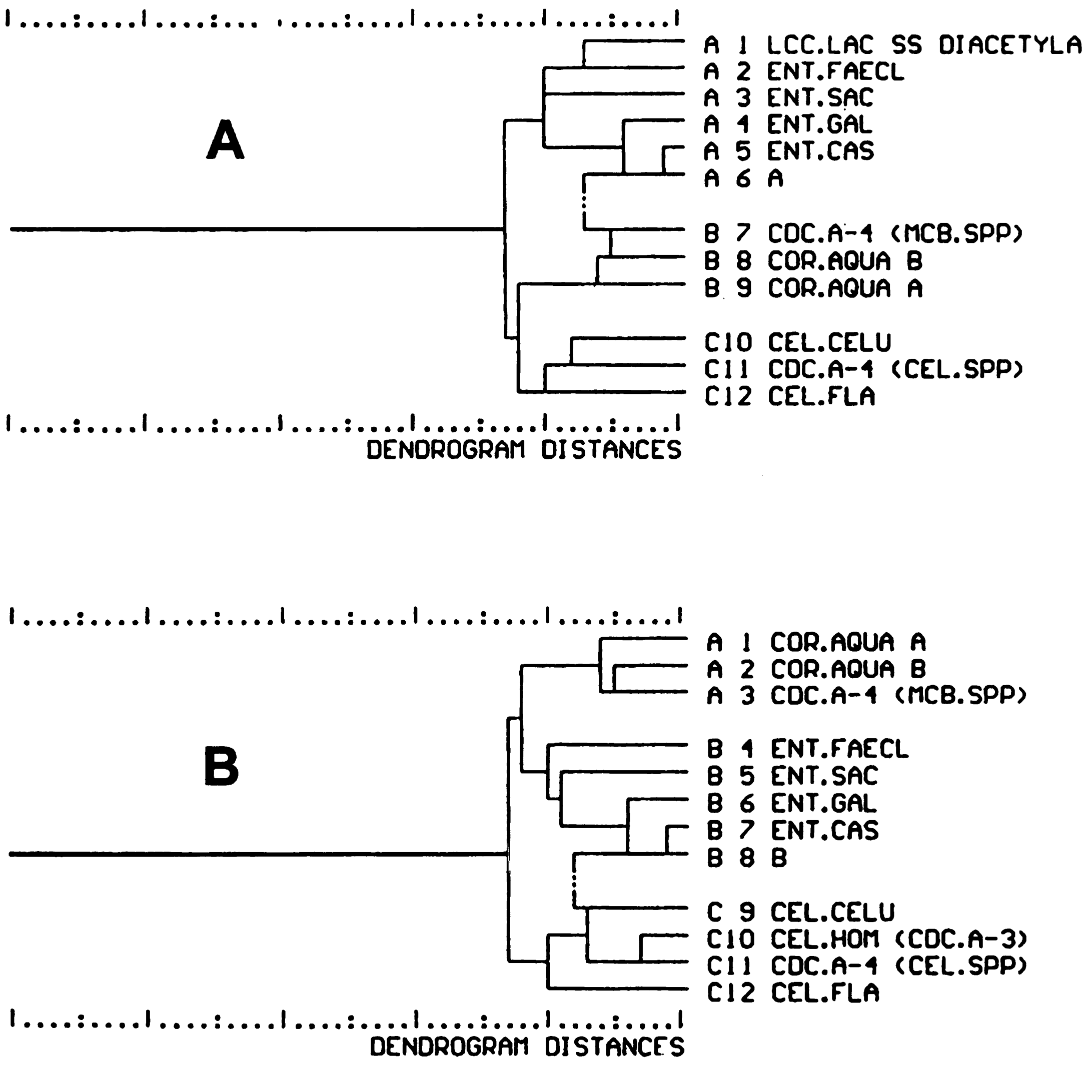
BIOLOG ™ was contacted regarding the conflicting identifications. BIOLOG ™ had released an upgraded database, Release 3.7 with changes in the criteria for *E. casseliflavus* and *E. gallinarum*. The new database was installed into the BIOLOG ™ program. The bionumbers of each of the four plates was entered into the new database. The resulting identification matched the API STREP identification; the isolate BAW #1 was identified as *E. casseliflavus* by BIOLOG ™ software version 3.7.

The BIOLOG TM system provides cluster analysis of isolates based on its metabolic data. The dendrograms produced with the isolate BAW #1 using release 3.7 are shown in Figure 3. Each dendrogram shows the isolate BAW #1 as closest to E. casseliflavus.

Figure 3. BIOLOG™ generated dendrograms of the isolate BAW#1 showing metabolic proximity to *E. casseliflavus* and *E. gallinarum.*

A) dendrogram produced with BIOLOG™ number from Plate A: isolate BAW#1 is designated A6 A .

B) dendrogram produced with BIOLOG™ number from Plate B: isolate BAW#1 is designated B8 B.



5. API STREP Test

The isolate BAW #1 tested positive for acetoin production, β-glucosidase, pyrrolidonylaryl-amidase, β-galactosidase, leucine arylamidase, arginine hydrolysis, ribose, L-arabinose, mannitol, lactose, trehalose, raffinose, and starch. The isolate tested negative for hippurate hydrolysis, α-galactosidase, β-glucuronidase, alkaline phospatase, sorbitol, inulin, glycogen and β-hemolysis (See Table 9). The Animal Health Diagnostic Lab at Michigan State University determined the isolate BAW #1 was an *E. faecium*, type two or three. To check the database for further information, I called the API STREP/ bioMerieux phone line. Based on the utilization profile, the seven digit profile 5157551 was produced. This profile was called in on two separate occasions to the API STREP/ bioMerieux phone line. Operators used the most recent version 3 to give a " good to the species" level identification of the isolate BAW #1 as *E. faecium 3* or *enterococcus faecium 2.* A supplemental Note 60 was accessed which suggests that if the isolate BAW #1 has a yellow pigment, it is to be considered a "good to the species level" identification as *E. casseliflavus* . The database includes *E. gallinarum*, but does not include *E. saccharolyticus*. The "good to the species level" identification indicates that the isolate BAW #1 matches at least 90% of what would be expected for the designated species. The API

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STREP data supplemented by my data concerning pigmentation, determined that the isolate BAW #1 is an *E. casseliflavus* strain.

6. Antibiotic resistance testing

The isolate BAW #1 tested sensitive to the antibiotics cephalothin, gentamicin, tetracycline, ciprofloxacin and nitrofurantoin. The isolate BAW #1 tested moderately susceptible to the antibiotics ampicillin and penicillin. The isolate BAW #1 tested resistant to the antibiotics cefoxitin, clindamycin, erythromycin, oxacillin and tribissen (Table 10).

7. Analysis of 16s rRNA sequence of the isolate BAW#1

Tree building using the ARB program Fast DNA ml for the enterococcal species show isolate BAW#1 very close to both *E. gallinarum* and *E. casseliflavus*, slightly closer to the latter (Figure 4). Seven strains made up the *E. gallinarum-E. casseliflavus* group which clearly branched separate from the other enterococcal species. The proximity of this entire group precludes identification to the species level using this data. The 16s rRNA data shows the *E. gallinarum-E. casseliflavus* group distinct from the *faecium-mundtii- hirae-durans* group, as well as the other enterococcal species. *E. saccharolyticus* is quite far from BAW#1.
The distance matrix shows the relationships between the strain BAW#1, and the *E. saccharolyticus, E. casseliflavus* and *E. gallinarum* strains. (Table 11). The isolate BAW#1 is closest to the *E. gallinarum* strain AFO

39900. *E. saccharolyticus* is clearly not in the same phylogenetic group as the *E. casseliflavus* and *E. gallinarum* strains, with a distance of greater than 0.022 from all strains. The entire *gallinarum- casseliflavus* group has distances an order of magnitude less, with values less than 0.0022. Based on the distance matrix, the isolate is clearly not a strain of *E. saccharolyticus*. This data is insufficient to determine the identity at the species level, however it is clear that the strain is a part of *the E. gallinarum-E. casseliflavus* group.

Table 9. Metabolic profile of the isolate BAW #1 and related species as determined by API Strep™. Numerical Data is the percent of strains testing positive for a given species. Data from the API STREP package insert. Isolate data is "+" or "-", Reaction data for the isolate is from the Animal Health Diagnostic Lab at Michigan State University.

	Isolate BAW #1	*E. faecium 2*	*E. faecium 3*	*S. bovis I*	*S. bovis II-1*	*S. bovis II-2*	*E. gallinarum*
acetoin production	+	99	100	99	99	100	100
Hippurate hydrolysis	-	96	1	0	0	3	100
β-glucosidase	+	100	100	100	93	100	100
Pyrrolidonylaryl-Amidase	+	100	100	0	0	0	100
α-galactosidase	-	90	90	76	93	78	100
β-glucuronidase	-	0	0	2	1	41	83
β-galactosidase	+	99	98	1	6	38	10
alkaline phosphatase	-	1	1	0	0	0	0
leucine arylamidase	+	99	99	97	100	100	100
arginine hydrolysis	+	99	66	0	0	0	100
Ribose	+	99	99	0	0	0	100
L-arabinose	+	60	99	14	10	1	100
Mannitol	+	99	99	97	1	3	100
Sorbitol	-	15	15	0	0	0	0
Lactose	+	99	99	100	96	99	100
Trehalose	+	99	99	100	26	79	100
Inulin	-	1	96	83	56	32	100
Raffinose	+	99	99	99	99	79	100
Starch	+	80	99	100	99	11	83
Glycogen	-	1	1	97	86	1	16
β-hemolytic	-	0	0	1	2	1	0

Table 10. Antibiotic Sensitivity of the isolate BAW #1.

Antibiotic	Class	Mechanism	Target organisms	Isolate BAW#1
Penicillin	β-lactam	inhibit cell wall synthesis	streptococcal and staphylococcal	Moderately susceptible
Ampicillin	β-lactam	inhibit cell wall synthesis	streptococci, staphylococci, E.coli, proteus, haemophilus	moderately susceptible
Cephalothin	β-lactam: first generation cephalosporin	inhibit cell wall synthesis	staphylococci, streptococci	sensitive
Cefotoxin	β-lactam third generation cephalosporin	inhibit cell wall synthesis	gram-, some β-lactamase producing gram + strains	resistant
Oxacillin (methicillin)	β-lactam- penicillinase resistant penicillin	inhibit cell wall synthesis	penicillinase producing staphylococci	resistant
Gentamicin	amino- glycoside	inhibit protein synthesis	enterococci, gram- bacillus	sensitive
Tetracycline	tetracyclines	inhibit protein synthesis	many gram- and gram + plasmid mediated resistance common	sensitive
Cipro- floxacin	flouroquinone	inhibits DNA gyrase	enterobacteriaceae neisseria sp., some streptococcus and staphylococcus	sensitive
Nitro- furantoin		inhibits DNA, RNA and cell wall synthesis	enterococci and enterobacteriaceae urinary infections	sensitive
Clindamicin	modified from lincomycin	inhibits protein synthesis	most gram+ cocci and anaerobes. enterococci are resistant	resistant
Erythromycin	macrolide	inhibits protein synthesis	some streptococcus, legionella, etc.	resistant
Tribissen/ trimethoprim sulfa	* Brand name is Bactrim	inhibits steps in microbial folic acid synthesis	enterobacteriaceae, staphylococcus, etc.	resistant

Data in Table 10 is drawn from Hindler et al 1994 and Physicians Desk Reference 1997.

Figure 4. Phylogenetic relationship of the enterococci and the isolate BAW#1 based on 16s rRNA sequence analysis by the ARB Fast DNA ml program.

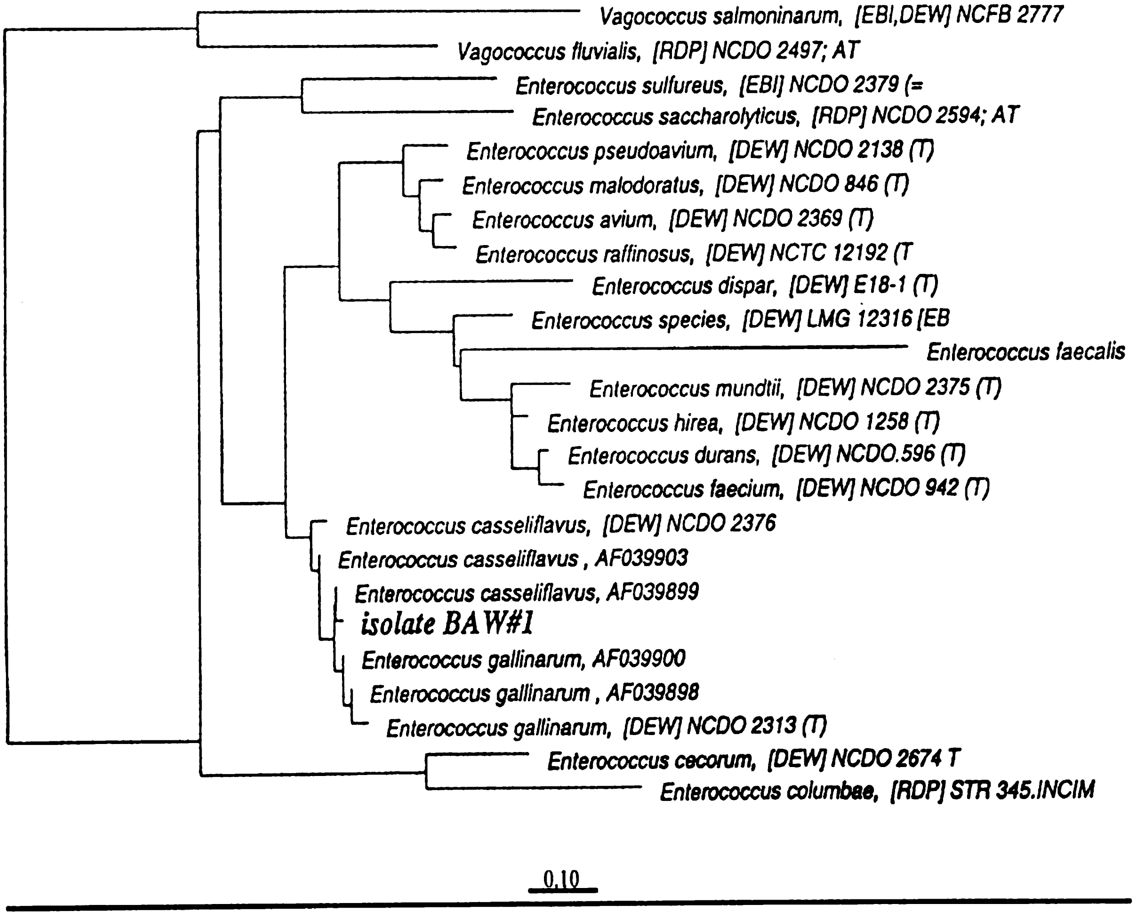


TABLE 11. Phylogenetic relationship of the enterococci and the isolate BAW#1 based on 16s rRNA sequence analysis by the ARB least squares distance matrix program.

E. Sac NCDO 2594							
E. Gal NCDO 2313	0.02446						
Isolate BAW#1	0.02596	0.00287					
E. Cas NCDO 2376	0.02446	0.00215	0.00430				
E. gal AFO39898	0.02667	0.00143	0.00215	0.00358			
E.cas AFO39899	0.02519	0.00215	0.00072	0.00358	0.00143		
E. gal AFO39900	0.02593	0.00215	0.00143	0.00430	0.00072	0.00072	
E. cas AFO39903	0.02519	0.00215	.00215	0.00215	0.00143	0.00143	0.00214
	E. Sac NCDO 2594	*E. gal* NCDO 2313	Isolate BAW#1	*E. cas* NCDO 2376	*E. gal* AFO 39898	*E. cas* AFO 39899	*E. cas* AFO 39903

Discussion

The isolate BAW #1 is a gram positive, facultatively anaerobic cocci which grows in chains of 2 to over 20 cocci. It is tolerant of high salt. The isolate grew on m-*enterococcus* media containing 0.04% sodium azide. Based on these tests, it is possible to identify the isolate as a member of the genus *Enterococcus.* The isolate is able to survive storage at 4 ° C for over 6 years. In rich media the isolate BAW #1 entered log phase within 2 to 4 hours, and attained a concentration of greater than 10^{-8} colony forming units per milliliter even under anaerobic conditions. The organism is clearly a survivor.

The isolate BAW #1produced a yellow pigment. Of the enterococci, only *E. casseliflavus, E. mundtii* and *E. sulfureus* produce a yellow pigment. *E. casseliflavus* is usually motile, *E. mundtii* and *E. sulfureus* are not motile (Table 12).

Phenotypic differentiation between *E. casseliflavus* and *E. mundtii* is generally based on motility. However, the type strain of *E. casseliflavus* is not motile. Recent research has determined that the motility test is not a reliable differentiator.

The conventional tests suggested the identification of the isolate BAW #1 is *E. casseliflavus, E. mundtii* or *E. sulfureus* (Table 12).

Table 12. Phenotypic differentiation of *E. casseliflavus* from closely related enterococci with test results of the BAW#1 isolate.

	BAW#1	E. cas	E. gal	E. mun	E. sul	E. fcm	E. sac
Motility	-	+/(-) (2) +* (6)	- (2) +* (6)	- (2)	- (4)	- (2)	- -(6)
Pigment	Yellow	Yellow (5) * (6)	None (5)	Yellow (5)	Yellow	None (5)	None (5)
Hippurate hydrolysis	- API	- (5)	+ (5)	- (5)		(+)(5)	- (5)
Tagatose	- *BIOLOG*	-/(+) (1)	+/(-) (1)	- (1)		-/(+) (2)	
Arabinose	+ API	+ (1)	+ (1)	+ (1)	- (4)	+ (1)	
Sorbitol	- API	- (5)	- (5)	d (5)	- (4)	- (5)	+ (5)
Ribose	+ API	+	+ (4)	+ (2)	+ (4)		
Raffinose	+ API	+ (3)	+ (3)	- (3)	+ (4)	- (3)	
Inulin	- API	+ (3)	+ (3)	- (3)		- (3)	
β-glucopyranosidase	No Data	+ (6)	+ (6)	- (6)	+ (6)	- (6)	+ (6)
Efrotomycin sensitivity	No Data	R (6)	R (6)	S (6)	R (6)	S (6)	R (6)

1) Cowan and Steele 1993
2) Bridge and Sneath 1982
3) Knudtston and Hartman 1993
4) Morrison et al 1997
5) Holt et al 1993
6) Carvalho et al 1998
7) Martinez-Murcia and Collins 1991

* denotes variability of strains

Further data on the metabolic profile of the isolate BAW #1 obtained through the use of BIOLOG™ and API Strep. These tests demonstrated that the isolate is capable of utilizing a broad range of substrates under aerobic and anaerobic conditions.

The metabolic based identification scheme BIOLOG ™ names the organism *E. casseliflavus*. The earlier BIOLOG ™ release 3.5 identified the organism as *E. gallinarum*. Release 3.7 changed the criteria for identification *of E. gallinarum* and *E. casseliflavus* , when the BIOLOG ™ Bionumber produced by the BIOLOG ™ plate reader was entered into the computer, the new software identified the isolate BAW #1 as *E. casseliflavus*. The distance criteria roughly approximates the number of mismatches, the isolate BAW #1 values were close to 1 for anaerobic conditions, and closer to two for aerobic conditions. The similarity index greatly exceeded the acceptable reading of 0.5.

The API STREP test kit identified the organism as *E. casseliflavus*. The database also required further interrogation to produce accurate results. The API STREP kit identification as determined by code 5157551 named the organism *E. faecium* type 2 or 3, and considered the identification as "good to the species level". This identification was questioned because it conflicted with identifications derived from other systems. This questioning pinpointed the significance of the pigment of the isolate BAW #1 , and based on this, the API STREP identification was changed to *E. casseliflavus*. The API STREP package insert contains no reference to criteria for differentiating *E. faecium* from *E. casseliflavus*. *E. casseliflavus* and *E. saccharolyticus* are not mentioned on the package insert.

The API Strep system is lacking in its ability to differentiate members of the faecium group of enterococcus.

Fermentation product analysis produced results which fit the profile for a member of the genus *Enterococcus*, this test provided further evidence that isolate is not a member of the genus *Ruminococcus*. Anaerobically, in the nutrient rich rumen fluid based media GCSX-RF, the isolate BAW #1 produced 95% lactate and 5% ethanol. This is representative of the classic homofermentative metabolic pathway common to enterococci and streptococci (Garg and Mital 1991, Neijssel et al. 97). Grown in aerobic conditions, the isolate BAW #1 produced a broader complement of fermentation products: acetic acid, lactate and ethanol. Enterococci are known to produce these components under nutrient limited conditions. Streptococcal and enterococcal metabolic pathways continue to be the subject of research, it is known that the fermentation products of these genera are dependent on pH, pO_2, carbohydrate source and overall nutrient limitation (Neijssel et al 1997).

Analysis of the 16s rRNA sequence data support the identification of isolate BAW#1 as a member of the *E. casseliflavus-E. gallinarum* group.

This analysis is insufficient to differentiate these two species. The data clearly shows that the isolate is not a strain of *E. saccharolyticus*. This data supports the phenotypic identification of the organism as *E. casseliflavus*. The importance of a full set of data when conducting analysis using databases is underscored by this analysis.

The identification of the isolate as *E. casseliflavus* by API Strep, BIOLOG ™, and ARB database sequence analysis contradicted the identification of the isolate as *E saccharolyticus* by Lawes and as *Ruminococcus albus* by Tadese. The characterization of the isolate in this study was multidimensional, including three databases in addition to the traditional approach using Bergey's and phenotypic data. The identification of the isolate to the species level by Lawes as *E saccharolyticus* primarily rested upon the RDP analysis. However, the database used in that inquiry was strikingly incomplete. The RDP database only included six enterococcal species: *E. cecorum, E. faecalis, E. hirae, E. sulfureus, E. columbae* and *E. saccharolyticus*. None of these are of the *E. faecium* group of enterococci that the isolate clearly belongs to. However, despite this lack of depth of the initial analysis, the isolate BAW#1 is listed in GENBANK as E. saccharolyticus, and has been given the accession number ESU30931 (Entrez Nucleotide Query).

The organism is of a genus which is known to be pathogenic, usually only of serious concern in patients with a severely suppressed immune system. The *E. casseliflavus* strain tested resistant to five of twelve antibiotics tested. It is only moderately susceptible to two more of these. It tested sensitive to penicillin and ampicillin. In each case, the isolate BAW #1 tested as would be expected of a member of the genus *enterococcus*. The isolate BAW #1 does not appear to have picked up resistance to any of the antibiotics for which it was tested. The isolate BAW #1 was not tested for vancomycin resistance. As a strain of *E. casseliflavus,* the isolate probably carries the VanC2 gene characteristic of that species (Coque et al 1996).

Summary

A bacterial isolate BAW #1 has been examined using BIOLOG™, API Strep™ and conventional analysis. Examination and updating the databases of BIOLOG™ and API Strep™ produced a consensus that the isolate BAW #1 is a strain of *E. casseliflavus*. 1995 versions of the BIOLOG™ database named the isolate BAW #1 incorrectly. The API Strep™ diagnosis was also incorrect, until further tests were made and the company called for consultation.

Analysis of 16s rRNA sequence data supports the identification of isolate BAW#1 as a member of the *E. casseliflavus-E. gallinarum* group. The 16s rRNA data does not contain enough information to make a decisive identification at the species level. The data does not support the identification of the isolate as a member of the genus *Rumincoccus* or as *E. saccharolyticus*.

The development of these databases has created a simple means of identifying commonly encountered bacteria. However, they may be deceptively simple and produce false results. At one point during this investigation, the well reputed databases API Strep™, BIOLOG™ and RDP were identifying the isolate BAW #1 as *E. faecium*, *E. gallinarum* and *E. saccharolyticus*, respectively. Each had a limited database. Ironically, the simple testing of the color of a swab of the isolate BAW #1 could have greatly limited the search. However, useful phenotypic information has been gathered through the use of BIOLOG™ and API Strep™. Anaerobically, the major fermentation product of the isolate BAW #1 is lactic acid. Aerobically acetic acid, lactic acid and ethanol were produced.

CHAPTER TWO

CHARACTERIZATION OF AN ENTEROCOCCAL BACTERIOPHAGE

Phage Literature Review

MORPHOLOGY

The tailed phage include Siphoviridae, Myoviridae, and Podoviridae. While they show considerable difference in detail, they share important morphological features. The phage in these classes have single linear dsDNA contained in a capsid (Figure 6d). The capsid is built of coat protein molecules arranged in icosahedrally symmetric arrays. All have a single host attachment apparatus (a tail) attached to one corner of the capsid. The tails may vary in contractibility and length but all are thought to contain a threefold or sixfold rotational symmetry axis that projects through the center of the capsid shell of the virion (Casjens and Hendrix 1988).

The capsid of the tailed phages may be isometric or elongated. The elongated capsid of the Myoviridae T4 phage has been studied in great detail. The T4 capsid is thought to be constructed from hemispherical caps having an icosahedral surface lattice and tubular sides with a matching helical surface lattice (Harrison et al 1996). The chemical interactions between the subunits of the tube are likely to be similar to those of the cap; the surface lattice of the tubes is thought to be related to that of the icosahedral capsids. This theory is supported by the presence of tubular mutants of icosahedral viruses (Harrison et al 1996).

DNA passes in and out of the capsid through the portal vertex during packaging and ejection. In phages where it has been studied, the portal vertex is composed of a dodecamer of a single polypeptide arranged in a ring with a 3 to 4 nm hole through its center. The hole is aligned with the long axis of the tail so that it acts as a pore (Casjens and Hendrix 1988).

The tail is composed of helically arranged subunits. This structure is flexible, and of varying length based on strain. In lambda, it is composed of 32 rings (Casjens and Hendrix 1988). A “tape measure protein” controls tail length. At the distal end of the tail is a base plate, which is frequently of 6 or 12 fold symmetry. This structure is frequently not seen in electron micrographs; its absence is most likely due to its destruction during preparation of specimens (Tikhonenko 1972). In some phages, the base plate has enzymatic activity, including lysozymes. Tail fibers attached to the end of the tail are the most important structural subunit for attachment to the host. The distal portion of the fibers is an important site of mutations determining host specificity. Fibers are also sometimes seen coming off of the sides of the tail (Casjens and Hendrix 1988).

TAXONOMY

Early taxonomic efforts by Bradley (1967) divided the tailed phages on the basis of capsid and tail morphology, as well as type of nucleic acid. According to this scheme, all phages with noncontractile tails were designated Group B. (Bradley 1967). Anna Tichonenko (1968) devised a similar scheme in which the same phages are considered group IV. In 1974, Ackermann and Eisensark (1974) subdivided the non-contractile tailed phages based on their capsid shape. Isometric phages in this scheme are in group B1, phages with moderately elongated capsids are group B2, and phages with very long capsids (length to width ratio 2.7 to 5.5) are group B3.

In 1991 the International Committee on the Taxonomy of Viruses (ICTV) moved to establish a universal database, the ICTVdB

(International Committee on the Taxonomy of Viruses Database). Its goal is to describe all viruses of animals, plants, bacteria, fungi and archaea from the family level down to strains and isolates. The database uses the DELTA system of programs for organization of virus. A decimal code hierarchy, similar to the system used for enzyme nomenclature, has been established. Families have been sorted and each assigned a number. The system can contain many more levels to accommodate strains and isolates. The decimal code 50.1.1. represents family 50, subfamily 1, genus 1 (Murphy et al 1995). In the ICTVdB the Siphoviridae is the family which contains the dsDNA, noncontractile tailed bacteriophages. The best known phage in this family is lambda. The decimal code for the Siphoviridae is 66; lambda is in the genus 66.0.1 "lambda-like phage".

INCIDENCE OF SIPHOVIRIDAE ASSOCIATED WITH STREPTOCOCCUS AND ENTEROCOCCUS.

Ackermann (1996) has catalogued the descriptions of viable, negatively stained phages that have been included in periodicals, books and dissertations. Ninety-six percent of the over 4500 phages described are tailed phages. Of these 61.7% (2708) are Siphoviridae. Only 15% of the tailed phages have elongated capsids, of these 418 (B2) and 58 (B3) are Siphoviridae. Phage that can infect gram positive cocci account for 238 B2's and 8 B3's. Eight of the most elongated B3 bacteriophages are infective of the bacterial genus enterococci. The genus streptococci are listed as infected by 182 isometric B1 bacteriophages and no B2 or B3 bacteriophages.

Phylogenetic relationships of the tailed phages to each other in unknown. However, protein and gene sequence data is beginning to accumulate. Tailed phage genomes apparently consist of gene blocks or modules that may be exchanged to produce new phage species (Ackermann et al 1995).

Siphoviridae have been associated with *S. bovis*. Styriak et al (1991) isolated twenty strains of *S. bovis* from rumen contents. They tested filtered rumen fluid on the twenty strains and found two that produced plaques. The phage isolates were strain specific with the *S. bovis* isolates. Both phage strains had prolate capsids. Some strains previously identified as *S. bovis* are now classified as Enterococci (Chapter 1).

A phage infective of *S. faecalis* (now *E. faecalis*) was also described as having an elongated "bacillus shaped" capsid and appears to be divided into three volumes. The tail had cross striations and ended in a baseplate with six-fold symmetry. The phage was designated 10C1 (Anderson 1973).

Ackermann, Caprioli and Kasatiya (1975) describe an enterococcal phage VD13. This phage was found while investigating urogenital group D Streptococci. It has a prolate capsid and a flexible noncontractile tail terminated in a base plate. Capsids mostly appeared oval, flattened, and up to 65 nm wide. However, capsids deeply embedded in stain appeared to have hexagonal outlines. Tails showed cross-striations after staining with uranyl acetate (UA). Preparations contained spirals 10 nm wide, of variable length and with a 12-nm periodicity.

The phage VD13 was tested against a battery of enterococci for plaque production. It lysed 37 out of 146 strains of *S. (E.) faecalis, S. (E.) faecium, S. (E.) durans* and *S. liquifaciens.* It was inactive on 10 strains each of group A Streptococci, Bacillus, Staphylococcus, Micrococcus, Eschericia, Pseudomonas and Salmonella (Ackermann et al 1975).

The ICTVdB lists only five Siphoviridae infective of streptococcal species (A25, a25 PEI, A25 VD13, A25 omega8 and a25 24), and no phages infective of enterococci.

METHODOLOGY OF PREPARATION OF PHAGE FOR TEM

The preferred method for examination of phage is negative staining, followed by transmission electron microscopy. This method is quick and shows fine detail of phage ultrastructure. Several stains may be used depending on specific needs. The most common are uranyl acetate, and phosphotungstic acid.

Staining with UA may produce negative and positive staining of adjacent areas, negative staining is considered superior in providing useful information. The capsid shape and tail striations are most easily seen on phages negatively stained with UA. Positively stained capsids are blackened and shrunken, therefore size measurements of positively stained phages may be inaccurate. Positive staining with UA is known to produce shrinkage of from 11 to 31% of phage capsids (Ackermann 1987). Negative staining with UA may cause flattening and therefore an increase in the size of the phages (Hayat and Miller 1990).

Phosphotungstic acid (PTA) produces only negative staining. It is known for producing rounded and flattened capsids, and is not the best for showing tail striations (Hayat and Miller 1990). PTA is not capable of contrasting specimen details smaller than 1 nm. UA has an ionic size of 0.4nm to 0.5nm, PTA has an ionic size of 0.9, and because of this UA can demonstrate finer details (Hayat and Miller 1990).

An alternative preparative technique is shadow casting. The Kleinschmidt technique (a variation of shadow casting) and its derivatives utilize cytochrome C in ammonium acetate to form a monolayer (Kleinschmidt 1968). This monolayer facilitates spreading of nucleic acids or particulate samples such as phages. The phage solution is deposited on a coated grid and shadowed with a thin film of platinum. Staining with a dilute UA solution to enhance contrast may proceed the shadowing. Resolution with shadow casting is limited by the granular structure of the shadowing film. Platinum granules deposited from an electron beam under optimum conditions may measure between one and four nanometers. Specimen detail which is not twice the diameter of the grain must be interpreted carefully. Specimen dimensions may be exaggerated by the accumulation of shadowing materials, forming what is known as a cap (Willison et al 1980). Shadow casting techniques are superior to negative staining for showing up fine fibrous structures such as the fiber of bacteriophage tails (Bradley 1967).

Thin sectioning of plastic embedded phage samples is useful in the study of intracellular phage development. It is considered less prone to the flattening and emptying artifacts frequently associated with negative staining (Kellenberger et al 1995). Ultrathin sections are between 70 and 90 nm thick, or approximately twice as thick as a viral particle, thereby obscuring morphological details (Ackermann 1987).

Objectives:

1) To examine the structure of the phage isolated from the isolate BAW#1 with transmission electron microscopy.
2) To test the phage lysate for plaque production on Streptococcus bovis
3) To isolate the phage DNA harvested from an infection of the isolate BAW#1.

Materials and Methods

1. Purification of lysate

Methods developed for isolation and purification of DNA from *Eschericia coli* and lambda phage were utilized in the study of the isolate and its bacteriophage. These standard methods were modified to accommodate the anaerobic metabolism of the isolate. Glucose-Cellobiose-Starch - Rumen Fluid media and TSA were used in these experiments. GCS-RF medium is the standard for the cultivation of anaerobic rumen bacteria. (See Appendix I). The media are prepared as broth. Soft agar GCS-RF contained 0.7% agar, and hard agar GCS-RF contained 2.0% agar in this research. For aerobic phage work, Trypticase Soy Broth (TSB), Trypticase Soy Agar (TSA) and TSA soft agar were used.

Lysate was diluted and used to infect the isolate as follows. The lysate was used to prepare a dilution series in sterile dH_20. Broth culture (0.3-ml) with an OD_{600} of 0.25 was added to the lysate and incubated 30 minutes. The solution was then added to 3 mls of soft agar, vortexed and poured onto a GCS-RF plate. The plates were incubated overnight. A well-isolated plaque was picked using a sterile pasteur pipette and deposited into 1 ml of dH_20. One drop of chloroform was added. The phages were allowed to diffuse out of the agar for one hour, and centrifuged for 20 min. at 10,000g. The supernatant obtained was then filter sterilized through a 0.45um Gelman filter and held at 4 °C. This phage picking procedure was repeated three times to ensure purification (Sambrook et al 1989).

The phage was tested for plaque production under aerobic conditions using the soft agar overlay technique (Sambrook et al 1989). TSA Plates were used with TSA soft agar. TSB was used as the broth to prepare a log phase culture of the isolate. Plates were incubated at 39° C.

2. Phage tested for plaque production on *Streptococcus bovis*

S. bovis ATCC str 3317 91-08 was grown in TSB. The culture was tested for plaque production using the soft agar overlay technique. Media used were TSA (agar 2.0%) and soft agar (0.7 % agar) in TSB. Plates were incubated at 39 °C. A dilution series of the lysate collected from an infection of the isolate BAW#1 was used for this experiment. The most concentrated in the dilution series produced 10^{-7} plaques per ml when plated on the Isolate BAW#1using the soft agar overlay technique (Sambrook et al 1989).

3. Purification and restriction digest of phage DNA

The phage was plated, lysate collected, and phage DNA isolated using a Promega Lambda purification kit. Briefly, the protocol of the PROMEGA DNA isolation kit for Lambda was followed. (Promega Insert, Sambrook et al 1989)

Lysate preparation: The isolate was used instead of *E. coli* in the protocol. The soft agar overlay method using GCS-RF media was used to produce plaques. After overnight incubations (anaerobic at 39° C) plaques were nearly confluent. 2 to 3 ml dH_20 was overlaid on each plate. The top agarose was scraped off and transferred to a centrifuge tube to be incubated with intermittent shaking for 30 min. The tube was then centrifuged at 10,000g for 10 min. at 4°C. The supernatant was removed, 0.3% chloroform was added and the lysate was stored at 4°C.

DNA extraction: 40 ul Nuclease was added to 10-ml lysate and incubated for 45 min. at 37°C. 4 ml Phage Precipitant was added, the tube was gently mixed and placed on ice for 30 min. The tube was centrifuged at 10,000g for 10 min. The supernatant was decanted and discarded. The pellet was resuspended in 500 ul of Phage Buffer and 3 mg Proteinase K was added. The resuspended phage were transferred to a 1.5-ml eppendorf tube and centrifuged for 10 sec. at 12,000g. The supernatant was transferred to a fresh tube. One ml of Purification Resin was added to the tube and mixed by gently inverting the tube. The solution was pipetted into a syringe barrel. The resin/lysate mixture was pushed into the Promega Minicolumn, then washed with 2 ml of 80% isopropanol. The Minicolumn was transferred to a fresh eppendorf tube in a microcentrifuge, 100 ul of 80°C double distilled water was added and the column was then immediately centrifuged for 20 sec. at 12,000g. The eluted DNA in doubly distilled water was stored at 20° C till further use.

Restriction digest: Phage DNA was digested with HindIII and run on a gel with a Lambda HindIII standard. Sambrook et al 1989)

4. Transmission electron microscopy of the phage

Phage lysate produced from soft agar overlay plates was used to prepare samples. The phage was prepared for electron microscopy by two methods:

A) Negative Staining with Uranyl Acetate (UA)

B) Phage spread on a monolayer of Cytochrome C and shadow casted.

Negative Staining with Uranyl Acetate (UA)

The negative stain samples were processed as follows: A drop of lysate (@ 5 ul) was deposited on a formvar coated grid and allowed to stand for 1 min. Excess lysate was removed by draining from below with filter paper. A 5-ul drop of 2% UA was deposited on the grid, allowed to stand for one minute, and drained. Grids were examined with the Philips CM 10 or JEOL 100CX II and micrographs recorded.

Phage spread on a monolayer of Cytochrome C and shadow casted.

The shadow cast samples were processed as follows: 40 ul 0.3M ammonium acetate was deposited on a sheet of parafilm. A 10-ul drop of 1 mg/ml 0.03% Cytochrome C and 20-ul lysate was added to the drop.

The drop was allowed to stand covered for 10 minutes. Coated grids were then touched to the drop, held for 30 seconds, held to a drop of 95% ethanol for 30 seconds and deposited face down on a filter paper. Grids were then removed from the filter paper and placed in the modified Balzer 510A freeze etch device. Grids were then shadow casted with 250 Hz of platinum evaporated in the electron beam gun. Platinum deposition was monitored with a Quartz Crystal Monitor, and controlled with a Control Unit EVM052. The angle of platinum deposition was 7 degrees. A TSR1000b rotary unit controlled the rotary stage. The stage was rotated at 80 rpm during platinum and carbon deposition. A thin layer of carbon was evaporated onto the specimens immediately after platinum deposition. Grids were examined with the Philips CM 10 and micrographs recorded.

For the second Kleinschmidt shadowing experiment, the grids were touched to a drop of 0.5% UA immediately after the grid was picked up from the sample drop. The samples were washed as above in ethanol, dried, and mounted on the stage. The samples were again shadowed with platinum at 7 degrees. A thin layer of carbon was evaporated onto the specimens immediately after platinum deposition, with the stage rotating at 80 rpm. Grids were examined with the Philips CM 10 and micrographs recorded.

Bacteriophage measurement: Prior to photographing the phage for measurement, the Philips CM10 was z-axis centered and aligned. Micrographs were taken of tobacco mosaic virus (TMV), known to have a width of 18 nm. The magnification of the phage micrographs was adjusted based on the TMV measurements.

Micrographs were digitized using a Kodak MEGAPLUS Camera Control Unit Model 1.4 and Kodak MEGAPLUS Camera, Model 1.4. The digitized images were imported into Photoshop 3.5 on a PowerMac 7100. On screen magnification was increased at least five fold, and the "get info" tool used to measure the size of the phages and TMV. Using this methodology twenty phages were measured and sized averaged. Phage with obvious malformations were not used in the determinations of size. The size of the phages were determined by the formula:

L_o = Length of object in nanometers
M = magnification of micrograph after calibration
S = Size in nanometers
and $L_o / M = S$

Periodicity of the unwound tail was determined by counting 16 spirals on the computer screen, as above.

5. Phage infection of isolate BAW#1 followed by electron microscopy.

The phage and isolate BAW#1 were prepared for electron microscopic examination as follows: 100 ul of phage lysate at a concentration of 10^{-7} pfu/ml was added to 1 milliliter of a log phase culture of isolate BAW#1. TSB was used as liquid media. The multiplicity of infection was assumed to be approximately 1 phage per 100 colony-forming units. The culture was sampled for electron microscopy by withdrawal of 2 ul at various time points. The 2-ul aliquot was deposited on a coated grid and allowed to stand for one minute. A 2-ul drop of dH_2O was added to the grid and removed from the side with filter paper after 15 seconds. A drop of uranyl acetate was immediately added to the grid and allowed to stain the phage/ enterococcal mixture on the grid for 15 seconds. The stain was removed from the side with a filter paper and the grid allowed to dry. Grids were examined in a Philips CM10 transmission electron microscope. Grids were scanned at low magnification (about 5000X) for enterococcal chains and at a higher magnification of 25000X for phage. The time points sampled were: prior to addition of phages, 15 minutes after addition of phages, 2 hours after addition of phages, 3 hours after addition of phages, 4 hours after addition of phages, 4.5 hours after addition of phages and 5 hours after addition of phages

Results

1. Purification of lysate and testing of the phage under aerobic conditions.

Methods developed for lambda phage and *Eschericia coli* were useful in the study of the isolate and its bacteriophage. Picking of a single plaque produced about 100,000 plaque-producing particles. When grown aerobically at 39° C, the phage was capable of producing plaques on a lawn of the isolate on TSA in less than five hours.

2. Phage tested for plaque production on *Streptococcus bovis*

The phage did not produce plaques on *S. bovis* ATCC str 3317 91-08 when grown on TSA at 39°C or 35°C. Examination of the plates with a 10X ocular revealed no microscopic plaques.

3. Purification and restriction digest of phage DNA

The Promega kit designed for lambda phage DNA extraction was used to extract DNA from the isolate BAW#1 bacteriophage. This DNA was sufficiently pure for restriction digestion (Figure 4). The DNA was cut with HindIII, producing a gel with nine clear bands. The size of the bands was determined by comparing them to a lambda DNA HindIII digest standard. The bands lengths were 5950, 4000, 3800, 2800, 2100, 1650, 1300, 1050, and 680bp. The total length of phage DNA based on this is 23330bp.

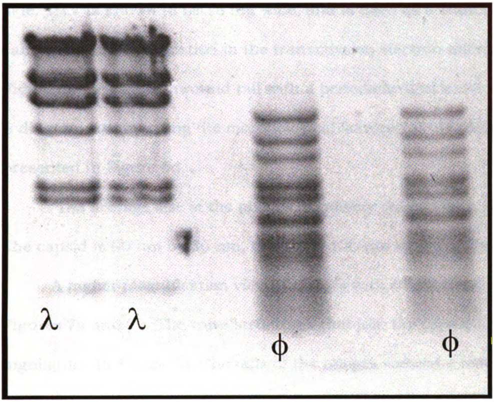
4. Transmission electron microscopy of the phage prepared for electron microscopy.

Negative Staining with Uranyl Acetate (UA)

Staining with UA allowed visualization of fine structures of the phages (Figure 6). Figures 6b, 6n, and 6o show negatively stained phages with visible baseplates. Ridges were visible on the edges of the phage capsid (Figure 6o) and in some micrographs, the top of the capsid was angular (Figure 6b). Note that the stain was pooled at the edges of the phage, and only lightly stained the phage itself. In contrast, positive staining of the phage capsid appeared dark. This demonstrates the affinity of UA for DNA (6a, 6g, 6m, and 6i). The positively stained capsids appeared angular, and have maintained their shape. Occasionally phages were seen with an intermediate staining reaction (Figures 6e and 6h). Two tail fibers and a base plate were visible on Figure 6i; this positively stained phage had an elongated head.

Phage stained with UA were seen with capsids that were empty headed and swollen. (Figures 6f and 6k), and with a visible valve structure. A longitudinal ridge was sometimes visible in the tails of

Figure 5. Bacteriophage DNA extracted from the isolate BAW#1 and cut with the restriction endonuclease HindIII (ϕ) The DNA of the phage lambda is used as a standard for size determination (λ).



phages with the empty capsids. Some phages appeared to have lost their DNA (Figure 6l).

Figure 6c shows a phage and tobacco mosaic virus (TMV) side by side. TMV is known to be 18 nm wide, and is used as a standard for calibration of magnification in the transmission electron microscope. Figure 6j shows an unwound tail with a periodicity and width of 10 nm. A diagram summarizing the morphological features of the phage is presented in Figure 6d.

The average size of the phages negatively stained with UA was: The capsid is 90 nm by 36 nm, the tail is 136 nm by 9 nm (Table 13).

A higher magnification view of phages with empty heads is seen in Figures 7a and 7b. The valve structures that join the capsid to the tail is highlighted in Figure 7a. The tails of the phages showed a longitudinal ridge. The perimeters of the phage capsid were balloon like and visible around a hollow core. A group of phages in Figure 7b shows one phage positioned approximately perpendicular to the image plane (see double arrow). Another phage in the group shows the empty head and valve structure (see single fat arrow). The tails appeared to be joined together in a knot (long arrow). The six pointed tailed structure in Figure 7c may be a phage capsid perpendicular to the image plane.

Table 13. Measurements of the phage infective of isolate BAW#1. Phage stained with uranyl acetate and examined in Philips CM10 Transmission Electron Microscope. All measurements are in nanometers.

	Capsid length	Capsid Width	Tail Width	Tail Length	Sides	Height/ Width
	86.6	38.5	9.6	125.0	62.5	2.3
	91.6	38.8	7.4	133.7	70.9	2.4
	93.3	38.5	11.4	134.3		2.4
	91.0	38.3	8.0	140.4	73.6	2.4
	86.3	35.3				2.5
	90.5	36.2	8.7	140.4	76.9	2.5
	88.3	35.4	8.7	140.4	66.9	2.5
	91.7	36.9				2.5
	86.8	34.7				2.5
	89.5	35.8				2.5
	88.2	34.7	8.7	134.3		2.5
	92.2	36.4				2.5
	91.7	35.8				2.6
	90.6	35.3				2.6
	90.9	35.4	8.7	140.4	76.2	2.6
	89.5	34.2	9.5	134.0		2.6
	89.5	34.2	9.5	137.0		2.6
	93.3	35.5				2.6
	88.2	33.1	8.7	135.7		2.7
	91.4	32.8				2.8
Mean	90.06	35.79	135.97	8.98		2.52
Standard Deviation	2.12	1.72	4.63	1.04		0.11
Minimum	86.3	32.8	125.0	7.4		2.25
Maximum	93.3	38.79	140.4	11.4		2.78
Count	20	20	11	11		20
Confidence Level	1.00	.8	3.11	.70		.05

Phage spread on a monolayer of Cytochrome C and shadow casted.

Micrographs of phages spread with cytochrome C and shadowed with platinum (the Kleinschmidt techniques) are shown in the Figure 8a through 8f. Micrographs of phages stained with UA prior to shadowing are shown in Figures 8a, 8d, and 8e. Angularity of the phage capsid was apparent, the angularity of the vertices of the phage was particularly evident in some cases (Figure 8a.) The apical vertex was 115 degrees. The base plate of the phage in these lightly shadowed micrographs was visible, and a tail fiber is seen extending from the thickened distal end of the tail to what appears to be a membranous particle (Figure 8d).

Phage spread with cytochrome C, unstained, and shadowed with platinum were also examined (Figures 8b, 8c, and 7f). The thicker platinum shadow produced a different image than in the more lightly shadowed preparations (Figures 8a, 8d and 8e). Phage capsids and tails appeared thicker. Tail fiber were visible (Figure 8b) and an unusual triangular thickening at the distal end of the tail was noted (Figure 8c).

The phages prepared by the shadow casting technique did not show valve structures or the longitudinal ridge of the tail. The end plate of the tail was exaggerated and appeared quite thick (Figures 8c and 8b). The image of the phage is longer and wider (120 nm by 57 nm) than other preparation methods. The tails in these micrographs were not longer, however they were wider. Background surface of the micrographs was irregular and splotchy in appearance. Phage in these photos appeared to lack angularity and definition.

Micrograph 8f shows a group of phage. The phage in the upper and lower right hand corners appeared to have a cap of platinum grain around a central core. The measurements of this central core (capsid: 100 nm by 33 nm) were in agreement with the size of the phage determined by the UA staining method.

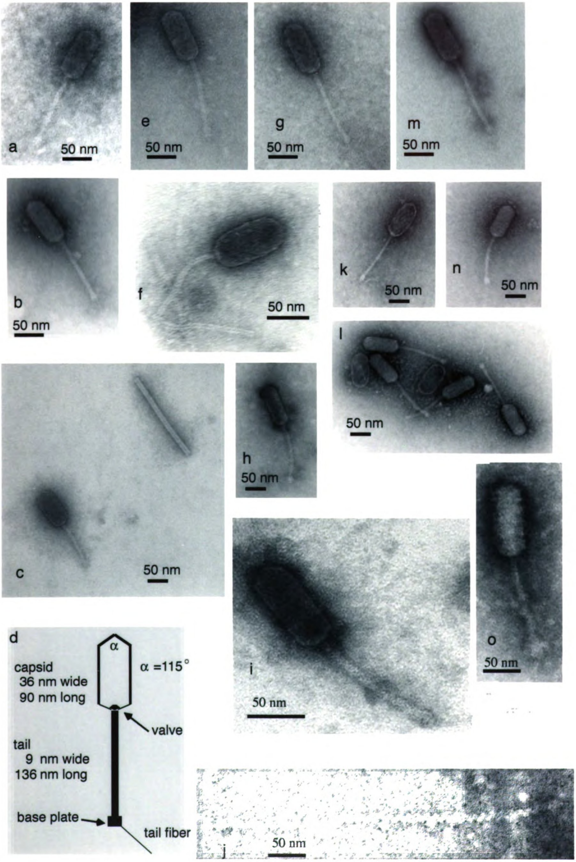
5. Phage infection of the isolate BAW#*1* followed by electron microscopy.

Micrographs were recorded of the isolate BAW#1 prior to infection with the phage. Isolate BAW#1 grows in chains, repeatedly dividing longitudinally to produce the characteristic chains. (Figures 9a and 9b). The separation of the chains was occasionally noted (Figure 9e). The size of individual cocci was approximately 0.9um by 1.1um. Most chains appeared elongated, with the maximum chain length over 20 cocci.

The phage was very difficult to find in samples taken during the first 3 hours of the experiment. After 4 hours, the phage was occasionally seen, but not in high concentrations. At 5 hours, the phage was seen near most enterococcal chains. The chains frequently appeared to be lined with phage (Figure 8b). In this image, the phage were noted externally all over the four cocci enterococcus. The enterococcal chains were examined to determine if certain positions within the chain were more frequently attacked, however, no differences were noted. The individual infected cocci were examined to determine if the phage was attacking a certain part of the cocci (i.e. new membrane near the septum or older membrane at the polar regions), again, no differences were noted. Phages were seen that appeared to attach to the outside of the bacterium (Figures 9f and 9c). An unwound tail was observed close to a bacterium under multiple attack, (Figure 9c). At five hours, bacteria were found which appeared to have burst, (Figure 9g) and in one image several phages were attached to the debris.

Figure 6. Phage harvested from the isolate BAW#1 and stained with uranyl acetate prior to examination in the transmission electron microscope. The phages in these micrographs show positive and negative staining reactions. Micrographs are lettered from top left to bottom right.

a) Positive staining of the phage capsid due to the affinity of UA for DNA. The positively stained capsid is angular.
b) Negatively stained phage with a visible baseplate. The top of the capsid is angular. Note that the stain is pooled at the edges of the phage, and only lightly stains the phage itself.
c) Phage and tobacco mosaic virus (TMV) side by side. TMV is known to be 18 nm wide, and is used as a standard for calibration of magnification in the transmission electron microscope.
d) A diagram summarizing the morphological features of the phage is presented.
e) A phage with an intermediate staining reaction
f) Phages stained with UA appears empty headed and swollen, the valve structure is visible. A longitudinal ridge is visible in the tails of these phages with empty capsids.
g) Positive staining of the phage capsid due to the affinity of UA for DNA. The positively stained capsid is angular.
h) A phage with an intermediate staining reaction.
i) A negatively stained phage with two tail fibers.
j) An unwound tail. The periodicity and width is 10 nm,
k) *A* phage with an empty and swollen head.
l) Several phages are visible in this micrograph. Note that two capsids are empty and swollen, the others are negatively stained.
m) Positive staining of the phage capsid due to the affinity of UA for DNA. The positively stained capsid is angular.
n) A negatively stained phage.
o) A negatively stained phage with ridges visible along the sides of the capsid.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c
50 nm
h
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i
50 nm
o
50 nm
d
α
capsid
36 nm wide
90 nm long
α =115°
valve
tail
9 nm wide
136 nm long
base plate
tail fiber
j
50 nm

Figure 7. Phages harvested from the isolate BAW#1and stained with uranyl acetate prior to examination in the transmission electron microscope. Micrographs are lettered from top left to bottom right.

a) Phages have tangled tails. In the phage with an empty capsid (see arrow), the valve is visible joining the capsid to the tail. The tail of this phage is thickened and has a lengthwise ridge. The other phages have capsids full of DNA, the capsids are angular.

b) Phages with tangled tails (see long arrow). Three phages have full capsids, one has an empty capsid with a visible valve (see medium arrow). Small arrows point to what may be a top view of another phage.

c) A long fine structured tail protrudes from what may be a phage perpendicular to the plane of view.

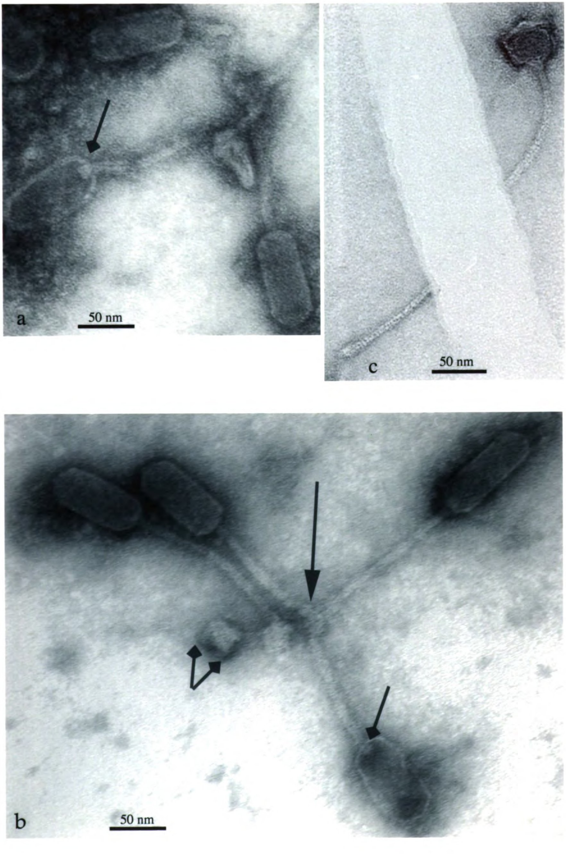
a
50 nm
c
50 nm
b
50 nm

Figure 8. Phages harvested from the isolate BAW#1and shadow cast with platinum prior to examination in the transmission electron microscope. The phages in Figures a, d, and e were lightly stained with uranyl acetate prior to shadowing, The phages in Figures b, c, and f were shadow cast with an excessive deposition of platinum. Micrographs are lettered from top left to bottom right.

a) Edges of the phage are clearly visible and have been measured. The top apex of the phage has an angle of 115 degrees.

b) Heavy shadow on phage, tail width approaches 25 nm, tail fiber is visible.

c) Phage is very heavily shadowed, it appears round and fat. The tail appears to end in an angular structure not characteristic of tail fiber or baseplate.

d) Phage with tail fiber attached to membranous particle (see arrow). Phage is evenly and lightly shadowed, tail baseplate is visible, capsid is angular and full.

e) Phage capsid shows significant staining due to affinity of DNA for uranyl acetate.

f) A large group of phages is joined together in a snarl of their tails

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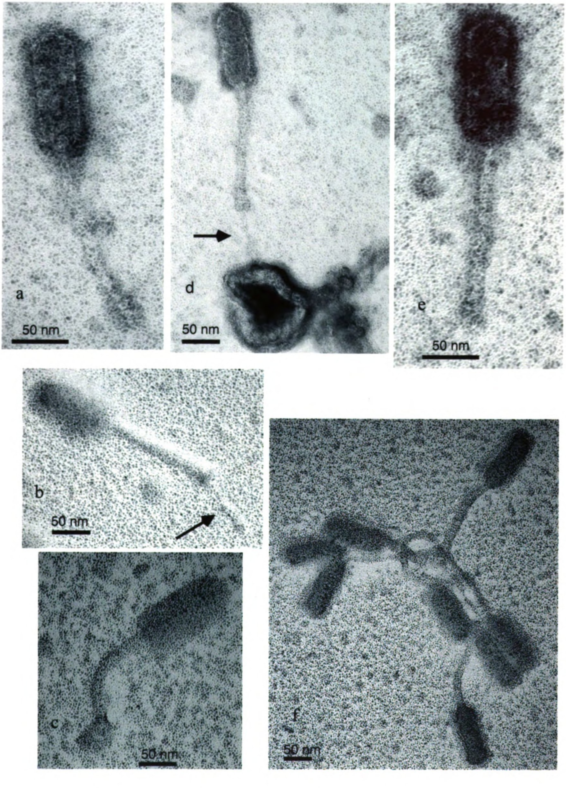


Figure 9. The micrographs show the bacterium prior to, during and after attack by the bacteriophage. All grids were stained with UA for 15 seconds. Micrographs are lettered from top left to bottom right.

a) Isolate BAW#*1* prior to infection with the phage. The arrows point to developing septa in dividing cells.

b) A large number of phages (see short arrows) are attached to this chain of four.

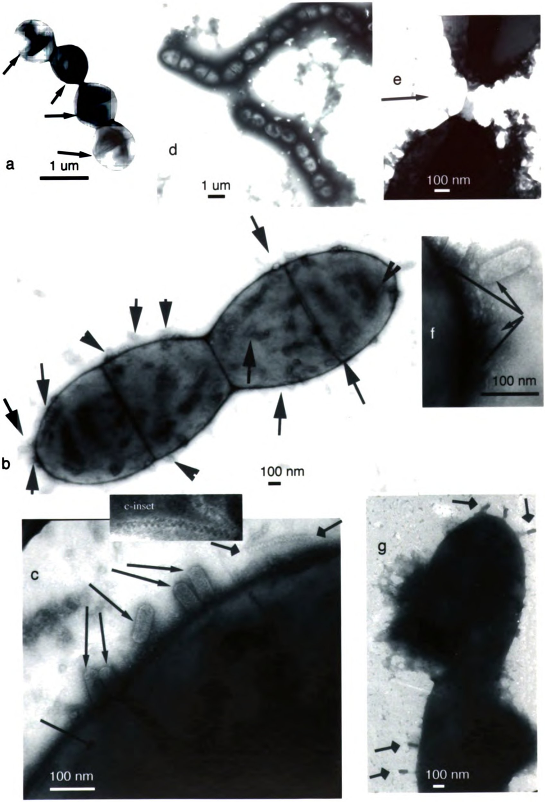
c) High magnification allows visualization of the phage ultrastructure on the surface of isolate BAW#1. Short arrows point to an unwound tail. The inset magnification is 286,000,and contrast has been inverted.

d) Two long chains of isolate BAW#1 show the usual elongation of cocci.

e) Two cocci separating.

f) High magnification shows the surface of isolate BAW#1 densely populated by phages.

g) A lysed isolate BAW#1. Arrows point to several phages attached to the bacterial debris.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b
100 nm
f
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c-inset
c
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g
100 nm

Discussion

Purification of the phage.

The phage was successfully purified after infection of the isolate BAW#1. A single plaque produced about one hundred thousand plaque forming units. Inoculation with phage lysate always produced plaques on a lawn of the isolate BAW#1 isolate. Colonies appearing within the margins of plaques were picked. These phage resistant strains were spotted on to a lawn of the isolate BAW#1 isolate, however, no plaques were produced. No evidence was found of lysogeny. The phage demonstrated insignificant reduction in plaque forming ability after treatment with 0.3% chloroform. The most rapid plaque production recorded was a log phase isolate BAW#1 culture inoculated into TSA supplemented with glucose, in this case, plaques were visible after 5 hours.

The restriction digest of the isolated phage nucleic acid demonstrated that the phage was a double stranded DNA phage. This is to be expected given the morphology of the phage. The length of the phage DNA by this analysis was 23330 bp, short in comparison with the tailed phage lambda.

The phage did not produce plaques on *S. bovis* ATCC str. 3317 91-08. This is not a surprise, considering that isolate BAW#1 and *S.*

Bovis are not in the same genus, and phage which are capable of infecting more than one species are rare (Ackermann 1987).

Electron microscopic examination of the phage.

The examination of the phage with the TEM showed the following features. The capsid was prolate, the tail was non-contractile. An endplate was visible on the tail in several micrographs (Figures 5I, 5k, 5l, 5n, 5o 7b, 7c and 7d). A fiber was seen extruding from the tip of the tail (Figures 5I, 7b and 7d). The shadow cast micrographs, while obscuring fine structure of the capsid, most clearly demonstrated the tail fiber. The tail fiber of the phage was visible in micrographs prepared with the Kleinschmidt technique and where the platinum shadow was light. The fiber was only seen once in a non-shadowed micrograph.

Frequently, phages were noted in groups that appear to be connected (Figures 6a, 6b and 7f). The phages in these groups may have been attacking a common bacterial receptor site, or this may be an artifact of specimen preparation.

Negative staining is a very useful method for providing detail of the capsid and valve structure, tail structure and baseplate of the phage. The tail fiber was only seen once using this technique. Phages which were negatively stained may have undergone osmotic shock, resulting in the loss of the capsid content, DNA. Phages with empty capsids were clearly misshapen and were not used in data collection for size determination. These shocked phages did, however, provide key information on the portal valve and tail structure.

Determination of the size of the phage after negative staining requires close attention to the osmotic state of the phage being examined. Negatively stained phage capsids appeared angular. The area surrounding the phages was more electron dense than the capsid or tail. These phages were used in size determination. Positively stained phages appeared angular, with the capsid core darkened. Several of the phages with positively stained capsids measured larger than the negatively stained phages, data from these phages were not used for size determination. Phages in all three forms, positively stained, negatively stained and osmotically shocked appeared on the same grid. Differences may have been due to differential concentrations of stain versus buffer or partial drying of grids.

Shadow casting of the phage produced variable results. The first of the variations was the apparent thickness of the shadow itself. Two runs of the shadow cast produced two distinctly different results. One of the runs laid a very heavy shadow on the specimen and support structure, the shadow here appears piled on itself and knobby (Figures 7b, 7c and 7f). In Figure 7f, the heavy coat of platinum created a cap on either side of two of the phages. The phage capsid measured 30 nm wider than in other preparation techniques. The fine structure of the phage was buried under a drift of platinum shadow. Contrast in these images was due solely to the platinum.

On the second shadow cast run, the phages were stained with uranyl acetate and rinsed in ethanol. The shadow layer was much lighter and finer, the outlines of the phage capsid were clearly visible. The uranyl acetate added contrast to the image. Measurements of the phages in this treatment were close to the measurement of negatively stained phages.

In the Balzer freeze fracture apparatus deposition of platinum was by electron beam gun, which can short out and frequently needs to be reset. This may have been one factor that may have caused a differing total deposition. Exact placement of the specimen within the gun was also a variable, as was the condition of the electron beam gun itself. It was also possible that the pre-shadowing UA treatment of the grids in the second run altered the dynamics of the shadowing process in an unknown manner.

The second anomaly of the shadow casting was that the rotary shadow casting of both runs produced some phages that appeared to be unidirectionally shadowed. Figure 8b is from the first, heavily shadowed run, and the phage appears to be unidirectionally shadowed. Phages in figures 8c and 8f are from the same run and appeared rotary shadowed.

This phenomenon was also seen on the second shadow cast (not shown). Because the angle of platinum shadow deposition was only seven degrees, a minor bend in a grid or its support structure on the rotary stage may have blocked the platinum from one direction.

The repetition of the shadow cast provided an important reality check for the size of the phage. The lightly shadowed phages resembled the phages stained with UA in size and shape. The heavily shadowed phage preparations provided insight into the tail fiber and base plate, for these structures, exaggeration of size provides useful information. The Kleinschmidt technique of spreading macromolecules with cytochrome C and subsequent shadow casting is frequently used to illuminate DNA structure. The DNA strand itself was only 2nm wide, it appeared about 20 nm wide after the Kleinschmidt process. It is likely that the tail fiber of the phages underwent a similar size enhancement in these experiments.

Summary

A phage infective of the isolate BAW#1 was examined by transmission electron microscopy (TEM). The phage has a long non-contractile tail and an elongated capsid. The valve joining the capsid to the tail was visible in preparations where the phage had lost its DNA. Preparation of the phage for TEM using shadowcasting and negative staining provided information on the morphology of tail fibers. The phage capsid is 90 nm by 36 nm, the tail is 136 nm by 9 nm. The phage did not look like the phage described by Tadese, however, it did have a similar morphology to one of the phage reported by Lawes. The phage has double stranded DNA. It is a member of the Siphoviridae family of phage, a B2 in Bradley's scheme (Bradely 1967).

Isolate BAW#1 grown in TSB showed a heavy phage infection four to five hours after addition of phage as demonstrated by plaque production and examination of infected cultures at high magnification. No evidence was found of lysogeny, the phage readily produces plaques on a lawn of the host.

The phage did not produce plaques on a lawn of S. *bovis* ATCC str 3317 91-08 grown on Trypticase Soy Agar.

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Appendix I

AEROBIC MEDIA

ATCC MEDIA 260: TRYPTICASE SOY AGAR (BBL 11043) WITH 5% DEFIBRINATED SHEEP BLOOD

Pancreatic digest of casein	15.0 g
Agar	15.0 g
Papaic digest of soybean meal	5.0 g
NaCl	5.0 g
Sheep Blood defibrinated	50.0 ml
dH_20	950.0 ml

pH: 7.3+/- 0.2 at 25C, makes 1000ml.

TRYPTICASE SOY BROTH WITH 5% DEFIBRINATED SHEEPS BLOOD

Bacto Tryptone	3.75 g
Bacto Soytone	1.25 g
NaCl	1.25 g
dH20	225 ml
Defibrinated sheep blood	25 ml

pH: 7.3+/- 0.2 at 25C, makes 250 ml

ANTIBIOTIC MEDIA (DIFCO) is 26.5 g/L

Bacto Beef Extract	1.5 g
Bacto-Yeast Extract	3.0 g
Bacto- Peptone	6.0 g
Bacto-Dextrose	1.0 g
Bacto Agar	15.0 g

MOTILITY SULFIDE MEDIA 104.4 g/L

Bacto beef extract	3.0 g
Protease peptone No 3	10.0 g
L-cystine	0.2 g
Ferric Ammonium Citrate	0.2 g
Sodium Citrate	2.0 g
Sodium Chloride	5.0 g
Bacto gelatin	80.0 g
Bacto agar	4.0 g

KF STREPTOCOCCUS AGAR 76.05 g/L

Bacto-Protease Peptone No 3	10.0 g
Bacto- Yeast Extract	10.0 g
Sodium Chloride	5.0 g
Sodium Glycerophosphate	10.0 g
Maltose	20.0 g
Lactose	1.0 g
Sodium Azide	0.04 g
Bacto Brom Cresol Purple	0.015 g
Bacto-agar	20.0 g

m- Enterococcus Agar 41.5 g/L

Bacto- Tryptone	20.0 g
Bacto-yeast extract	5.0 g
Bacto dextrose	2.0 g
Dipotassium Phosphate	4.0 g
Sodium Azide	0.4 g
Bacto Agar	10.0 g
2,3,5, Triphenyl Tetrazolium Chloride	0.1 g

ANAEROBIC MEDIA

RUMEN FLUID

Rumen fluid is passed through two layers of cheesecloth into large flasks. After overnight incubation at 39C, fluid is centrifuged at 15000rpm for 30 min. The supernatant is then autoclaved at 15psi for 20 min. After cooling the bottles are stores at 4C. Immediately prior to use in media, the fluid is re-centrifuged for 30 minutes at 15000rpm.

SODIUM CARBONATE SOLUTION 8%

Na_2CO_3	24.0 g
dH_20	300 ml

Boil water under CO_2 to push O_2 out. Add Na_2 CO_3 and mix thoroughly. Gas with CO_2 for 15 min. Tube up under CO_2, and clamp in press. Autoclave at 248°C, 15psi for 20min.

MINERAL 1 SOLUTION

K_2HPO_4	6.0 g
dH_20	1000 mls

Place solution in 1.5 liter bottle. Autoclave at 248°C, 15psi for 20min. When solution is cool, tighten lid and store at 4C.

MINERAL 2 SOLUTION

KH_2PO_4	6.0 g
$(NH_4)_2SO_4$	6.0 g
NaCl	12.0 g
$MgSO_4{:}7H_20$	2.45 g
$CaCl_2{*}2H_20$	1.59 g
dH_20	to 1000 mls

Place solution in 1.5 liter bottle. Autoclave at 248°C, 15psi for 20min. When solution is cool, tighten lid and store at 4C.

BRANCHED CHAIN FATTY ACID SOLUTION

Isobutyric acid	30 ul
Isovaleric acid	30 ul
2-methylbutyric acid	30 ul
n-valeric	30 ul

Branched Chain Fatty Acids are filter sterilized through a 0.22um sterile Acrodisk and stored at 4C.

CYSTEINE SULFIDE SOLUTION (2.5%) REDUCING AGENT

cysteine HCl	7.5 g
Na_2S*9H_2O	5.0 g
dH_20	100 ml
10 N NaOH	to pH 10.0
dH_20	to 300 mls

Weigh out and dissolve cysteine-HCL and Na_2S*9H_20 in small amount of dH_20. Adjust pH to 10.0 with 10 NaOH. Boil under $C0_2$ and wire down in a boiling flask. Autoclave at 248°C, 15psi for 20min. Pipette under $C0_2$ and incubate for 24 H to check sterility.

ANAEROBIC DILUTION MEDIA

Mineral solution 1	11.2 ml
Mineral solution 2	11.2 ml
Resazurin	0.3 ml
dH_20	256.3 ml
Cysteine Sulfide	6.0 ml
Sodium Carbonate	15.0 ml

Combine first 4 components, adjust pH to 6.7-6.8 range. Boil under CO_2 and wire down in a boiling flask. Autoclave at 248°C 15psi for 20min. When solution has cooled add sodium carbonate and cysteine sulfide solutions. Pipette under CO_2 and incubate for 24 H to check sterility.

GCS-RF BROTH, SOFT AND HARD AGAR

Glucose	0.2 g
Cellobiose	0.2 g
Starch	0.2 g
Yeast Extract	0.6 g
Trypticase	1.5 g
Rumen Fluid	90 ml
Mineral 1	11.2 ml
Mineral 2	11.2 ml
Resazurin	0.3 ml
dH_20	163.6ml
HCl to adjust pH	as necessary
Cysteine Sulfide	6.0 ml
Sodium Carbonate	15.0 ml
Agar: for soft agar	2.05 g
Agar: for plates and slants	6.0 g
Net Volume to:	300 mls

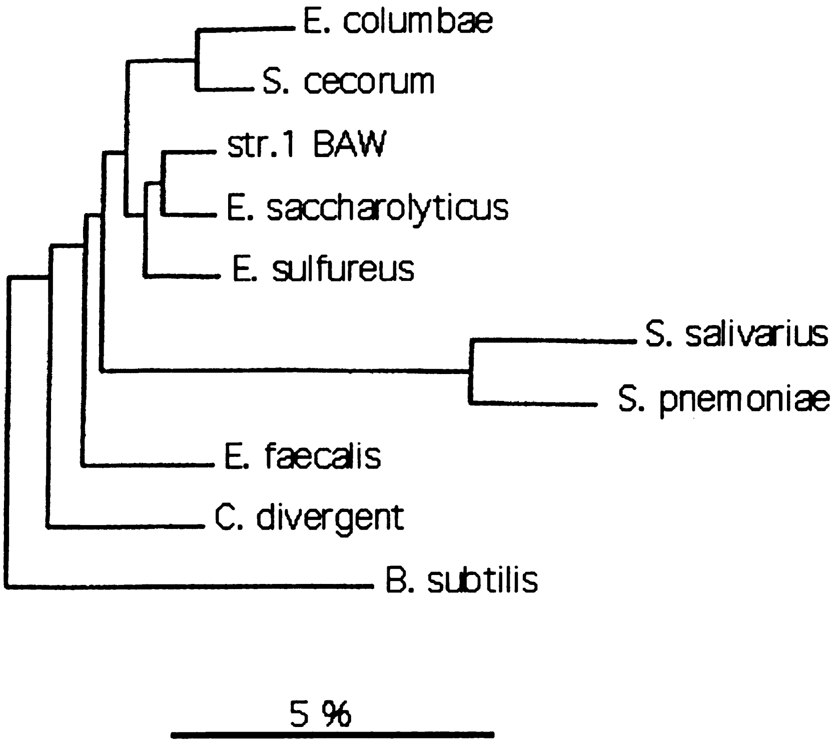
- GCS-RF BROTH: Weigh out and dissolve solids in small amount of H_2O. Add rumen fluid and resazurin. Adjust pH to 6.7 to 6.8 range with HCl. Boil under CO_2 and wire down in a boiling flask and autoclave.
- GCS-RF Soft Agar: Soft agar is 0.65% agar added to pH adjusted solution prior to boiling down. Cysteine sulfide and sodium carbonate are added to solution at the end of the boiling down process. 2.5 ml tubes are then prepared under CO_2, and put into a press to autoclave.
- GCS-RF Hard Agar: For plates add 2.0% agar to pH adjusted solution. Boil under CO_2 and wire down in a boiling flask and autoclave.
- GSCX-RF: As GCS-RF Hard Agar, add 15ml glycerol (5%) as substitute for 15ml of water.

  All GCS-RF formulations are autoclaved at 248°C 15psi for 20 min. All component addition and pipetting is conducted under CO_2. All media is incubated for 24 H to insure sterility.

Appendix II

Dendrogram showing phylogenetic relationship of some of the most similar organisms to isolate BAW#1 (str. 1 BAW) based on 16s rRNA sequence comparison. From Angela Lawes 1993.

This tree was built with the sequences from the following enterococcal species: *E. columbae, E. saccharolyticus, E. sulfureus, E. faecium, E. cecorum and E. hirae.* Note that the RDP did not contain *E. casseliflavus, E. gallinarum, E. mundtii, E. durans, E. malodoratus, E. pseudoavium, E. raffinosus* or *E. faecium.*



Appendix III

Alignment of 16s rRNA sequence of isolate BAW#1 with closely related sequences of *E. casseliflavus and E. gallinarum* strains.

```
Name: EnrGalli NDCO 2313          Len:  1554  Check: D6735E1F
Name: IslBAW#1                    Len:  1554  Check: ECB6E486
Name: EnrCasse NCDO 2376          Len:  1554  Check: 3B4AE986
Name: AF039898 E. gall            Len:  1554  Check: 1E04D5C6
Name: AF039899 E. cass            Len:  1554  Check: 8ED8DD06
Name: AF039900 E. gall            Len:  1554  Check: 82BC850A
Name: AF039903 E. cass            Len:  1554  Check: D34FC5E0

      1          11         21         31         41       50
      |          |          |          |          |        |
Enr   UUAUGAGAGU UUNAUCCUGG CUCAGGACGA ACGCUGGCGG CGUGCCUAAU    50 Enr
Isl   ...U....GU UUGAUCCUGG CUCAGGACGA ACGCUGGCGG CGUGCCUAAU    43 Isl
Enr   UUAUGAGAGU UUNAUCCUGG CUCAGGACGA ACGCUGGCGG CGUGCCUNAU    50 Enr
AF0   .......... .......... ........ga acgctggcgg cgtgcctaat    22 AF0
AF0   .......... .......... ....ggacga acgctggcgg cgtgcctaat    26 AF0
AF0   .......... .......... .......... .......... ......taat     4 AF0
AF0   .......... .......... .....gacga acgctggcgg cgtgcctaat    25 AF0

      51         61         71         81         91       100
      |          |          |          |          |        |
Enr   ACAUGCAAGU CGAACGCUNN UUCUNUCACC GGAGCUNGCU CCACCGAAAG   100 Enr
Isl   ACAUGCAAGU CGAACGCUUU UUCUUUCACC GGAGCUUGCU CCACCGAAAG    93 Isl
Enr   ACAUGCAAGU CGAACGCUNN NNCUNUCACC GGAGCUNGCU CCACCGAAAG   100 Enr
AF0   acatgcaagt cgaacgcttt ttctttcacc ggagcttgct ccaccgaaag    72 AF0
AF0   acatgcaagt cgaacgcttt ttctttcacc ggagcttgct ccaccgaaag    76 AF0
AF0   acatgcaagt cgaacgcttt ttctttcacc ggagcttgct ccaccgaaag    54 AF0
AF0   acatgcaagt cgaacgcttt ttctttcacc ggagcttgct ccaccgaaag    75 AF0

      101        111        121        131        141      150
      |          |          |          |          |        |
Enr   AAAAAGAGUG GCGAACGGGU GAGUAACNCG UGGGUNACCU NCCCAUCAGA   150 Enr
Isl   AAAAAGAGUG GCGAACGGGU GAGUAACACG UGGGUAACCU GCCCAUCAGA   143 Isl
Enr   AAAAAGAGUG GCGAACGGGU GAGUAACNCG UGGGNNACCU NCCCAUCAGA   150 Enr
AF0   aaaaagagtg gcgaacgggt gagtaacacg tgggtaacct gcccatcaga   122 AF0
AF0   aaaaagagtg gcgaacgggt gagtaacacg tgggtaacct gcccatcaga   126 AF0
AF0   aaaaagagtg gcgaacgggt gagtaacacg tgggtaacct gcccatcaga   104 AF0
AF0   aaaaagagtg gcgaacgggt gagtaacacg tgggtaacct gcccatcaga   125 AF0

      151        161        171        181        191      200
      |          |          |          |          |        |
Enr   AGGGGAUAAC ACUUGGAAAC AGGUGCUAAU ACCGUAUAAC ACUAUNNUCC   200 Enr
Isl   AGGGGAUAAC ACUUGGAAAC AGGUGCUAAU ACCGUAUAAC ACUAUUUUCC   193 Isl
Enr   AGGGGAUAAC ACUUGGAAAC AGGUGCUNAU ACCGUAUAAC ACUNUNNUCC   200 Enr
AF0   aggggataac acttggaaac aggtgctaat accgtataac actattttcc   172 AF0
AF0   aggggataac acttggaaac aggtgctaat accgtataac actattttcc   176 AF0
AF0   aggggataac acttggaaac aggtgctaat accgtataac actattttcc   154 AF0
AF0   aggggataac acttggaaac aggtgctaat accgtataac actattttcc   175 AF0

      201        211        221        231        241      250
      |          |          |          |          |        |
Enr   GCAUGGAAGA AAGUUGAAAG GCGCUNUUGC GUNACUNAUG GAUGGACCCG   250 Enr
Isl   GCAUGGAAGA AAGUUGAAAG GCGCUUUUGC GUCACUGAUG GAUGGACCCG   243 Isl
Enr   GCAUGGAAGA AAGUUGAAAG GCGCUNUUGC GUNACUNAUG GAUGGACCCG   250 Enr
AF0   gcatggaaga aagttgaaag gcgcttttgc gtcactgatg gatggacccg   222 AF0
AF0   gcatggaaga aagttgaaag gcgcttttgc gtcactgatg gatggacccg   226 AF0
AF0   gcatggaaga aagttgaaag gcgcttttgc gtcactgatg gatggacccg   204 AF0
AF0   gcatggaaga aagttgaaag gcgcttttgc gtcactgatg gatggacccg   225 AF0

      251        261        271        281        291      300
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Appendix III-cont'd

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     |          |          |          |          |         |
Enr  CGGUGCAUUA GCUNGUUGGU GAGGUAACGG CUCACCAAGG CNACGAUGCA    300 Enr
Isl  CGGUGCAUUA GCUAGUUGGU GAGGUAACGG CUCACCAAGG CAACGAUGCA    293 Isl
Enr  CGGUGCAUUA GCUGGUUGGU GAGGUAACGG CUNACCAAGG CAACGAUGCA    300 Enr
AF0  cggtgcatta gctagttggt gaggtaacgg ctcaccaagg ccacgatgca    272 AF0
AF0  cggtgcatta gctagttggt gaggtaacgg ctcaccaagg caacgatgca    276 AF0
AF0  cggtgcatta gctagttggt gaggtaacgg ctcaccaagg ccacgatgca    254 AF0
AF0  cggtgcatta gctagttggt gaggtaacgg ctcaccaagg caacgatgca    275 AF0

     301        311        321        331        341       350
     |          |          |          |          |         |
Enr  UAGCCGACCU GAGAGGGUNA UCGGCCACAN UGGGACUGAG ACACGGCCNN    350 Enr
Isl  UAGCCGACCU GAGAGGGUGA UCGGCCACAC UGGGACUGAG ACACGGCCCA    343 Isl
Enr  UAGCCGACCU GAGAGGGUNA UCGGCCACAC UGGGACUGAG ACACGGCCCN    350 Enr
AF0  tagccgacct gagagggtga tcggccacac tgggactgag acacggccca    322 AF0
AF0  tagccgacct gagagggtga tcggccacac tgggactgag acacggccca    326 AF0
AF0  tagccgacct gagagggtga tcggccacac tgggactgag acacggccca    304 AF0
AF0  tagccgacct gagagggtga tcggccacac tgggactgag acacggccca    325 AF0

     351        361        371        381        391       400
     |          |          |          |          |         |
Enr  GACUCCUACG GGAGGCAGCC GUAGGGAAUC UUCGGCAAUG GACGAAAGUC    400 Enr
Isl  GACUCCUACG GGAGGCAGCA GUAGGGAAUC UUCGGCAAUG GACGAAAGUC    393 Isl
Enr  GACUCCUACG GGAGGCAGCA GUAGGGAAUC UUCGGCAAUG GACGAAAGUC    400 Enr
AF0  gactcctacg ggaggcagca gtagggaatc ttcggcaatg gacgaaagtc    372 AF0
AF0  gactcctacg ggaggcagca gtagggaatc ttcggcaatg gacgaaagtc    376 AF0
AF0  gactcctacg ggaggcagca gtagggaatc ttcggcaatg gacgaaagtc    354 AF0
AF0  gactcctacg ggaggcagca gtagggaatc ttcggcaatg gacgaaagtc    375 AF0

     401        411        421        431        441       450
     |          |          |          |          |         |
Enr  UGACCGAGCA ACGCCGCGUG AGUGAAGAAG GUNNUCGGAU CGUAAAACUC    450 Enr
Isl  UGACCGAGCA ACGCCGCGUG AGUGAAGAAG GUUUUCGGAU CGUAAAACUC    443 Isl
Enr  UGACCGAGCA ACGCCGCGUG AGUGAAGAAG GUNNUCGGAU CGUNAAACUC    450 Enr
AF0  tgaccgagca acgccgcgtg agtgaagaag gttttcggat cgtaaaactc    422 AF0
AF0  tgaccgagca acgccgcgtg agtgaagaag gttttcggat cgtaaaactc    426 AF0
AF0  tgaccgagca acgccgcgtg agtgaagaag gttttcggat cgtaaaactc    404 AF0
AF0  tgaccgagca acgccgcgtg agtgaagaag gttttcggat cgtaaaactc    425 AF0

     451        461        471        481        491       500
     |          |          |          |          |         |
Enr  NNUUGUUAGA GAAGAACAAG GAUGAGAGUN AAACGUNCAU CCCUNNACGG    500 Enr
Isl  UGUUGUUAGA GAAGAACAAG GAUGAGAGUA GAACGUUCAU CCCUUGACGG    493 Isl
Enr  UNUUGUUAGA GAAGAACAAG GAUGAGAGUN AAAUNUNNAU CCCUNNACGG    500 Enr
AF0  tgttgttaga gaagaacaag gatgagagta aaacgttcat cccttgacgg    472 AF0
AF0  tgttgttaga gaagaacaag gatgagagta gaacgttcat cccttgacgg    476 AF0
AF0  tgttgttaga gaagaacaag gatgagagta gaacgttcat cccttgacgg    454 AF0
AF0  tgttgttaga gaagaacaag gatgagagta aaatgttcat cccttgacgg    475 AF0

     501        511        521        531        541       550
     |          |          |          |          |         |
Enr  UAUCUAACCA GAAAGCCACG GCUAACUACG UGCCAGCAGC CGCGGUNAUA    550 Enr
Isl  UAUCUAACCA GAAAGCCACG GCUAACUACG UGCCAGCAGC CGCGGUAAUA    543 Isl
Enr  UAUCUAACCA GAAAGCCACG GCUNACUACG UGCCAGCAGC CGCGGUNAUA    550 Enr
AF0  tatctaacca gaaagccacg gctaactacg tgccagcagc cgcggtaata    522 AF0
AF0  tatctaacca gaaagccacg gctaactacg tgccagcagc cgcggtaata    526 AF0
AF0  tatctaacca gaaagccacg gctaactacg tgccagcagc cgcggtaata    504 AF0
AF0  tatctaacca gaaagccacg gctaactacg tgccagcagc cgcggtaata    525 AF0

     551        561        571        581        591       600
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Appendix III-cont'd

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     |          |          |          |          |         |
Enr  CGUAGGUGGC NAGCGUNNUC CGGAUNNAUU GGGCGUNAAG CGAGCGCAGG   600 Enr
Isl  CGUAGGUGGC AAGCGUUGUC CGGAUUUAUU GGGCGUAAAG CGAGCGCAGG   593 Isl
Enr  CGUAGGUGGC NAGCGUUGUC CGGAUUUAUU GGGCGUAAAG CGAGCGCAGG   600 Enr
AF0  cgtaggtggc aagcgttgtc cggatttatt gggcgtaaag cgagcgcagg   572 AF0
AF0  cgtaggtggc aagcgttgtc cggatttatt gggcgtaaag cgagcgcagg   576 AF0
AF0  cgtaggtggc aagcgttgtc cggatttatt gggcgtaaag cgagcgcagg   554 AF0
AF0  cgtaggtggc aagcgttgtc cggatttatt gggcgtaaag cgagcgcagg   575 AF0

     601        611        621        631        641       650
     |          |          |          |          |         |
Enr  CGGUUUCUUA AGUCUNAUGU GAAAGCCCCC GGCUCAACCG GGNNGGGUCA   650 Enr
Isl  CGGUUUCUUA AGUCUGAUGU GAAAGCCCCC GGCUCAACCG GGGAGGGUCA   643 Isl
Enr  CGGUUUCUUA AGUCUNAUGU GAAAGCCCCC GGCUCAACCG GGNNGGGUCA   650 Enr
AF0  cggtttctta agtctgatgt gaaagccccc ggctcaaccg gggagggtca   622 AF0
AF0  cggtttctta agtctgatgt gaaagccccc ggctcaaccg gggagggtca   626 AF0
AF0  cggtttctta agtctgatgt gaaagccccc ggctcaaccg gggagggtca   604 AF0
AF0  cggtttctta agtctgatgt gaaagccccc ggctcaaccg gggagggtca   625 AF0

     651        661        671        681        691       700
     |          |          |          |          |         |
Enr  UUGGAAACUG GGAGACUNGA GUGCAGAAGA GGAGAGUGGA AUUCCAUGUG   700 Enr
Isl  UUGGAAACUG GGAGACUUGA GUGCAGAAGA GGAGAGUGGA AUUCCAUGUG   693 Isl
Enr  UUGGAAACUG GGAGACUNGA GUGCAGAAGA GGAGAGUGGA AUUCCAUGUG   700 Enr
AF0  ttggaaactg ggagacttga gtgcagaaga ggagagtgga attccatgtg   672 AF0
AF0  ttggaaactg ggagacttga gtgcagaaga ggagagtgga attccatgtg   676 AF0
AF0  ttggaaactg ggagacttga gtgcagaaga ggagagtgga attccatgtg   654 AF0
AF0  ttggaaactg ggagacttga gtgcagaaga ggagagtgga attccatgtg   675 AF0

     701        711        721        731        741       750
     |          |          |          |          |         |
Enr  UAGCGGUGAA AUGCGUAGAU AUAUGGAGGA ACACCAGUGG CGAAGGCGGC   750 Enr
Isl  UAGCGGUGAA AUGCGUAGAU AUAUGGAGGA ACACCAGU.G CGAAGGCGGC   742 Isl
Enr  UAGCGGUGAA AUGCGUAGAU AUAUGGAGGA ACACCAGUGG CGAAGGCGGC   750 Enr
AF0  tagcggtgaa atgcgtagat atatggagga acaccagtgg cgaaggcggc   722 AF0
AF0  tagcggtgaa atgcgtagat atatggagga acaccagtgg cgaaggcggc   726 AF0
AF0  tagcggtgaa atgcgtagat atatggagga acaccagtgg cgaaggcggc   704 AF0
AF0  tagcggtgaa atgcgtagat atatggagga acaccagtgg cgaaggcggc   725 AF0

     751        761        771        781        791       800
     |          |          |          |          |         |
Enr  UCUCUNGUCU GUNACUGACG CUNAGGCUCG AAAGC-GUGG GGAGCGAACA   799 Enr
Isl  UCUCUGGUCU GUAACUGACG CUGAGGCUCG AAAGCCGUGG GGAGCGAACA   792 Isl
Enr  UCUCUNGUCU GUAACUGACG CUNAGGCUCG AAAGC-GUGG GGAGCGAACA   799 Enr
AF0  tctctggtct gtaactgacg ctgaggctcg aaagc-gtgg ggagcgaaca   771 AF0
AF0  tctctggtct gtaactgacg ctgaggctcg aaagc-gtgg ggagcgaaca   775 AF0
AF0  tctctggtct gtaactgacg ctgaggctcg aaagc-gtgg ggagcgaaca   753 AF0
AF0  tctctggtct gtaactgacg ctgaggctcg aaagc-gtgg ggagcgaaca   774 AF0

     801        811        821        831        841       850
     |          |          |          |          |         |
Enr  GGAUUAGAUA CCCUNGUAGU CCACGCCGUA AACGAUGAGU GCUNAGUGUU   849 Enr
Isl  GGAUUAGAUA CCCUGGUAGU CCACGCCGUA AACGAUGAGU GCUAAGUGUU   842 Isl
Enr  GGAUUAGAUA CCCUNGUAGU CCACGCCGUA AACGAUGAGU GCUAAGUGUU   849 Enr
AF0  ggattagata ccctggtagt ccacgccgta aacgatgagt gctaagtgtt   821 AF0
AF0  ggattagata ccctggtagt ccacgccgta aacgatgagt gctaagtgtt   825 AF0
AF0  ggattagata ccctggtagt ccacgccgta aacgatgagt gctaagtgtt   803 AF0
AF0  ggattagata ccctggtagt ccacgccgta aacgatgagt gctaagtgtt   824 AF0

     851        861        871        881        891       900
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Appendix III-cont'd

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       |          |          |          |          |        |
Enr    GGAGGGUUUC CGCCCUUCAG UGCUGCAGCN AACGCAUUAA GCACUCCGCC    899 Enr
Isl    GGAGGGUUUC CGCCCUUCAG UGCUGCAGCA AACGCAUUAA GCACUCCGCC    892 Isl
Enr    GGAGGGUUUC CGCCCUNCAG UGCUGCAGCA AACGCAUUAA GCACUCCGCC    899 Enr
AF0    ggagggtttc cgcccttcag tgctgcagca aacgcattaa gcactccgcc    871 AF0
AF0    ggagggtttc cgcccttcag tgctgcagca aacgcattaa gcactccgcc    875 AF0
AF0    ggagggtttc cgcccttcag tgctgcagca aacgcattaa gcactccgcc    853 AF0
AF0    ggagggtttc cgcccttcag tgctgcagca aacgcattaa gcactccgcc    874 AF0

       901        911        921        931        941      950
       |          |          |          |          |        |
Enr    UGGGGAGUAC GACCGCAAGG UUNAAACUCA AAGGAAUUGA CGGGGGC.CG    948 Enr
Isl    UGGGGAGUAC GACCGCAAGG UUGAAACUCA AAGGAAUUGA CGGGGGCCCG    942 Isl
Enr    UGGGGAGUAC GACCGCAAGG UUGAAACUCA AAGGAAUUGA CGGGGGC.CG    948 Enr
AF0    tggggagtac gaccgcaagg ttgaaactca aaggaattga cgggggcccg    921 AF0
AF0    tggggagtac gaccgcaagg ttgaaactca aaggaattga cgggggcccg    925 AF0
AF0    tggggagtac gaccgcaagg ttgaaactca aaggaattga cgggggcccg    903 AF0
AF0    tggggagtac gaccgcaagg ttgaaactca aaggaattga cgggggcccg    924 AF0

       951        961        971        981        991      1000
       |          |          |          |          |        |
Enr    CACAAGCGGU GGAGCAUGUN GUUUAAUUCG AAGNAACGCG AAGAACCUNA    998 Enr
Isl    CACAAGCG.U GGAGCAUGUG GUUUAAUUCG AAGCAACGCG AAGAACCUUA    991 Isl
Enr    CACAAGCGGU GGAGCAUGUN GUUUAAUUCG AAGNAACGCG AAGAACCUNA    998 Enr
AF0    cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg aagaacctta    971 AF0
AF0    cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg aagaacctta    975 AF0
AF0    cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg aagaacctta    953 AF0
AF0    cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg aagaacctta    974 AF0

       1001       1011       1021       1031       1041     1050
       |          |          |          |          |        |
Enr    CCAGGUCUUG ACAUCCUNUG ACCACUCUAG AGAUAGAGCU UN.CCUUCGG   1047 Enr
Isl    CCAGGUCUUG ACAUCCUUUG ACCACUCUAG AGAUAGAGCU UCCCCUUCGG   1041 Isl
Enr    CCAGGUCUUG ACAUCCUNUG ACCACUCUAG AGAUAGAGCU UU.CCUUCGG   1047 Enr
AF0    ccaggtcttg acatcctttg accactctag agatagagct tccccttcgg   1021 AF0
AF0    ccaggtcttg acatcctttg accactctag agatagagct tccccttcgg   1025 AF0
AF0    ccaggtcttg acatcctttg accactctag agatagagct tccccttcgg   1003 AF0
AF0    ccaggtcttg acatcctttg accactctag agatagagct tccccttcgg   1024 AF0

       1051       1061       1071       1081       1091     1100
       |          |          |          |          |        |
Enr    GGGCAAAGUG ACAGGUGGNG CAUNGUUGUC GUCAGCUCGU GUCGUGAGAU   1097 Enr
Isl    GGGCAAAGUG ACAGGUGGUG CAUGGUUGUC GUCAGCUCGU GUCGUGAGAU   1091 Isl
Enr    GGGCAAAGUG ACAGGUGGNG CAUNGUUGUC GUCAGCUCGU GUCGUGAGAU   1097 Enr
AF0    gggcaaagtg acaggtggtg catggttgtc gtcagctcgt gtcgtgagat   1071 AF0
AF0    gggcaaagtg acaggtggtg catggttgtc gtcagctcgt gtcgtgagat   1075 AF0
AF0    gggcaaagtg acaggtggtg catggttgtc gtcagctcgt gtcgtgagat   1053 AF0
AF0    gggcaaagtg acaggtggtg catggttgtc gtcagctcgt gtcgtgagat   1074 AF0

       1101       1111       1121       1131       1141     1150
       |          |          |          |          |        |
Enr    GUUGGGUNAA GUCCCGCAAC GAGCGCAACC CUNAUNGUUA GUUGCCAUCA   1147 Enr
Isl    GUUGGGUUAA GUCCCGCAAC GAGCGCAACC CUUAUUGUUA GUUGCCAUCA   1141 Isl
Enr    GUUGGGUNAA GUCCCGCAAC GAGCGCAACC CUNNUNGUUA GUUGCCAUCA   1147 Enr
AF0    gttgggttaa gtcccgcaac gagcgcaacc cttattgtta gttgccatca   1121 AF0
AF0    gttgggttaa gtcccgcaac gagcgcaacc cttattgtta gttgccatca   1125 AF0
AF0    gttgggttaa gtcccgcaac gagcgcaacc cttattgtta gttgccatca   1103 AF0
AF0    gttgggttaa gtcccgcaac gagcgcaacc cttattgtta gttgccatca   1124 AF0

       1151       1161       1171       1181       1191     1200
```

Appendix III-cont'd

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         |          |          |          |          |          |
Enr   UUUAGUUGGG CACUCUAGCG AGACUGCCGG UGACAAACCG GAGGAAGGUG  1197 Enr
Isl   UUUAGUUGGG CACUCUAGCG AGACUGCCGG UGACAAACCG GAGGAAGGUG  1191 Isl
Enr   UUUAGUUGGG CACUCUAGCG AGACUGCCGG UGACAAACCG GAGGAAGGUG  1197 Enr
AF0   tttagttggg cactctagcg agactgccgg tgacaaaccg gaggaaggtg  1171 AF0
AF0   tttagttggg cactctagcg agactgccgg tgacaaaccg gaggaaggtg  1175 AF0
AF0   tttagttggg cactctagcg agactgccgg tgacaaaccg gaggaaggtg  1153 AF0
AF0   tttagttggg cactctagcg agactgccgg tgacaaaccg gaggaaggtg  1174 AF0

      1201       1211       1221       1231       1241      1250
      |          |          |          |          |         |
Enr   GGGAUGACGU CAAAUCAUCA UGCCCCUUAU GACCUGGGCU ACACACGUGC  1247 Enr
Isl   GGGAUGACGU CAAAUCAUCA UGCCCCUUAU GACCUGGGCU ACACACGUGC  1241 Isl
Enr   GGGAUGACGU CAAAUCAUCA UGCCCCUUAU GACCUGGGCU ACACACGUGC  1247 Enr
AF0   gggatgacgt caaatcatca tgccccttat gacctgggct acacacgtgc  1221 AF0
AF0   gggatgacgt caaatcatca tgccccttat gacctgggct acacacgtgc  1225 AF0
AF0   gggatgacgt caaatcatca tgccccttat gacctgggct acacacgtgc  1203 AF0
AF0   gggatgacgt caaatcatca tgccccttat gacctgggct acacacgtgc  1224 AF0

      1251       1261       1271       1281       1291      1300
      |          |          |          |          |         |
Enr   UACAAUGGGA AGUACAACGA GUUGCGAAGU CGCGAGGCUN AGCUAAUCUC  1297 Enr
Isl   UACAAUGGGA AGUACAACGA GUUGCGAAGU CGCGAGGCUA AGCUAAUCUC  1291 Isl
Enr   UACAAUGGGA AGUACAACGA GUUGCGAAGU CGCGAGGCUN AGCUNAUCUC  1297 Enr
AF0   tacaatggga agtacaacga gttgcgaagt cgcgaggcta agctaatctc  1271 AF0
AF0   tacaatggga agtacaacga gttgcgaagt cgcgaggcta agctaatctc  1275 AF0
AF0   tacaatggga agtacaacga gttgcgaagt cgcgaggcta agctaatctc  1253 AF0
AF0   tacaatggga agtacaacga gttgcgaagt cgcgaggcta agctaatctc  1274 AF0

      1301       1311       1321       1331       1341      1350
      |          |          |          |          |         |
Enr   UNAAAGCUGC UCUCAGUUCG GAUUGUAGGC UGCAACUCGC CUNCAUGAAG  1347 Enr
Isl   UUAAAGCUUC UCUCAGUUCG GAUUGUAGGC UGCAACUCGC CUACAUGAAG  1341 Isl
Enr   UNNAAGCUNC UCUCAGUUCG GAUNGUAGGC UGCAACUCGC CUNCAUGAAG  1347 Enr
AF0   ttaaagcttc tctcagttcg gattgtaggc tgcaactcgc ctacatgaag  1321 AF0
AF0   ttaaagcttc tctcagttcg gattgtaggc tgcaactcgc ctacatgaag  1325 AF0
AF0   ttaaagcttc tctcagttcg gattgtaggc tgcaactcgc ctacatgaag  1303 AF0
AF0   ttaaagcttc tctcagttcg gattgtaggc tgcaactcgc ctacatgaag  1324 AF0

      1351       1361       1371       1381       1391      1400
      |          |          |          |          |         |
Enr   CCGGAAUCGC UNGUAAUCGC GGAUCAGCAC GCCGCGGUNA AUACGUUCCC  1397 Enr
Isl   CCGGAAUCGC UAGUAAUCGC GGAUCAGCAC GCCGCGGUGA AUACGUUCCC  1391 Isl
Enr   CCGGAAUCGC UAGUAAUCGC GGAUCAGCAC GCCGCGGUNA AGACGUUCCC  1397 Enr
AF0   ccggaatcgc tagtaatcgc ggatcagcac gccgcggtga atacgttccc  1371 AF0
AF0   ccggaatcgc tagtaatcgc ggatcagcac gccgcggtga atacgttccc  1375 AF0
AF0   ccggaatcgc tagtaatcgc ggatcagcac gccgcggtga atacgttccc  1353 AF0
AF0   ccggaatcgc tagtaatcgc ggatcagcac gccgcggtga atacgttccc  1374 AF0

      1401       1411       1421       1431       1441      1450
      |          |          |          |          |         |
Enr   GGGCNUNGUA CACACCGCCC GUCACACCA- CGAGAGUUUG UAACACCCGA  1446 Enr
Isl   GGGCCUUGUA CACACCUCCC GUCACACCAC CGAGAGUUUG UAACACCCGA  1441 Isl
Enr   GGGCCNNGUA CACACCGCCC GUCACACCA- CGAGAGUUUG UAACACCCGA  1446 Enr
AF0   gggccttgta cacaccgccc gtcacacca- cgagagtttg taacaccc..  1418 AF0
AF0   gggccttgta cacaccgccc gtcacacca- cgagagtttg taacacccga  1424 AF0
AF0   gggccttgta cacaccgccc gtcacacca- cgagagtttg taacacccga  1402 AF0
AF0   gggccttgta cacaccgccc gtcacacca- cgagagtttg taacacccga  1423 AF0

      1451       1461       1471       1481       1491      1500
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Appendix III-cont'd

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     |          |          |          |          |         |
Enr  AGUCGGUGAG GUNACCNNUU UGGAGCCAGC CGCCUNAGGU GGG.......  1489 Enr
Isl  AGUCGGUGAG GUAACCUUUU UGGAGCCAGC CGCCUAAGGU GGGUUAGAUG  1491 Isl
Enr  .......... .......... .......... .......... ..........  1446 Enr
AF0  .......... .......... .......... .......... ..........  1418 AF0
AF0  agtcggtgag gtaacctttt tggagccagc cgcctaaggt gggatagatg  1474 AF0
AF0  agtcggtgag gtaacctttt tggagccagc cgcctaaggt gggatagatg  1452 AF0
AF0  agtcggtgag gtaacctttt tggagccagc cgcctaaggt gggatagatg  1473 AF0

     1501       1511       1521       1531       1541     1550
     |          |          |          |          |         |
Enr  .......... .......... .......... .......... ..........  1489 Enr
Isl  AUUGGGGUGA AGUCGUAACA AGGUAACCGU .......... ..........  1521 Isl
Enr  .......... .......... .......... .......... ..........  1446 Enr
AF0  .......... .......... .......... .......... ..........  1418 AF0
AF0  attggggtga agtcgtaaca aggtagccg- ---------- -------tat  1506 AF0
AF0  attggggtga agtcgtaaca aggtagccgt atcggaaggt gcggctggat  1502 AF0
AF0  attggggtga agtcgtaaca aggtagccgt ---------- --------at  1505 AF0

     1551
     |
Enr  ....                                                    1489 Enr
Isl  ....                                                    1521 Isl
Enr  ....                                                    1446 Enr
AF0  ....                                                    1418 AF0
AF0  cg..                                                    1508 AF0
AF0  cacc                                                    1506 AF0
AF0  cgga                                                    1509 AF0
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Appendix IV.

Endnotes

While my Master's Thesis research did not land on the cover of Science magazine, my graduate research was very fruitful.

Entering graduate school after almost a 10-year absence from science was a serious challenge. Sixty hours a week reviewing undergraduate algebra and chemistry ended with 4.0's in Biochemistry. Coursework in molecular biology, rumen microbiology and microbial ecology provided a foundation for my research.

I had the opportunity to use a variety of methods to investigate the bacterium and phage. I learned and applied a variety of late twentieth century microbiological methods. I used BIOLOG; API Strep and 16s rRNA based databases to identify a bacterial isolate. I explored the strengths and weaknesses of these databases. HPLC analysis of the chemical byproducts of the bacterium provided important supporting data and an introduction to a powerful analytical tool.

To study the genetic make up of the phage, I applied molecular biology techniques, and used the data to interrogate earlier data on the phage and its hosts. To study the ultrastructure of the phage, I was introduced to the electron microscope, which led directly to a position as an electron microscopist at the Center for Electron Optics at MSU.

I learned a fundamental caution for scientific work. Decisions are made about what receives the attention of the scientists eye. High hopes regarding a phage capable of infecting important rumen species led to dismissal of data that didn't "fit". The three different microbial databases produced an analysis based on partial data for the genus *Enterococcus.*

The most important point, however, is the confirmation of the 60's axiom to "Question Authority". I found this crucial in determining validity of results from both world-renowned databases and laboratory cohort. Science can't move forward without a rigorous interrogation of data, data analysis methods and data collectors. "Objective science" only approximates objectivity if the subjective factor is made conscious.

Future research

Changes in medicine and the efficacy of antibiotics are bringing the microbiologists eye back to phage therapy. Phage of the species group *E. casseliflavus* and *E. gallinarum* are worthy of investigation for use in phage therapy. Multiply drug resistant enterococci are increasingly becoming feared pathogens.

Phage therapy was attempted and discarded earlier this century in the US. The rapid development of bacterial resistance to phage makes the development of phage therapy complex. In the former Soviet satellite of Georgia, phage therapy was developed and successfully used. The methods involved using many strains of phage infective of a pathogen. An interesting discussion of these efforts and phage therapy in general was published on the web by Elizabeth Kutter, at Evergreen State College. see
http://www.evergreen.edu/user/T4/PhageTherapy/Phagethea.html

Collection of a battery of strains in the *E. gallinarum-E. casseliflavus* and a battery of phage infectious to them could be the beginning of an interesting research initiative. An initial investigation would be to test pathogen survival when challenged with single and multi-phage batteries. Phage receptor sites on enterococci have not been investigated. A bacterial strain that has mutations for resistance may have altered viability and pathogenecity. A thorough investigation into these questions could lead to important medical breakthroughs.