MULTIPLE ANTIBIOTIC RESISTANCE OF SURFACE MUCUS DWELLING BACTERIAL POPULATIONS IN FRESHWATER FISH

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MULTIPLE ANTIBIOTIC RESISTANCE OF SURFACE MUCUS DWELLING BACTERIAL POPULATIONS IN FRESHWATER FISH

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ABSTRACT

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Surface mucus of a freshwater fish, *Alburnus alburnus* (bleak), caught from Lake Mogan, situated in south of Ankara, was collected in different seasons. The total cultivable bacteria were enumerated by spread plate method on nine different media. Bacteria were isolated based on colony morphologies and pigmentation. A total of sixty bacterial isolates obtained. The mucus-dwelling bacteria were first tested for resistance against ampicillin and kanamycin; then streptomycin and chloramphenicol were added to the experimental set up. The resistance levels of isolates were determined in terms of four antibiotics by tube dilution method. About 90% of the isolates were resistant to chloramphenicol, about 84% to kanamycin, about 88% to streptomycin and about 98% to ampicillin. These high levels of antibiotic resistance are rather interesting from a standpoint that the lake has no record of antibiotics exposure of any sort. The plasmid isolations were carried out to determine if the multiple antibiotic resistance could be attributed to plasmids for starting assumption. But we found no direct relationship

between the presence of plasmids and multiple antibiotic resistance. Our study indicated that multiple antibiotic resistance at high levels is among the current phenotypes of the fish mucus-dwelling bacterial populations in Lake Mogan.

Key Words: fish mucus-dwelling bacteria, viable count, freshwater, multiple antibiotic resistance, ampicillin, kanamycin, chloramphenicol, streptomycin

ÖZ

TATLI SU BALIKLARININ YÜZEY MUKUSUNDA YAŞAYAN BAKTERİ POPULASYONLARININ ÇOKLU ANTİBİYOTİK DİRENÇLERİ

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Farklı mevsimlerde Ankara ilinin güneyinde bulunan Mogan Gölü'nden yakalanan bir tatlı su balığından, *Alburnus alburnus* (gümüş), yüzey mukusu örnekleri alınmıştır. Toplam kültüre edilebilen bakteri sayımı dokuz farklı besiyerinde yayma yöntemi kullanılarak yapılmıştır. Bakteriler koloni morfolojileri ve renkleri temel alınarak izole edilmiştir. Toplam altmış bakteri izolatı elde edilmiştir. Mukusta yaşayan bakterilerin direnci ilk önce ampisilin ve kanamisine karşı denenmiştir; daha sonra deneylere streptomisin ve kloramfenikol ilave edilmiştir. İzolatların dört antibiyotiğe karşı olan direnç seviyeleri tüp dilüsyon yöntemi ile belirlenmiştir. Buna göre izolatların yaklaşık %90'nı kloramfenikole, yaklaşık %84'ü kanamisine, yaklaşık %88'i streptomisine ve yaklaşık %98'i ampisiline karşı dirençli bulunmuştur. Bu yüksek seviyedeki antibiyotik direnci hiçbir antibiyotiğe maruz bırakıldığı kaydedilmemiş bir göl için oldukça ilginçtir. Çoklu antibiyotik direncinin, başlangıç varsayımındaki gibi, plazmit ilişkili olup olmadığını anlamak amacıyla

plazmit izolasyonları gerçekleştirilmiştir. Fakat plazmit varlığı ve çoklu antibiyotik direnç arasında direkt bir ilişki bulunamamıştır. Bu çalışmamız Mogan Gölü'ndeki balık mukusunda yaşayan bakteri populasyonları için yüksek seviyedeki çoklu antibiyotik direncinin yeni bir fenotip olduğunu göstermiştir.

Anahtar Kelimeler : balık mukusunda yaşayan bakteriler, tatlı su, canlı sayım, çoklu antibiyotik direnci, ampisilin, kanamisin, kloramfenikol, streptomisin

To My Parents

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LIST OF ABBREVIATIONS

Am	:	Ampicillin
Cfu	:	Colony forming unit
Cm	:	Chloromphenicol
EMB	:	Eosin methylene blue
fs	:	Fish surface
Kc	:	Kanamycin
LB	:	Luria- Bertani agar
M9	:	M9 minimal agar
MS	:	MS agar
NA	:	Yeast extract, Van Niel's agar
PA	:	P agar
PBS	:	Phosphate-buffered saline
Sm	:	Streptomycin
TSA	:	Tryptic soy agar
TSB	:	Trypticase soy broth
YE	:	Yeast extract

CHAPTER 1

INTRODUCTION

1.1 Bacterial Diversity in Freshwater Lakes

In the lake environment, different species of pelagic bacteria position themselves in the water column in relation to local conditions of light intensity, oxygen level, and nutrient concentrations. Attached bacteria also occupy specific niches within the lake or river environment, being associated with particular pelagic and benthic algae, macrophytes, and sediments. Bacterial biofilms form important microbial communities in lakes (air-water interface at lake surface, sediments) and in streams (air-water interface, stone and plant surfaces). The physiology of attached bacteria is different in many respects from pelagic organisms (e.g., growth characteristics, resistance to antimicrobial compounds), and the close proximity of bacterial cells within dense attached communities is important for activities such as gene transfer (Sigee, 2004).

Most aquatic bacteria are gram-negative, with relatively few gram-positive representatives (Sigee, 2004). Members of the genera *Achromobacter*, *Brevibacterium*, *Micrococcus*, *Bacillus*, *Pseudomonas*, *Nocardia*, *Streptomyces*, *Micromonospora*, *Cytophaga*, *Spirillium*, and *Vibrio* are reported as occurring widely in lake water (Taylor, 1942; Rheinheimer, 1991). Stalked bacteria, such as *Caulobacter*, *Hyphomicrobium*, and other genera, are associated with submerged surfaces (Rheinheimer, 1991).

Autotrophic bacteria are autochthonous members of the microbiota of lakes and play an important role in nutrient cycling (Caldwell, 1977). Photoautotrophic bacteria, normally found in lakes include the cyanobacteria and in anoxic zones the purple and green anaerobic photosynthetic bacteria (Rheinheimer, 1991). Chemolithotrophic bacteria have important roles in nitrogen, sulfur, and iron cycling within lakes; members of the genera *Nitrosomonas*, *Nitrobacter*, and *Thiobacillus* are essential members of freshwater microbial communities (Atlas and Bartha, 1998).

Microorganisms found in the sediment of freshwater lakes are usually different from those in the overlying waters. Bacteria capable of anaerobic respiration are important members of sediment microbiota and include *Pseudomonas* species capable of denitrification activities. Within the sediment, obligately anaerobic bacteria occupy important niches. These bacteria include endospore-forming *Clostridium* species, methanogenic bacteria that produce methane gas, and *Desulfovibrio* species that produce hydrogen sulfide (Atlas and Bartha, 1998).

In addition to autochthonous microbial populations, many allochthonous terrestrial microorganisms are carried by erosion and runoff from soils into freshwater aquatic ecosystems. Allochthonous microorganisms also enter when leaves from adjacent plants fall into these water bodies and when municipal sewage enters freshwater environments together with high amounts of organic matter. Heterotrophic microbial populations in areas that receive high amounts of organic matter are generally elevated, but as the amounts of important organic matter decrease, populations of heterotrophic microorganisms also decline (Atlas and Bartha, 1998).

1.2 Bacteria Associated with Surface-Mucus of Fish

Various skin surfaces and mucosal membranes in animals have a normal bacterial flora with a characteristic composition. The composition of the normal flora may vary with the animal species, feed, and housing conditions including population density (Sørum and Sunde, 2001).

Fish spend all their life stages in an aqueous environment that allows intimate contact with a variety of microflora, including pathogenic and opportunistic bacteria, which may colonize their various external and internal body surfaces. The epidermal mucus layer constitutes the primary biological interface between fish and the aqueous environment (Hansen and Olafsen, 1999). The mucus coat may be an adhesion site for bacteria (Conway, 1989; Speare and Mirsalimi, 1992), but it has also been suggested that it may function to prevent firm attachment of bacteria to the skin (Crouse-Eisnor *et al*., 1985).

Among several biological functions, the epidermal mucus of fish may play an important role in host defense, particularly in the prevention of colonization by parasites, bacteria and fungi (Ebran *et al*., 1999).

Bacterial adhesion to mucosal surfaces is a crucial stage in the establishment of a protective “normal” microflora, and also the first step in an infective process. The microflora is to some extent affected by the bacterial flora in the water (Hansen and Olafsen, 1999). According to a survey (Austin, 1982) of bacteria isolated from the skin of a healthy coastal marine fish exhibit greater taxonomic diversity than the bacterial flora in the ambient water. On the external surfaces of freshwater fish the same taxonomic bacterial groups seem to prevail, with *Aeromonas* being

more and *Vibrio* less abundant (Crouse-Eisnor *et al*., 1985; Horsley, 1977; Horsley, 1973; Sakata, 1990).

Generally, the range of bacterial genera isolated is related to the aquatic habitat of the fish and varies with factors such as the salinity of the habitat and the bacterial load in the water. In many investigations, identification of isolates to the genus level only makes it difficult to determine the precise relationships of aquatic and fish microflora. Bacteria recovered from the skin and gills may be transient rather than resident on the fish surfaces (Cahill, 1990).

1.3 Genetic Interactions between Bacteria in Freshwater Environment

Genetic diversity in freshwater bacteria, as with bacteria generally, is characterized by the presence of two separate genetic systems, composed of chromosomal and extra-chromosomal DNA. Extra-chromosomal DNA is present in the bacterial cell as separate, relatively short-sequence fragments, and includes phages, plasmids, transposons, and insertion sequences. This DNA typically encodes for non-essential characteristics such as resistance to antibiotics. These elements are able to replicate independently of the cell cycle (Sigee, 2004).

In most part, genetic diversity in bacteria is promoted by the active transfer of genes between organisms, which involves the transport and incorporation of extra-chromosomal DNA (containing the transferring gene), from one cell to another (Sigee, 2004). Three main mechanisms for bacterial gene transfer have been demonstrated in the freshwater

environment (Pickup, 1992; Van Elsas, 1992; Ehlers, 2000) - transformation, transduction, and conjugation.

Evidence for gene transfer between bacteria in the freshwater environment comes from three major sources –retrospective analysis, laboratory (*in vitro*), and field (*in situ*) experimental studies (Pickup, 1992). These studies indicate that plasmid transfer between bacteria is frequently encountered within the freshwater environment (Sigee, 2004).

1.3.1 R Plasmids as Gene Mobility Mechanism

The R (resistance) plasmids confer resistance to antibiotics and various other growth inhibitors. Most of the time, the strains carrying the resistance genes could transfer them to sensitive strains via cell-to-cell contact. The infectious nature of the conjugative R plasmids permits their rapid spread through cell populations. (Madigan and Martinko, 2006).

Several antibiotic resistance genes can be carried by an R plasmid. In general, these genes encode proteins that either inactivate the protein or prevent its uptake into the cell. Many drug-resistant elements on R plasmids are transposable elements and also many of these plasmids are conjugative (Madigan and Martinko, 2006).

Since the first R plasmid was detected in the 1950s, plasmids carrying drug resistance genes have been detected from most bacterial groups, indicating a large pool of R plasmids among antibiotic-resistant bacteria. However, as the number of characterized R plasmids increases, it seems that the new ones are often variants of previously described plasmids. The situation may actually be that the number of different R

plasmids is relatively limited, but the number of variants of each R plasmid may increase concurrent with the use of antibiotics in various ecological niches (Sørum and L'Abée-Lund, 2002). One example in support of this hypothesis is the occurrence of IncU plasmids with varying resistance region contents but with an identical plasmid backbone structure. They have been found in *Aeromonas* a fish pathogen, in environmental aeromonads and in enterobacteria isolates from human clinical samples (Rhodes et al., 2000; L'Abe´e-Lund and Sørum, 2001).

1.3.2 The Ecological Importance of Gene Transfer in Freshwater Systems

The transfer and spread of genes (including natural mutations) within aquatic bacterial populations are important for a number of key processes that affect the ability of bacteria to adapt to changes in the freshwater environment and compete with other organisms (Young,1992).

The discharge of bacteria with plasmids containing antibiotic resistance genes (originating from clinical and agricultural sources) from sewage and waste-treatment facilities is one of the main reasons for introduction and spread of novel genes in freshwater environments (Sigee, 2004). For example, oxytetracycline is the most frequently used antibiotic in the Chilean salmonid industry because of its broad spectrum of activity and low cost. However, the intensive use of this antibiotic has resulted in an increased frequency of oxytetracycline-resistant microorganisms and of oxytetracycline-resistant fish pathogens. On the other hand, the high percent of oxytetracycline-resistant bacteria also

was observed in Chilean salmon farms with no recent history of antibiotic use (Miranda and Zemelman, 2002).

1.4 Antimicrobial Drug Resistance

Antimicrobial drug resistance is the acquired ability of a microorganism to resist the effects of a chemotherapeutic agent to which it is normally susceptible. Antibiotic producers are microorganisms, and in order to survive, the antibiotic-producing microorganisms developed resistance mechanisms to neutralize or destroy their own antibiotics. In addition, genes encoding these resistance mechanisms can be transferred to other, usually related, organisms. As a result, most antimicrobial drug resistance involves resistance genes that are transferred between and among microorganisms by genetic exchange (Madigan and Martinko, 2006).

A series of antibiotic resistance genes have been sequenced and found to be identical or nearly identical in various ecological environments. Similarly, genetic vectors responsible for assembly and mobility of antibiotic resistance genes, such as transposons, integrons and R plasmids of similar or identical type are also widespread in various niches of the environment (Sørum and L'Abée-Lund, 2002).

Each gene cassette can provide resistance to a chemically distinct class of antimicrobial agents. The first undesired aspect of multiple-resistance transfer agents, such as plasmids, transposons, and integrons, is that they can collect and recombine existing resistance gene cassettes in almost any combination (Collis and Hall, 1992; Recchia and Hall, 1995). Consequently, treatment with any given antimicrobial agent

can result in selection for bacteria resistant not only to that specific agent, but by genetic linkage of resistance genes, to other unrelated antimicrobial agents (Summers, 2002).

For some decades, the focus on the prevalence, spread and mechanisms involved in antibiotic resistance in bacteria was concentrated on the pathogens. However, an increasing effort has now been directed into the study of the mobility and transfer of resistance genes between the nonpathogenic normal flora of animals and those of humans or between the environmental flora and medically important bacteria (Sørum and Sunde, 2001). Due that R plasmids may also be isolated from normal bacterial flora of healthy food-producing animals (Sunde and Sørum, 2001).

1.4.1 Antibiotic Resistance of the Normal Flora

An important preventive factor against antibiotic resistance in bacteria causing community-acquired infections is to keep the level of antibiotic-resistant bacteria in the normal flora at a low level. With the established genetic mechanisms for exchange of DNA between bacteria, the normal flora is capable of supplying drug resistance genes to the pathogens, depending on their needs (Sørum and L'Abée-Lund, 2002).

The normal bacterial flora contains antibiotic resistance genes to various degrees, even in individuals with no history of exposure to commercially prepared antibiotics (Sørum and Sunde, 2001).

Testing the "normal flora" for occurrence of antibiotic resistance is not straightforward compared to testing the susceptibility of single

isolates of bacterial pathogens from infections. Studies on the antibiotic resistance of the normal flora should take into consideration that several species of bacteria with various levels of antibiotic resistance exist at the same site. Many normal flora species cannot be cultivated and many species occur in low numbers resulting in problems with isolation (Sørum and Sunde, 2001).

1.4.2 Resistance Mechanisms

Some microorganisms are naturally resistant to certain antibiotics. There are several reasons why microorganisms may have an inherent natural resistance to an antibiotic: lack of antibiotic-inhibited structure, reduced permeability, alteration and subsequent inactivation of the antibiotic, target modification, developing a resistant biochemical pathway by genetic change or physiological switch, or efflux of the antibiotic (Madigan and Martinko, 2006).

R plasmid resistance is usually due to the presence in the R plasmid of genes encoding new enzymes that inactivate the drug or genes that encode enzymes that either prevent drug uptake or actively pump it out. Many R plasmids confer multiple antibiotic resistance because a single R plasmid may contain several different genes, each encoding a different antibiotic-inactivating enzyme (Madigan and Martinko, 2006). According to Hermansson *et al*. (1987) some strains of bacteria resistant to antibiotics do not contain any plasmids. In such a case bacterial resistance to antibiotics depends on the mobile genetic elements, called transposons (Herwig *et al*., 1997).

1.4.3 Factors Contributing to Acquired Antibiotic Resistance

A large number of bacteria and actinomycetales occurring in aquatic ecosystems are capable of synthesising antimicrobial compounds (Klein and Alexander, 1986; Lemos *et al.*, 1985). These inhibitory substances are molecules between 2,000-15,000 dalton range and their concentration in water could reach upto 1 μg/cm^3 (Lemos *et al.*, 1985; van Duck *et al.*, 1976*)*. Bacteria slowly but steadily synthesize and secrete into water a number of antimicrobial substances, namely phenazines, pyrrolnitrin, bacteriocins, glycolipids and bromopyrrolic compounds (Barja *et al.*, 1989; Dakhama *et al.*, 1993; Lemos *et al.*, 1991; Williams and Vickers, 1986). A lot of algae also produce antimicrobial substances, which inhibit the growth of bacteria (Klein and Alexander, 1986). In aquatic systems beside autochthonous sources antibiotics also come from anthropogenic activities (Mudryk, 2002). It seems that R plasmids existed in the natural microbial population before antibiotics were discovered and used in medicine or agriculture (Madigan and Martinko, 2006). However the impact of the intensive use of antimicrobial agents worldwide for prophylactic and therapeutic purposes has been associated with the increase of bacterial resistance in the exposed microbial environment (Sarter *et al*., 2007).

Several different kinds of antibiotics are used in fish farms to control bacterial and fungal diseases by incorporation into the feed (Herwig *et al.*, 1997; Jones *et al.*, 1986), for example. The fish absorbs not all antibiotics; some are released into the water and sediments (Mudryk, 2002).

It is reasonable to conclude that resistant bacteria found in surface waters have not been selected by the vanishingly small amounts

of antimicrobial agents in those waters but have traveled there via animal or insect vectors, in airborne dusts (Levetin,1997; Stetzenbach, 1997) or simply in the flow of the waters after being released from some antimicrobial-rich setting. However, given the stability of plasmids and other resistance replicons, thousands of bacterial generations may have taken place since the actual selective exposure (Summers, 2002).

1.4.4 The Records on Antibiotic Resistance in Freshwater Environments

In a study conducted on *Aeromonas* and *Pseudomonas* isolates from farm-cultured rainbow trouts in Australia (Akinbowale *et al.*, 2007), considerable antibiotic resistance to several antibiotics was noted. For example, the percentage of resistant *Aeromonas* isolates was 33.3% for streptomycin and 2.1% for chloramphenicol. The percentage of resistant *Pseudomonas* isolates was 43.2% for streptomycin and 65.9% for chloramphenicol.

Currently, wide range multiple antibiotic resistance has been reported among fish pathogens (Schmidt *et al*., 2001; Teuber, 2001). For example, two major fish pathogens (*Flavobacterium psychrophilum* isolates and Yersinia *ruckeri* isolates) and motile *Aeromonas* were isolated from the four freshwater fish farms in western Denmark. These isolates were investigated in terms of antimicrobial agents and high levels of individual and multiple antimicrobial resistances were demonstrated within the collected flavobacteria and aeromonads (Schmidt *et al.*, 2000).

In another study, the incidence of antibiotic resistance was compared in bacteria isolated from natural pond water and pond

sediment versus water and sediment from different shrimp-culture ponds. Most of the bacteria isolated in both water and sediment whether natural or shrimp farm were *Vibrios*. As a result, they found that multiple antibiotic resistance to at least two antimicrobials was highest in ponds currently using oxolinic acid (24% of bacteria isolated from such ponds) and the lowest was those from ponds that have not used any antimicrobials (17%). The antibiotic resistance observed in ponds that have not used any antimicrobials was 41% of the total isolates. Among the individual antibiotics, incidence of resistance to oxytetracycline was highest (4.3% of the total number of isolates) followed by furazolidone(1.6%), oxolinic acid (1%) and chloramphenicol (0.66%) (Tendenci and de la Pena, 2001).

Also Lima-Bittencourt *et al.* (2007) studied with a freshwater enterobacterial population for antimicrobial and mercury resistance patterns and they reported that there was no correlation between antimicrobial resistance patterns of isolates and bacterial genera, but resistance patterns varied among water samples and between seasons. Resistance to multiple antimicrobials was common (61%). Resistance to β-lactams and chloramphenicol was the most frequent and resistance to amikacin, gentamicin and kanamycin was less frequent. They also examined the main water for abiotic factors pH and temperature and biotic factor chlorophyll *a* concentration but they did not found any influence for antimicrobial resistance.

In the other study, the gram-negative bacteria isolated from farmed catfish were analysed for multiple antibiotic resistance (Sarter *et al.,* 2007). It was reported that the average rates of resistance were 69.6% ampicillin, 60.9% for each of oxytetracycline and trimethoprim-

sulphamethoxazole, 51.6% nalidixic acid, 37.5% nitrofurantoin and 32.6% chloramphenicol.

1.5 Antibiotics Used in The Study

Antibiotics are natural compounds. They are produced by a wide range of fungi and bacteria for inhibiting or killing other microorganisms. Many natural antibiotics have been structurally modified in the laboratory to enhance their efficacy. These are said to be semisynthetic antibiotics (Madigan and Martinko, 2006). In this study four antibiotics; ampicillin, chloramphenicol, kanamycin and streptomycin were used.

1.5.1 Action Mechanisms of Antibiotics

The susceptibility of microorganisms to individual antibiotics varies significantly. Important targets of antibiotics in bacteria are ribosomes (translation), the cell wall, the cytoplasmic membrane, and the DNA replication and transcription (Madigan and Martinko, 2006).

1.5.1.1 Ampicillin

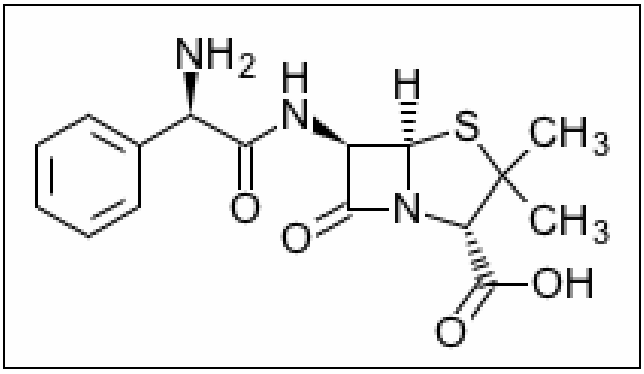


Figure 1.1 The chemical structure of ampicillin

Ampicillin, a β-lactam antibiotic, is the semisynthetic penicillin (Madigan and Martinko, 2006). It binds to and inhibits a number of enzymes in the bacterial membrane that are involved in the synthesis of the cell wall (Waxman and Strominger, 1983).

The ampicillin resistance (*amp*^{r}) gene is carried on the plasmid codes for an enzyme that is secreted into the periplasmic space of the bacterium, where it catalyzes hydrolysis of the β-lactam ring, with concomitant detoxification of the drug (Sykes and Mathew, 1976). In gram-negative bacteria, several β-lactam resistance genes are located as cassettes in class 1 integrons (Sørum and L'Abée-Lund, 2002). Normally *bla* genes in gram-positive bacteria such as staphylococci are transposon located on small transferable or mobilizable plasmids (Yazdankhah *et al.*, 2000; Sidhu *et al*., 2001). Besides**,** the *bla* genes have been found in staphylococci from humans, dairy cattle and pet animals and have also been found in the environment of a small animal clinic (Sørum and L'Abée-Lund, 2002).

1.5.1.2 Chloramphenicol

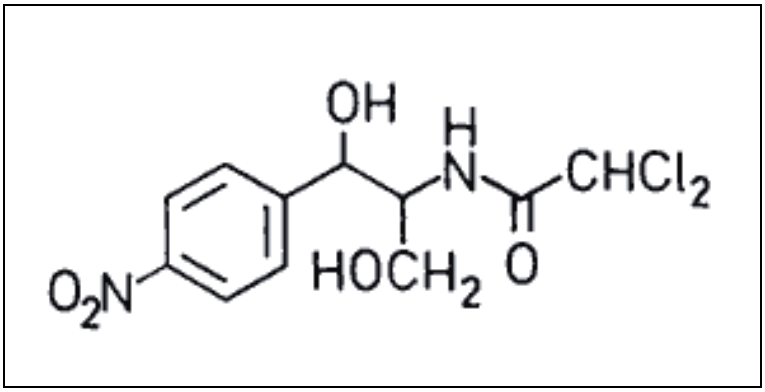


Figure 1.2 The chemical structure of chloramphenicol

Chloramphenicol is an aromatic compound (Madigan and Martinko, 2006). It binds to the ribosomal 50S subunit and inhibits protein synthesis (Maniatis *et al*., 1989). Thus it is specific for ribosomes of bacteria (Madigan and Martinko, 2006). The chloramphenicol resistance (*cat*) gene codes for a tetrameric, cytosolic protein that, in the presence of acetyl coenzyme A, catalyzes the formation of hydroxyl acetoxy derivatives of chloramphenicol that are unable to bind to ribosomes (Shaw *et al*., 1979). This protein is an R plasmid-encoded enzyme (Madigan and Martinko, 2006).

1.5.1.3 Kanamycin and Streptomycin

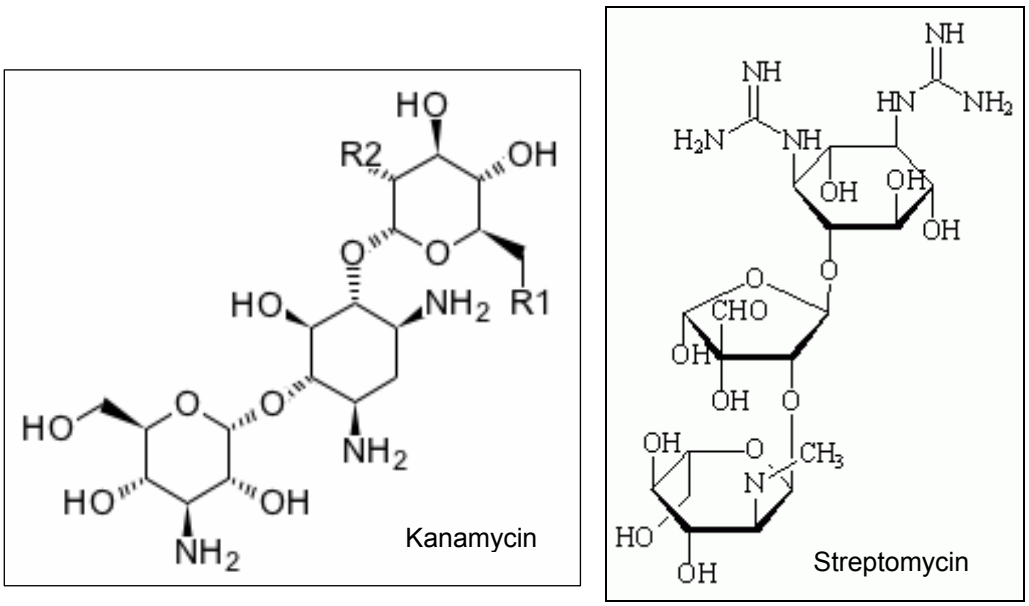


Figure 1.3 The chemical structures of kanamycin and streptomycin

Kanamycin and streptomycin are aminoglycoside antibiotics containing amino sugars bonded by glycosidic linkages to other amino sugars. The aminoglycosides inhibit protein synthesis at the 30S subunit of the ribosome (Madigan and Martinko, 2006).

Resistance towards aminoglycosides is caused by enzymes that chemically modify the aminoglycoside molecule. These enzymes may be encoded by a variety of gene cassettes in class 1 integrons. They encode acetyltransferases and at least one phosphotransferase that modify the aminoglycoside molecule. The aminoglycoside-modifying gene cassettes are as widespread as the class 1 integrons (Sørum and L'Abée-Lund, 2002). As an example the *aadA1* (e.g. *aad* A series, and B) cassette can be found in human *E. coli* (Naas *et al.*, 2000), in *E. Coli* from domesticated birds (Bass *et al.*, 1999), in *Salmonella* isolates from wild

birds in Southeastern USA (Hudson *et al.*, 2000), in *V. cholerae* O1 El Tor from Albany and Italy (Falbo *et al.*, 1999) and from the fish pathogen *A. salmonicida* (L'Abe´e-Lund and Sørum, 2001).

The aminoglycoside resistance gene pair *strA–strB* is not a gene cassette, and consequently is found independently from class 1 integrons. This gene pair occurs in bacteria from various environments. In human- and animal-associated bacteria, *strA–strB* are often carried by small RSF1010-like plasmids (Rådström *et al.*, 1991), while in plant pathogenic bacteria (Schnabel and Jones, 1999) and in a fish pathogenic bacterium (L'Abe´e-Lund and Sørum, 2000) *strA– strB* are included in Tn5393-like transposons that reside on large conjugative plasmids. This indicates genetic communication between such distinctly different environments as the human being, the soil and the marine ecosystem.

1.6 Aim of this study

Our aim was to investigate the extent of antibiotic resistance in fish mucus dwelling bacteria in a freshwater environment. This particular curiosity arose from a previous joint research conducted in Gözen's and Beklioğlu's laboratories. Gözen found that it was not possible to eliminate fish surface associated bacteria with the application of standardized concentrations of ampicillin and kanamycin. She administered the antibiotics directly into the fish tank. In this thesis the antibiotic resistance studies was conducted on individual isolates and the isolates were administered additional two antibiotics (streptomycin and chloramphenicol). We explored the possibility of attributing the multiple antibiotics resistance to plasmids as well.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Table2.1 Materials and suppliers

Chemicals	Suppliers	Chemicals	Suppliers
Tryptone	Fluka	Chloramphenicol	Sigma
Yeast extract	Difco	Trizma Base	Sigma
NaCl	Merck	EDTA	Sigma
Agar	Pronadisa	Lysozyme	Applichem
Peptone	Pronadisa	NaOH	Aldrich
Glucose	Sigma	Eosin methylene blue agar	Applichem
$MgSO4.7H_2O$	Merck	Ethanol	Riedel-de Haen
K_2HPO_4	Sigma-Aldrich	Glycerol	Merck
Beef extract	Pronadisa	SDS	Sigma
Tryptic soy broth	Difco	$C_2H_3KO_2$	Applichem
Na_2HPO_4	Sigma	Glacial acetic acid	Merck
KH_2PO_4	Merck	Phenol:Chloroform:Isoamyl Alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA	Sigma
NH_4Cl	Applichem	Ribonuclease A (DNAase free)	Applichem
Ampicillin sodium salt	Applichem	Agarose low EEO	Applichem
Streptomycin sulfate salt	Sigma-Aldrich	Ethidium Bromide	Applichem
Kanamycin sulfate	Applichem	Bromophenol blue	Applichem
DNA ladder (fragment size in 10 kbp - 0.5 kbp)	Applichem	Plasmid miniprep purification kit	GeneMark

2.2 Equipments

Table 2.2 Equipments and suppliers

Equipments	Suppliers
Analytical balance	Precisa
pH meter	Orion
Autoclave	Nüve
Laminar flow cabinet	Teknomar
Deep freezer (-70ºC)	Nuaire
Incubator	Binder
Shaker	Medline
Magnetic stirrer	Velp scientifica
Electrophoresis tank and accessories	Biometra
Centrifuge	Sigma
Vortex	Nüve
Microwave owen	Arçelik
Power supply	Biometra
Gel illuminating system	Vilber lourmat
Waterbath	Nüve

2.3 Media

The components of all media used in this study were given in Appendix A.

NA, PA and YE media was prepared according to Atlas and Parks (1993); LB, M9 and MS media was prepared according to Maniatis *et al.* (1989).

2.4 Antibiotics

Table 2.3 Antibiotic solutions

	Stock solution	
	Concentration	Storage
Ampicillin (Am)	1) 50 mg/ml in H_2O 2)100 mg/ml in H_2O	-20 ºC
Chloramphenicol (Cm)	1) 34 mg/ml in ethanol 2) 68 mg/ml in ethanol	-20 ºC
Kanamycin (Kc)	1)10 mg/ml in H_2O 2) 20 mg/ml in H_2O	-20 ºC
Streptomycin (Sm)	1)10 mg/ml in H_2O 2) 20 mg/ml in H_2O	-20 ºC

The stock solutions of antibiotics dissolved in H_2O were sterilized by filtration through 0.22-micron filters. For chloramphenicol which is dissolved in alcohol, the alcohol effect bioassays were also conducted. Ethanol had no growth inhibitory effect in the concentrations applied throughout the experiments. The solutions were stored in darkness.

All antibiotics were added to the media (broth or agar) after autoclaving and cooling to 45-50ºC from stock solutions under laminar flow.

2.5 Collection of Fish Mucus

The morphologically similar freshwater fish species, *Alburnus alburnus* (bleak), were obtained from the Lake Mogan, Ankara. Before collecting epidermal mucus, fish were kept in lake water, which was

filtered through 0.45-µm filters (millipore), for at least 24 hours in the laboratory (during this time the water was continuously air-bubbled).

Mucus was scraped from the dorsal body of 3 or 4 fish (on the average 10-14 cm) using a sterile plastic spatula. Ventral skin mucus was not collected to avoid intestinal and sperm contamination. The skin mucus harvest was accumulated in sterile distilled water. It was mixed by vortex and kept on ice during the experiments. The weight of the scraped mucus specimen was recorded.

2.6 Processing of the Fish Mucus Sample

The studies were carried out immediately after the mucus was collected. The specimen in distilled water was diluted up to 10^4 times. The bacterial suspension in dilutions and without dilution were inoculated in 100 µl volumes onto 9 different solid media (LB, LB with 1/10 NaCl, LB without NaCl, P agar, Van Niel's yeast extract agar, Yeast extract agar, TSA, MS, M9 minimal agar) with spread plate method in triplicates. The plates stayed in an incubator for 1-7 days at 28 ºC.

2.7 Enumeration of Total Cultivable Mucus Dwelling Bacteria

The bacteria belonging to various populations dwelling in fish mucus grew on agar plates during the period of incubation. The numbers of colony-forming units (cfu) were recorded by regarding the plates containing cfu's between 30-300. The bacterial densities then expressed in terms of per mg of mucus.

2.8 Obtaining Isolates

Colonies appeared in different morphologies and colors following the incubation. Representative colonies of similar colonial morphology were picked up by using an inoculating loop and streaked onto corresponding media for purification. Colonies to be isolated were selected from all 9 media tested in this study. Pure cultures were kept at -70°C in PBS (phosphate-buffered saline) including 30% glycerol for long term storage (see Appendix B).

2.9 Enumeration of Antibiotic Resistant Bacteria

The specimen collected from fish mucus was tested for antibiotic sensitivity to ampicillin, kanamycin, chloramphenicol, and streptomycin. Also the sensitivity to cocktail of these four antibiotics was assayed. Antibiotic stock solutions incorporated into 9 different solid media (LB, LB with 1/10 NaCl, LB without NaCl, P agar, Van Niel's yeast extract agar, Yeast extract agar, TSA, MS, M9 minimal agar). The final concentrations of antibiotics in plates were 20 μg/ml for ampicillin, 25 μg/ml for chloramphenicol, 10 μg/ml for kanamycin and streptomycin (Maniatis *et al*., 1989). The specimen was diluted up to 10^4 times. The bacteria in dilutions and without dilution were inoculated in 100 µl volumes onto plates with spread plate method in triplicates. The inoculated solid media were incubated at 28 ºC for 24-48 hours in an incubator. After incubation the number of colony forming units was recorded and expressed as per mg of mucus.

2.10 Gram Reactions of Bacterial Isolates

The isolates were differentiated in terms of wall structures as Gram-negative or Gram-positive. The isolates were inoculated onto EMB agar which permits the growth of gram negative bacteria only. The growth was recorded as Gram-negative.

2.11 Determination of Antibiotic Sensitivity of Isolates

Antibiotic sensitivity of each isolate was determined by tube dilution technique (Madigan and Martinko, 2006). The fresh cultures of the isolates grown in 9 different agar media tested were inoculated into corresponding broth media amended with increasing concentrations of each antibiotic. The starting concentrations of antibiotics were decided according to Kannan *et al*. (2006) of ampicillin and streptomycin in media were 3.1, 6.25, 12.5, 25, 50 and 100 μg/ml, kanamycin was 2.15, 4.35, 8.75, 17.5, 35 and 70 μg/ml and chloramphenicol was 1.85, 3.75, 7.5, 15, 30 and 60 μg/ml. Later the tubes were incubated at 28ºC for 24-48 hours at 200 rpm and observed for bacterial growth. Upon recording the cfu's the antibiotic concentrations were increased further. The applied antibiotic doses were shown in Table 2.4.

Table 2.4 Antibiotic doses for tube dilution method

Antibiotic	**Antibiotic doses as μg/ml**									
Ampicillin	3.1	6.25	12.5	25	50	100	200	400	800	1600
Streptomycin										
Kanamycin	2.15	4.35	8.75	17.5	35	70	140	280	560	1120
Chloramphenicol	1.85	3.75	7.5	15	30	60	120	240	480	960

2.12 Isolation of Plasmid DNA

All isolates were screened for the presence of plasmids. Mini preps were made according to standard procedure described by Maniatis *et al*. (1989) with minor modifications as lysozyme and phenol:chloroform:isoamyl alcohol inclusion.

2.12.1 Small-Scale Preparations of Plasmid DNA by Using the Alkaline Lysis Method

The principle of the method is selective alkaline denaturation of high molecular weight chromosomal DNA while covalently closed circular DNA remains double-stranded. Large and small plasmid DNAs have been extracted with this method (Birnboim and Doly, 1979).

The preparation of buffers used in plasmid isolation was explained in Appendix C.

2.12.1.1 Harvesting of Bacteria

A single bacterial colony was transferred into 2 ml of growth medium containing the appropriate antibiotic in a loosely capped 15-ml tube. The culture was incubated for 24-48 hours at 28 ºC with vigorous shaking. The 1.5 ml of the culture was transferred into an Eppendorf tube. It was centrifuged at 12,000 g for 30 seconds at 4 ºC in a Sigma 1-14 micro centrifuge. The supernatant was drawn off and bacterial pellets obtained.

2.12.1.2 Alkaline Lysis of Bacteria

The bacterial pellet was resuspended in 100 μl ice-cold Solution I containing lysozyme (2.5 mg/ml), freshly prepared from crystalline lysozyme. The bacterial pellet was completely dispersed by vortexing and incubated in 37ºC incubator for 30 minutes for complete lysis. At the end of this time, the 200 μl of freshly prepared Solution II was added and mixed by inverting the tubes rapidly 5 times after closing the tube tightly. The tube was stored on ice. The 150 μl of ice-cold Solution III was added and tubes were vortexed gently in an inverted position for 10 seconds to disperse Solution III through the viscous bacterial lysate. The tubes were stored on ice for 3-5 minutes. After centrifugation at 12,000 g for 5 minutes at 4 ºC, the supernatants were transferred to fresh Eppendorf tubes. An equal volume of phenol:chloroform:isoamyl alcohol was added to the supernatant. It was mixed by vortexing and centrifuged at 12,000 g for 2 minutes at 4 ºC. The upper phase was transferred to a fresh Eppendorf tube. The ds DNA were precipitated with 2 volumes of ethanol at room temperature. It was mixed by vortexing. The mixture was allowed to stand for 2 minutes at room temperature and centrifuged at 12,000 g for 5 minutes at 4 ºC. The supernatant was removed. The tube was stood in an inverted position on a paper towel to allow all of the fluid to drain away. The pellet of ds DNA was rinsed with 1 ml of 70% ethanol at 4 ºC. Again the supernatant was removed. The pellet of nucleic acid was allowed to dry in the air for 10 minutes. In the last stage, the nucleic acids were redissolved in 50 μl of TE (ph 8.0) containing DNase free pancreatic RNase (20 μg/ ml). The DNA was vortexed briefly and stored at -20 ºC.

Pancreatic RNase (RNase A) at a concentration of 10 mg/ml was dissolved in 10 mM Tris.Cl (pH 7.5), 15 mM NaCl. It was heated to 100 ºC for 15 minutes and allowed to cool slowly to room temperature. Then it was dispensed into aliquots and stored at -20 ºC.

2.13 Agarose Gel Electrophoresis

Electrophoresis of plasmid DNA was performed 0.9% agarose gel run at 80 V for 90 minutes.

2.13.1 Preparation of an Agarose Gel

Agarose gel was prepared by adding 0.9 g to 100 ml of TAE (Tris-Acetate-EDTA) buffer. The slurry in the Erlenmeyer flask was heated in a microwave oven until all of the grains of agarose dissolved.

The solution was cooled to approximately 60 ºC and ethidium bromide was added from a stock solution of 10 mg/ml in distilled water to a final concentration of 0.5 μg/ml and the solution was mixed thoroughly.

The warm agarose solution was poured into the mold. The gel was allowed to solidify completely for approximately 20-40 minutes at room temperature.

The electrophoresis tank was filled with approximately 1200 ml of TAE buffer. The comb was removed from the gel and the gel in the plastic tray was mounted in the tank so that the slots of the gel faced towards the negative pole-cathode. TAE buffer was added to the tank until the gel was covered to a depth of about 1mm.

The 10 μl of plasmid DNA sample was mixed with 2 μl of gel-loading buffer by sucking in and out of a micropipette. The mixture was slowly loaded into the wells of the submerged gel. The power supply was

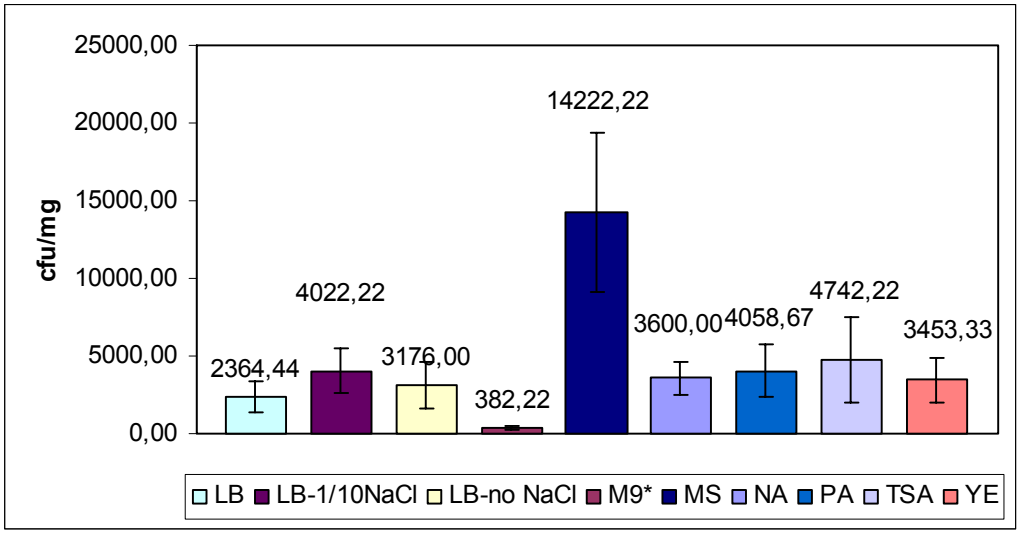
set to the constant voltage (80 volts). The gel was run for 90 minutes until the bromophenol blue dye-front have migrated to the bottom of the gel and then examined under UV light.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Enumeration of Total Cultivable Bacteria

The fish mucus samples were inoculated onto 9 different solid media (LB, LB with 1/10 NaCl, LB without NaCl, P agar, Van Niel's yeast extract agar, Yeast extract agar, TSA, MS, M9 minimal agar) to obtain total counts of mucus-dwelling bacteria. The comparisons of the total numbers as cfu/mg between the 9 media according to sampling season are illustrated in Figure 3.1 and 3.2. Also Figure 3.3 demonstrates the comparison of total numbers of bacteria in spring vs. autumn.

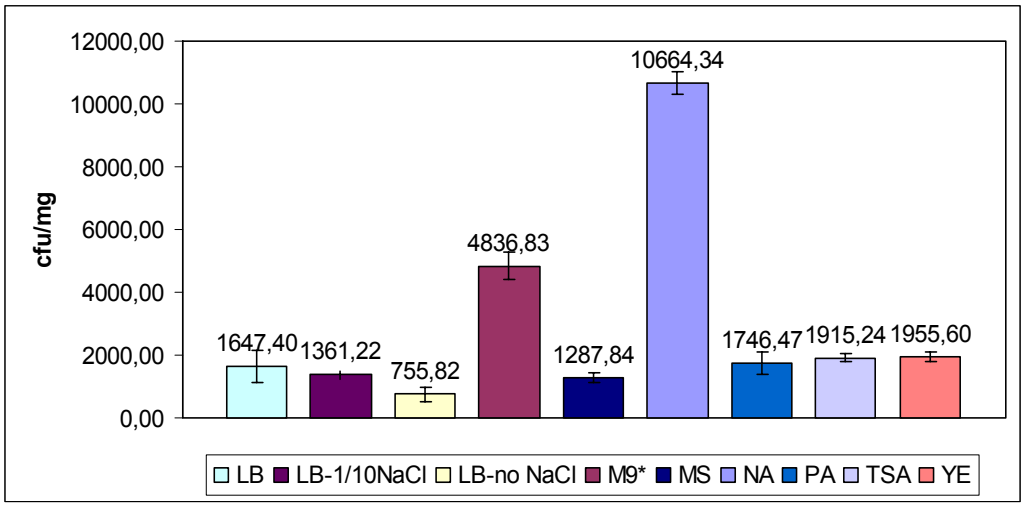


* Indicates that the plate count contained the data out of 30-300 numbers

Figure 3.1 The results of total counts (cfu/mg) of fish mucus-dwelling bacteria on 9 different solid media for samples collected in spring.

There were distinct differences between MS and M9 media at spring counts. MS had the highest and M9 had the lowest cfu/mg of

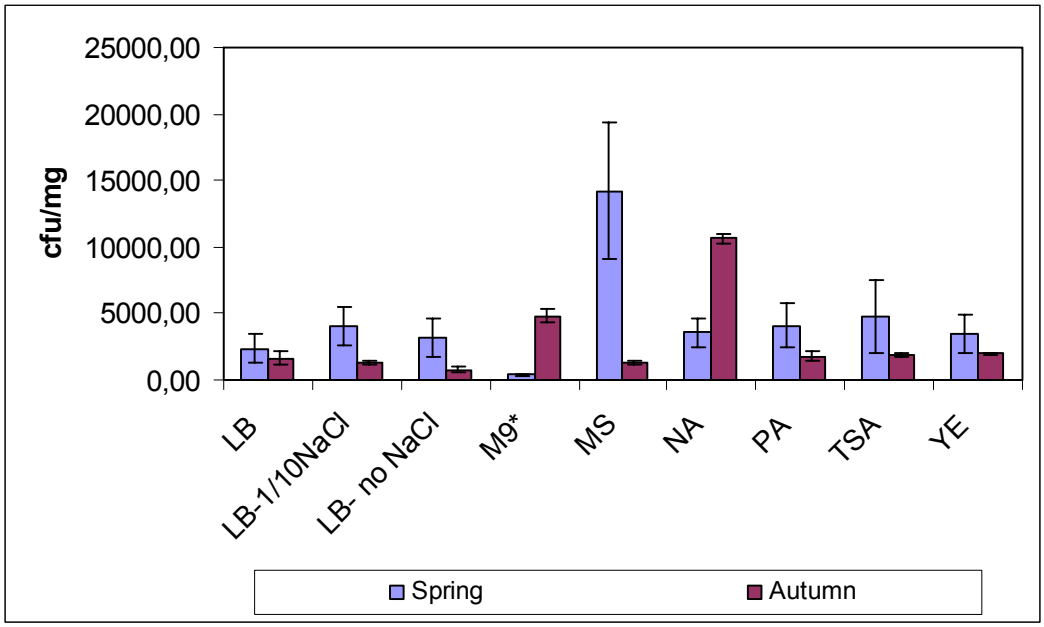
counts. The LB, LB-1/10 NaCl, LB-no NaCl, NA, PA, TSA and YE had similar cfu/mg of counts (Figure 3.1).



* Indicates that the plate count contained the data out of 30-300 numbers

Figure 3.2 The results of total counts (cfu/mg) of fish mucus-dwelling bacteria on 9 different solid media for samples collected in autumn.

According to the results of autumn counting (Figure 3.2), NA had the highest cfu/mg of counts. Also the value of M9 was higher than the others. The cfu/mg values of other seven media were close to each other. But the lowest value belonged to the LB without NaCl.



* Indicates that the plate count contained the data out of 30-300 numbers

Figure 3.3 Comparison between the total counts (cfu/mg) of fish mucus-dwelling bacteria on 9 different solid media in spring vs. autumn samples.

The total counts were higher in the spring sample in seven of the nine media. Also MS had the highest and M9 had the lowest cfu/mg at spring counts when compared to autumn counts (Figure 3.3).

In this study, seven different media (LB, M9, MS, NA, PA, TSA, YE) and also two different NaCl composition (1/10 NaCl and no NaCl) for LB medium were used to increase the diversity of cultivable bacteria. It is pointed out the importance of the use of the multiple-media set for plate counting efficiency in the study of Balestra and Misaghi (1997). The variations in numbers are clearly observed especially at the M9, MS and NA media. This diversity may come from the nutritional differences of among the cultivable bacterial populations. The MS and M9 are minimal media. But M9 is further enriched in terms of inorganic salts. NA is a true rich medium and also has variety of inorganic salts. Although the TSA is similar to NA according to salt composition, there is no difference in the

value of cfu/mg from other complex media. YE, LB, PA are complex media contain just NaCl as inorganic salt.

Furthermore, the direct comparison of antibiotic resistance to typical antibiotics even for the same bacteria is very difficult because of the different growth conditions, especially medium composition. For example, Ray and Newton (1991) monitored the stability of tetracycline and chlortetracycline grown in trypticase soy broth by high performance liquid chromatography. Chlortetracycline appeared to be actively degraded in less than 5 days in TSB, while both tetracyclines proved far more stable in water. It was inferred that in this case, the cause for inactivation of the drugs was to be found in the medium composition.

There are several studies about the increasing bacterial density (Lamberti and Resh, 1983; Baulch *et al*., 2005) and total microbial biomass (Pernthaler *et al*., 1998) with temperature increase in freshwaters. However, Yannarell *et al*. (2003) found that the composition of the lake bacterial communities was stable in the spring and the fall. Also results obtained from Lake Jeziorak Mały (Walczak and Donderski, 2004) indicate that the maximum number of neustonic bacteria was found in autumn. We also did our sampling of fish in spring and in autumn. However, our results reflect the number of fish surface mucus-dwelling bacteria, while former reports were related to planktonic bacteria (Figure 3.3).

3.2 Isolates

The 60 isolates were selected from skin mucus of freshwater fish according to morphology and colors. The morphological profiles of all

isolates and corresponding media are presented in Table 3.1. After isolation, eleven isolates (fs03, fs07, fs11, fs25, fs29, fs39, fs41, fs46, fs47, fs51, and fs60) lost their viability during storage. Therefore the following values reflect the experiments on 49 isolates.

There was different number of isolates that could be cultivated on media used in the experiments. The highest morphological differences among isolates were obtained from MS, PA and NA solid media. Apparently, most bacteria exhibit different nutritional requirements (Garland, 1995; Misaghi and Grogan, 1969), which are not met on one single medium. Furthermore, fast growing bacteria may exclude the slow growing ones on a solid culture by depleting nutrients and by applying antagonistic measures (Balestra and Misaghi, 1997).

Table 3.1 The morphological profiles of all the isolates with corresponding media

name of isolates	colonial surface charecteristics					colony edge charecteristics			colonial elevations					color of colony	name of medium
	concentric	contoured	radiated	smooth	wrinkled	circular	irregular	Flamentous	Raised	flat	Pulvinate	Convex	Umbonate		
FS01	√						u			√				White	MS
FS02	√					√						√		Bold White	MS
FS03 **vl**				√		√					√			Light White	MS
FS04	√					√						√		Salmon	MS
FS05				√		√			√					Yellow	MS
FS06				√			l			√				Yellow	MS
FS07 **vl**							u			√				Light Yellow	MS
FS08					√		l						√	Bold White	MS
FS09	√					√						√		Transparent & White Center	MS MS
FS10	√					√					√			Transparent-Yellow-Transparent	MS
FS11 **vl**		√					√						√	Yellow	MS
FS12				√		√			√					Dark Yellow	LB
FS13	√						u			√				Light Yellow	LB
FS14				√		√					√			Orange	LB
FS15	√						s			√				Transparent& Light Yellow Center	LB
FS16	√						u			√				Transparent	LB
FS17				√			u			√				White	TSA
FS18				√		√					√			Orange	TSA
FS19				√		√				√				Yellow	TSA
FS20			√				u			√				Transparent & Yellow Center	TSA
FS21				√			s					√		White	TSA
FS22				√		√						√		White	LB-NaCl*
FS23				√			u		√					Yellowish	LB-NaCl*
FS24				√		√						√		Orange	LB-NaCl*
FS25 **vl**				√		√					√			Dark Yellow	LB-NaCl*
FS26	√						s			√				Transparent-White-Transparent	LB-NaCl*

Table 3.1 (Cont.) The morphological profiles of all the isolates with corresponding media

name of isolates	colonial surface charecteristics					colony edge charecteristics			colonial elevations					color of colony	name of medium
	concentric	contoured	radiated	smooth	wrinkled	circular	irregular	Flamentous	Raised	flat	Pulvinate	Convex	Umbonate		
FS27	√						u			√				Transparent & Yellow Center	LB-NaCl*
FS28	√					√				√				Transparent-Yellowish-Transparent	PA
FS29 **vl**				√		√							√	White	PA
FS30	√					√						√		Orange	PA
FS31					√		r	√					√	Orange	PA
FS32	√					√						√		Orange& Yellowish Center	PA
FS33				√			u			√				Transparent-Yellowish-Transparent	PA
FS34	√					√				√				Dark Yellow	PA
FS35	√						s			√				Transparent & Yellow Center	PA
FS36					√		u			√				Transparent-Yellowish-Transparent	PA
FS37	√						s			√				Transparent & Orange Center	1/10NaCl**
FS38				√			u			√				White	1/10NaCl**
FS39 **vl**				√		√				√				Orange	1/10NaCl**
FS40	√						u			√				Transparent & Yellow Center	1/10NaCl**
FS41 **vl**				√		√						√		Bold White	1/10NaCl**
FS42				√		√						√		Yellow	NA
FS43	√						u			√				Yellow	NA
FS44			√				u		√					White & Pink Center	NA
FS45					√			√					√	Bold White	NA

Table 3.1 (Cont.) The morphological profiles of all the isolates with corresponding media

name of isolates	colonial surface charecteristics					colony edge charecteristics			colonial elevations					color of colony	name of medium
	concentric	contoured	radiated	smooth	wrinkled	circular	irregular	Flamentous	Raised	flat	Pulvinate	Convex	Umbonate		
FS46 **vl**				√		√						√		Dark Pink	NA
FS47 **vl**								√						flamentous through medium	NA
FS48				√		√						√		Orange	NA
FS49	√					√				√				Transparent & Yellow Center	NA
FS50				√		√						√		Dark Yellow	NA
FS51 **vl**					√	√			√					Transparent-Light Yellow	NA
FS52				√			u			√				Transparent-Yellowish	YE
FS53		√				√				√				White	YE
FS54	√						u			√				Transparent & Yellow Center	YE
FS55				√		√						√		Orange	YE
FS56	√					√				√				Transparent	YE
FS57				√		√						√		Bold White	YE
FS58				√		√			√					Transparent-Yellowish	NA
FS59				√		√			√					Yellow & Orange Center	PA
FS60 **vl**				√		√			√					Orange & Yellow Center	YE

u : undulate
s : serrated
r : rocky
l : lobate
vl : viability lost
* LB-NaCl refers to LB without NaCl
**1/10-NaCl refers to LB with 1/10 NaCl

3.3 Enumeration of Antibiotic Resistant Bacteria

Antibiotic resistance comparisons firstly were made of each of the two antibiotics (ampicillin and kanamycin) and mixture of them on the solid media. Figure 3.4 demonstrates the results of counts as cfu/mg on NA, NA with ampicillin, NA with kanamycin and NA with the mixture of ampicillin and kanamycin media. The comparison of counts as cfu/mg between the media without antibiotic and the media with the mixture of ampicillin and kanamycin on NA, LB and YE is illustrated in Figure 3.5.

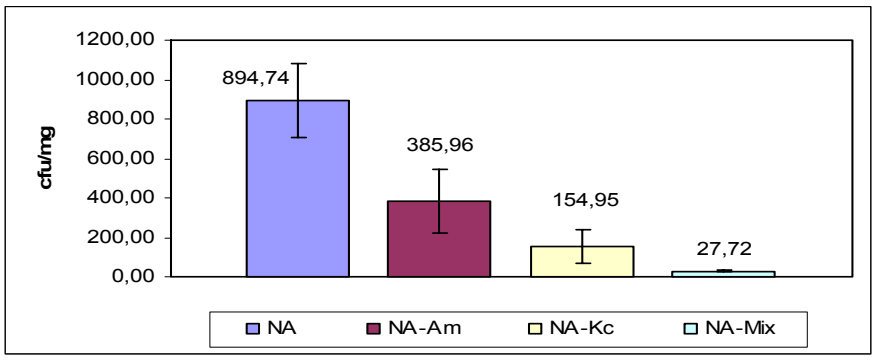


Figure 3.4 Comparison between the resistant counts of Am (20 µg/ml), Kc (10 µg/ml), mixture of Am and Kc and control (no antibiotic) counts on NA medium.

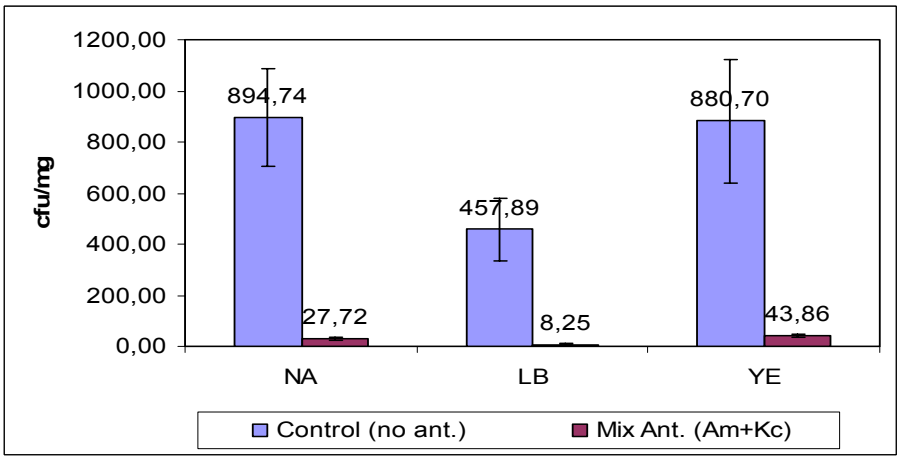


Figure 3.5 Comparison between the resistant counts of mixture of Am and Kc and control (no antibiotic) counts on NA, LB and YE media.

The bacterial counts were clearly lower on the antibiotic containing medium compared with the counts of control medium which had no antibiotic. This decrease is nearly two times on the ampicillin containing medium, six times on the kanamycin containing medium and thirty two times on the antibiotic mixture containing medium. Although the mixture of two antibiotics clearly decreased the counts, it did not completely eliminate the cultivable mucus-dwelling bacteria of freshwater fish (Figure 3.4). The similar results also were obtained from the LB and YE media (Figure 3.5).

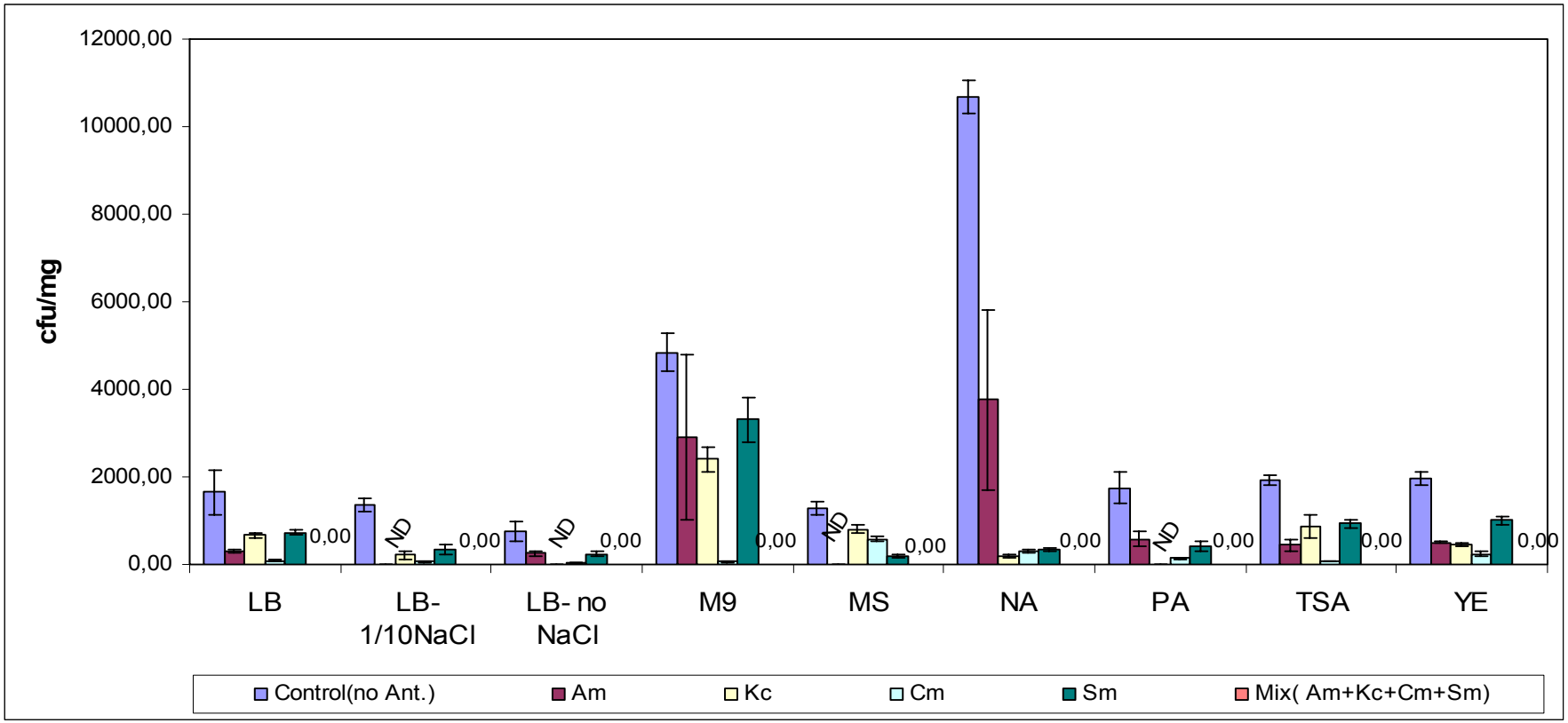
These results are in agreement with those of Beklioğlu *et al.* (2006) in terms of the decrease in numbers as a trend. They added the ampicillin and kanamycin mixture directly into the water and then sampled bacteria from fish surface mucus. We, however, in this study applied antibiotics directly in to the growth media followed by the sampling from fish mucus.

Secondly, antibiotic resistance comparisons were made each of the four antibiotics (ampicillin, kanamycin, chloramphenicol and streptomycin) and the mixture of them on the 9 different solid media (Figure 3.6).

The bacterial counts were clearly lower on the antibiotic containing media compared with the counts of corresponding control media which had no antibiotics supplemented. Chloramphenicol containing media had the lowest cfu/mg values for all of the media except that MS and NA. For MS streptomycin containing medium and for NA kanamycin containing medium had the lowest cfu/mg values. The highest cfu/mg values for LB, LB-1/10NaCl, M9, TSA and YE obtained in the presence of streptomycin. For LB-noNaCl, NA and PA, the highest cfu/mg values obtained in the

presence of ampicillin. Kanamycin containing MS had the highest cfu/mg of counts. Growth was not detected in the mixture of four antibiotics.

Beklioğlu *et al.* (2006) used ampicillin and kanamycin mixture, to more effectively remove the mucus dwelling bacteria of fish. Streptomycin and chloramphenicol was added to the mixture of antibiotics in this study with the expectation to find a cocktail composition for total kill. Our results showed that we indeed met the expectation. We clearly demonstrated that the mixture of ampicillin, kanamycin, streptomycin and chloramphenicol can be applied for the total elimination of the cultivable mucus-dwelling bacteria of freshwater fish.



ND indicates that no data could be obtained for corresponding media at the first inoculation. The average values of cfu/mg and standard deviations for second inoculations, respectively, were: LB-1/10Nacl-Am 3053,61 and 445,83; LB-noNaCl-Kc 1480,19 and 164,54; MS-Am 2071,68 and 1080,07; PA-Kc 2797,20 and 684,06.

Figure 3.6 Comparison between the resistant counts of Am (20 µg/ml), Kc (10 µg/ml), Cm (25 µg/ml), Sm (10 µg/ml), mixture of Am, Kc, Cm and Sm and control (no antibiotic) counts on 9 different solid media.

3.4 Differentiating the Bacterial Isolates as Gram-Positive and Gram-Negative

The isolates were inoculated onto EMB agar to differentiate in terms of wall structures as Gram-negative or Gram-positive.

Table 3.2 The results of growth onto the EMB solid medium

isolates	growth	isolates	growth	isolates	growth	isolates	growth	isolates	growth
fs01	+	fs14	+	fs24	+	fs36	+	fs50	-
fs02	+	fs15	+	fs26	+	fs37	+	fs52	+
fs04	+	fs16	+	fs27	+	fs38	+	fs53	+
fs05	+	fs17	+	fs28	+	fs40	+	fs54	+
fs06	+	fs18	-	fs30	-	fs42	+	fs55	+
fs08	+	fs19	+	fs31	+	fs43	+	fs56	+
fs09	+	fs20	+	fs32	-	fs44	+	fs57	-
fs10	+	fs21	+	fs33	+	fs45	+	fs58	+
fs12	+	fs22	+	fs34	+	fs48	+	fs59	+
fs13	+	fs23	+	fs35	+	fs49	+		

The growth of isolates on EMB media showed that about the 10% of isolates (5 isolates) were Gram-positive and the 90% of isolates (42 isolates) were Gram-negative.

This was not surprising, as most of the isolates of mucus-dwelling bacteria were identified as gram-negative species in several reports as well as many species of planktonic bacteria (Sigee, 2004; Atlas and Bartha, 1997).

3.5 Determination of Antibiotic Sensitivity of Isolates

Antibiotic resistance level studies were first initiated by using predetermined concentrations and later on based on the first set of results the concentrations applied were increased, the results are shown in the Table 3.3.

Table 3.3 Resistance to antibiotics of surface-mucus associated isolates of a freshwater fish

Name of Isolates	ANTIBIOTICS (µg/ml)			
	Cm	Kc	Sm	Am
fs01	S	S	S	>1600
fs02	30	4,35	50	>1600
fs04	240	>1120	50	>1600
fs05	S	S	3,1	200
fs06	15	S	S	800
fs08	60	S	12,5	>1600
fs09	1,85	S	S	12,5
fs10	>960	>1120	800	>1600
fs12	480	560	400	S
fs13	240	140	400	>1600
fs14	30	8,75	800	100
fs15	120	35	200	400
fs16	60	8,75	50	>1600
fs17	1,85	>1120	800	>1600
fs18	7,5	8,75	100	200
fs19	>960	>1120	>1600	100
fs20	7,5	140	100	>1600
fs21	3,75	280	800	>1600
fs22	240	140	400	>1600
fs23	S	70	50	100
fs24	1,85 **v**	S	S	S
fs26	15	8,75	3,1	400
fs27	60	2,15	S	25
fs28	15	140	200	>1600
fs30	60	35	S	50
fs31	3,75	2,15	3,1	100
fs32	1,85	S	3,1	100
fs33	240	280	200	>1600
fs34	30	1120	>1600	50
fs35	240	70	200	100
fs36	480	2,15	25	800

Table 3.3(Cont.) Resistance to antibiotics of surface-mucus associated isolates of a freshwater fish

Name of Isolates	ANTIBIOTICS (µg/ml)			
	Cm	Kc	Sm	Am
fs37	240	17,5	>1600	>1600
fs38	S	35	50	>1600
fs40	480	4,35	50	100
fs42	480	1120	800	1600
fs43	120	S	6,25	25
fs44	7,5	35	50	25
fs45	>960	>1120	>1600	>1600
fs48	7,5	8,75	25	200
fs49	1,85	35	400	100
fs50	30	2,15	6,25	200
fs52	S	280	>1600	>1600
fs53	30	280	200	800
fs54	480	4,35	6,25	1600
fs55	480	140	6,25	50
fs56	60	4,35	100	>1600
fs57	>960	140	>1600	800
fs58	>960	8,75	400	800
fs59	>960	>1120	>1600	>1600

v : variable result
S : sensitive

According to the data, about 90% of the isolates were resistant to chloramphenicol, about 84% to kanamycin, about 88% to streptomycin and about 96% to ampicillin. Except fs24 and fs12, all the isolates were resistant to ampicillin. Six of the isolates (fs10, fs19, fs45, fs57, fs58, fs59) exhibited high resistance to chloramphenicol (more than 960 µg/ml); six of isolates (fs04, fs10, fs17, fs19, fs45, and fs59) to kanamycin (more than 1120 µg/ml); seven of isolates (fs19, fs34, fs37, fs45, fs52, fs57, fs59) to streptomycin (more than 1600 µg/ml); nineteen of isolates (fs01, fs02, fs04, fs08, fs10, fs13, fs16, fs17, fs20, fs21, fs22, fs28, fs33, fs37, fs38, fs45, fs52, fs56, fs59) to ampicillin (more than 1600 µg/ml). Also among these highly resistant isolates two of them (fs45 and fs59) were resistant to all antibiotics tested at high levels, so that they were still

exhibiting growth at the highest concentrations tested. At this point even higher concentrations were not tried.

Among the isolates only one (fs24) was sensitive to all antibiotics tested. This particular isolate exhibited inconsistent growth from batch to batch at 1.85 µg/ml chloramphenicol. Since the tried concentration was low and the growth was not reproducible, we considered this isolate sensitive to chloramphenicol. Also just one isolate (fs01) was sensitive to three antibiotics namely chloramphenicol, kanamycin and streptomycin. One isolate (fs05) was sensitive to chloramphenicol and kanamycin; two isolates (fs06 and fs09) were sensitive to kanamycin and streptomycin. Three isolates (fs08, fs32, fs43) were shown sensitivity to just kanamycin; two (fs27 and fs30) to just streptomycin; and finally two (fs38 and fs52) to just chloramphenicol.

The data presented in Table 3.3 show that fish surface-mucus associated isolates show in most part high level of resistance towards the four antibiotics tested. A report with similar results for resistance to ampicillin was obtained from bacteria inhabiting Lake Gardno, about 90% in the study of Mudryk (2002), (about 96% in our study). But the resistance of streptomycin was quite different. In our study the streptomycin resistance was 88%, while it was less than 20% in the study of Mudryk (2002). Our results imply that the bacterial populations with high level of resistance to streptomycin can preferentially populate fish surface mucus. Therefore their frequency was found to be higher than that of planktonic counterparts. Or else a more plausible explanation could be that in the lake Gardno the actual frequency of streptomycin resistant bacteria was low. In both lakes, however, the resistant populations were similar in percentage for both in planktonic and mucus-dwelling bacteria.

According to the study of Tendenci and de la Pena (2001), when antimicrobial agents are used more often in an environment, the higher will be the occurrence of resistant microorganisms in that site; this is because exposure to antimicrobials may also result to cross-resistance between other aquaculture antibacterials. The frequency and rates of ascent and dissemination of antibiotic resistance in bacterial population are anticipated to be directly related to the volume of antibiotic use (Anderson and Levin, 1999). But it is an interesting thing in our study that there is high level of resistance at fish mucus dwelling isolates from Lake Mogan where the usage for any antibiotics have not been recorded.

Different studies have reported the incidence of antibiotic and heavy metal resistance in bacteria of fish from various metal-contaminated environments (Miranda *et al.,* 1998; Pathak *et al.*, 2005). Also a substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance (Summers, 2002; Alonso *et al.*, 2001). Bhattacherjee *et al.* (1988) also reported that under the environmental condition of metal stress, organisms adapt faster by the spread of R-factors than by mutation and selection. Additionaly, according to Akinbowale *et al.* (2007) there was a tendency towards a higher frequency of streptomycin resistance among all the metal-resistant *Pseudomonas* isolates compared with the metal-sensitive isolates. This may be due to the streptomycin and metal resistance determinants being co-located in *Pseudomonas* spp. According to this information it may be said that the high resistance towards the antibiotics tested in our study recorded in the fish mucus-dwelling bacteria sampled from Lake Mogan is likely to be related with heavy metal contamination.

3.6 Plasmid DNA Profiles of Isolates

Plasmid isolations were carried out for all viable isolates (49 isolates).

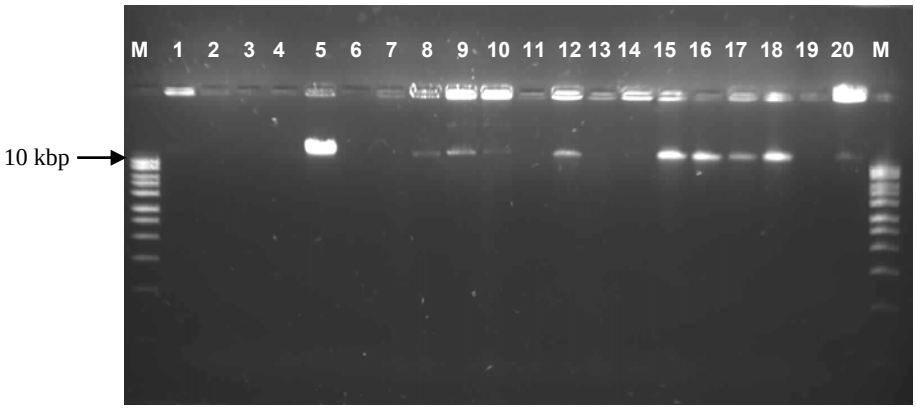


Figure 3.7 Agarose gel electrophoresis of plasmid DNAs from fish mucus-surface isolates: **M-** DNA size marker (10kbp, 8kbp, 6kbp, 5kbp, 4kbp, 3kbp, 2.5kbp, 2kbp, 1.5kbp,1 kbp, 0.5kbp), **1-** fs01, **2-** fs02, **3-** fs04, **4-** fs05, **5-** fs06, **6-** fs08, **7-** fs09, **8-** fs10, **9-** fs12, **10-** fs13, **11-**fs14, **12-** fs15, **13-** fs16, **14-** fs17 ,**15-** fs18, **16-** fs19, **17-** fs20, **18-** fs21, **19-** fs22, **20-** fs23.

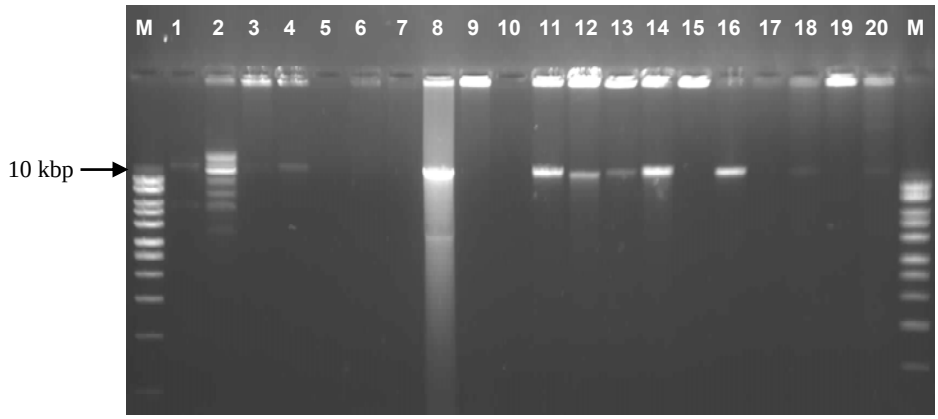


Figure 3.8 Agarose gel electrophoresis of plasmid DNAs from fish mucus-surface isolates: **M-** DNA size marker (10kbp, 8kbp, 6kbp, 5kbp, 4kbp, 3kbp, 2.5kbp, 2kbp, 1.5kbp,1 kbp, 0.5kbp), **1-** fs24, **2-** fs26, **3-** fs27, **4-** fs28, **5-** fs30, **6-** fs31, **7-** fs32, **8-** fs33, **9-** fs34, **10-** fs35, **11-** fs36, **12-** fs37, **13-** fs38, **14-** fs40, **15-** fs42, **16-** fs43, **17-** fs44, **18-** fs45, **19-** fs48, **20-** fs49.

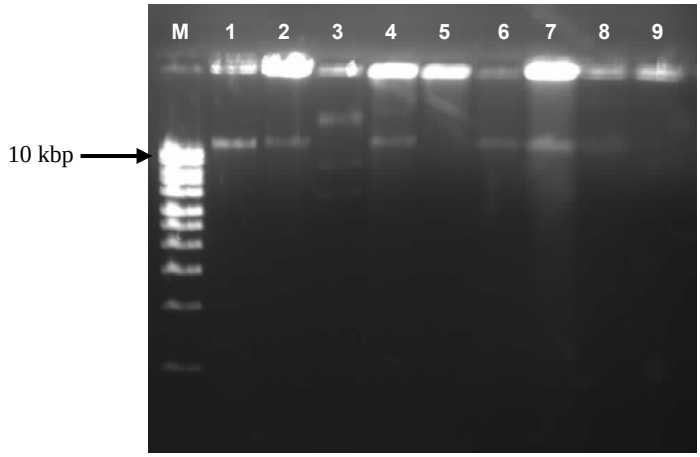


Figure 3.9 Agarose gel electrophoresis of plasmid DNAs from fish mucus-surface isolates: **M-** DNA size marker (10kbp, 8kbp, 6kbp, 5kbp, 4kbp, 3kbp, 2.5kbp, 2kbp, 1.5kbp, 1kbp, 0.5kbp), **1-** fs50, **2-** fs52, **3-** fs53, **4-** fs54, **5-** fs55, **6-** fs56, **7-** fs57, **8-** fs58, **9-** fs59.

As a result of plasmid isolations from 49 bacterial isolates, 28 isolates showed the presence of bands corresponding to high molecular weight fragments on the agarose gels (Figure 3.7, 3.8, 3.9). The multiplicity of these high molecular weight similar bands led us to think that they may not be plasmids but rather fragments of chromosomal DNA appearing as DNA extraction artifacts. Therefore additional electrophoreses were carried out. This time the plasmid isolations were conducted by including reference bacteria containing plasmid or not harboring. These were *Escherichia coli* DH5α harboring pGEMTphrC vector as positive control for Gram-negative isolates and *Bacillus thuringiensis* harboring pHT315Chi vector as positive control for Gram-positive isolates. The same bacterial strains not harboring plasmid DNA were as the negative controls.

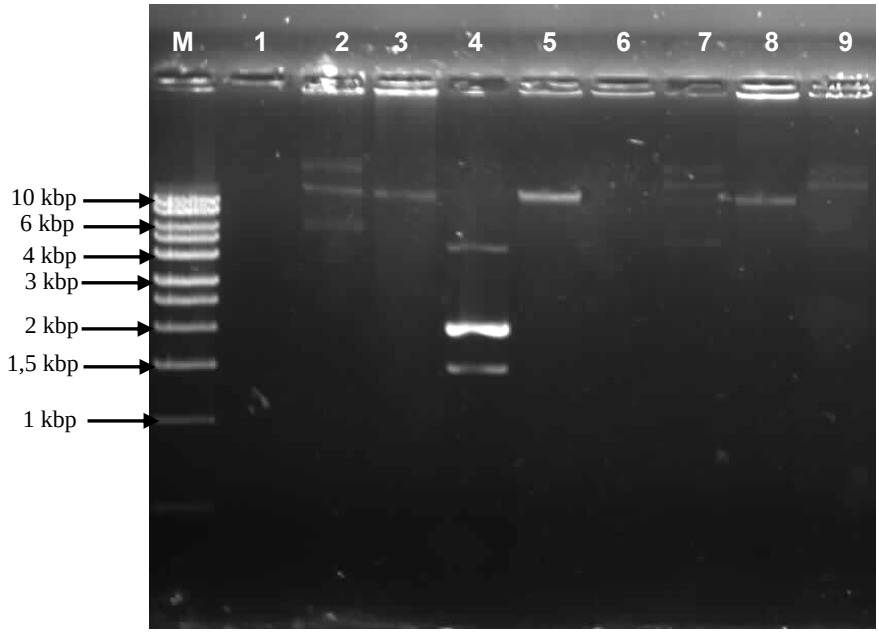


Figure 3.10 Agarose gel electrophoresis of plasmid DNAs from control bacteria and fish mucus-surface isolates: **M-** DNA size marker (10kbp, 8kbp, 6kbp, 5kbp, 4kbp, 3kbp, 2.5kbp, 2kbp, 1.5kbp, 1kbp, 0.5kbp), **1-** *B. thuringiensis*, **2-** *B. thuringiensis* with plasmid, **3-** *E. coli* DH5α, **4-** *E. coli* DH5α with plasmid, **5-** fs06, **6-** fs13, **7-** fs26, **8-** fs52, **9-** fs53.

The electrophoresis of plasmids isolated by alkaline lysis method with freshly prepared reagents on control groups showed again a high molecular weight band in *Escherichia coli* DH5α without plasmid. Followed by this observation we decided to use a commercial plasmid isolation kit. The electrophoresis of plasmids isolated by using a kit is shown in Figure 3.11.

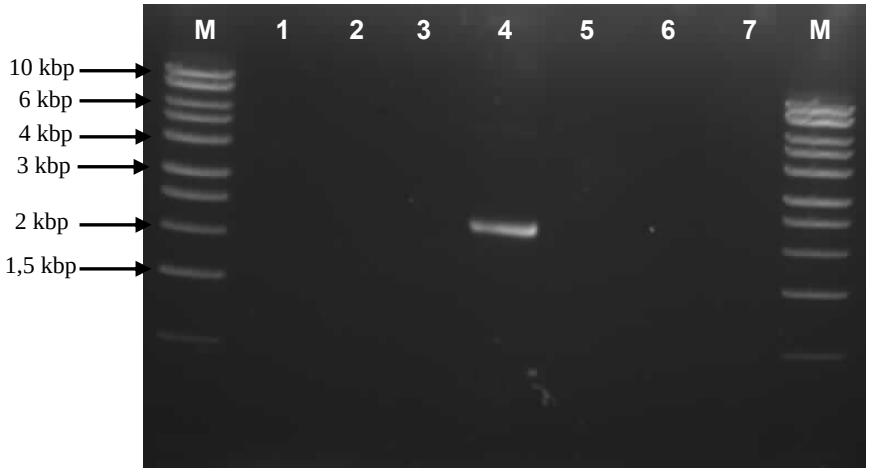


Figure 3.11 Agarose gel electrophoresis of plasmid DNAs from control bacteria and fish mucus-surface isolates: **M-** DNA size marker (10kbp, 8kbp, 6kbp, 5kbp, 4kbp, 3kbp, 2.5kbp, 2kbp, 1.5kbp, 1kbp, 0.5kbp), **1-** *B. thuringiensis*, **2-** *B. thuringiensis* with plasmid, **3-** *E. coli* DH5α, **4-** *E. coli* DH5α with plasmid, **5-** fs26, **6-** fs52, **7-** fs57.

As a result of plasmid isolation by using a commercial kit, just *Escherichia coli* DH5α harboring pGEMTphrC vector was visualized. We could not detect the high molecular weight band appearing in the former gels representing our fish mucus bacterial isolates. In addition, there was not any plasmid band in *Bacillus thuringiensis* harbouring pHT315Chi vector. However, the presence of this vector was confirmed in electrophoresis of plasmid isolation carried out with laboratory prepared reagents (Figure 3.10). Both of the positive control vectors appeared approximately at expected molecular sizes (pGEMTphrC 3.3 kb, pHT315Chi 8.6 kb) in the gels. The results indicated that our isolates do not harbor plasmids detectable with the methods we used (alkaline lysis with lysozyme reagents prepared in the laboratory or by using commercial kit). It appeared that the high molecular weight bands visualized in the lanes of our bacterial isolates were chromosomal fragments.

In this study, even though most of the isolates have high resistance against four antibiotics, there is no correlation between high resistance to antibiotics tested and the presence of plasmids. Indeed, we could not show the presence of plasmids at all. Possibly, some of the resistance genes for antibiotics are located on the chromosomes. In order to attribute the resistance genes to chromosomes or plasmids, we also performed plasmid curing experiments. It was carried out by incubating the isolates overnight at 32°C in corresponding broth medium containing 25 µg/ml of acridine orange. After appropriate dilution, the plates were incubated at 32°C. To select the strains that lost antibiotic resistance, the colonies were transferred to corresponding agar plates amended with the respective antibiotics in appropriate concentrations by replica plating method (Kanan *et al.*, 2006). Although, we tried many times, we were not able to find any isolate that lost ampicillin or chloramphenicol resistance. This also supports that the genes are not be plasmid encoded. Our results suggest that the resistance cassettes may be linked to mobile genetic elements. But of course, we did not carry out any analysis regarding such sequences.

Screening for antibiotic-resistant bacteria in natural ecosystems is highly relevant, and worldwide studies have been carried out to identify environmental reservoirs of bacterial antibiotic-resistance in wild-animal populations and natural water supplies (McKeon *et al.*, 1995; Gõni-Urriza *et al.*, 2000; Sherley *et al.*, 2000; Nascimento *et al.*, 2003). The information derived from studies of pathogenic bacteria may not be representative of all bacteria because it is known that commensal bacteria are also common reservoirs of antibiotic resistance genes (Levy and Marshall, 2004). By and large, our study stresses the importance of the high antibiotic resistance in normal flora rather than individual pathogenic species

CHAPTER 4

CONCLUSION

The total cultivable surface mucus associated bacteria of freshwater fish, *Alburnus alburnus*, obtained from Lake Mogan were enumerated onto nine different solid media. Since the values of cfu/mg varied according to the sampling season and the growth media, the overall higher counts observed in MS growth media for spring sample and in NA growth media for autumn sample.

- The number of bacteria cultured varied in different media. Therefore, certain media compositions favored different bacterial populations. MS medium was found to be consistently giving the highest cfu.

The occurrence of antibiotic resistance to ampicillin, kanamycin and cocktail of both antibiotics of mucus-dwelling bacteria were determined. The results of enumeration showed that the ampicillin resistance was higher than kanamycin resistance. Then streptomycin and chloramphenicol were added to the study and the total elimination of fish mucus associated bacteria was observed. Also, we have demonstrated that chloramphenicol produced the lowest counts in most media when compared with other antibiotics.

- Most of the bacterial isolates from fish mucus exhibited multiple-antibiotic resistance.

A total of sixty isolates were obtained from surface mucus of fish. Highest numbers of isolate were selected in MS, PA and NA solid media. Based on the results of the EMB growth, five isolates were Gram-positive and forty two isolates were Gram-negative within forty nine viable isolates. The forty nine isolates were tested to determine antibiotic resistance to ampicillin and kanamycin, as well as streptomycin and chloramphenicol. Based on these results, it was reported that all the isolates except one were resistant to at least one antibiotic. According to the data, about 90% of the isolates were resistant to chloramphenicol, about 84% to kanamycin, about 88% to streptomycin and about 96% to ampicillin. Also, high level of multiple resistance was observed in most isolates.

- Broth culture antibiotic tests indicated high levels of resistance towards all four antibiotics in most of the isolates.

To determine whether the resistances are plasmid-born or chromosomal, the plasmid isolations were carried out. Electrophoretic analyses hinted that our isolates were not harboring plasmid that could be detected with the methods we used. Therefore, we could not establish a correlation between the plasmid presence and antibiotic resistance.

Finally, regarding the results of our studies we can confidently say that if one needs to eliminate the bacterial populations for one reason or another an inclusive cocktail of antibiotics should be used; otherwise the resistant bacteria soon will be enriched in the experimental setting.

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APPENDIX A

COMPONENTS OF MEDIA USED

1. LB (Luria-Bertani) Agar

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Agar	15.0 g

All components were dissolved in distilled water to have a final volume of 1L. pH was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

2. LB (Luria-Bertani) Agar with 1/10 NaCl

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	1.0 g
Agar	15.0 g

All components were dissolved in distilled water to have a final volume of 1L. pH was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

3. LB (Luria-Bertani) Agar without NaCl

Tryptone	10.0 g
Yeast extract	5.0 g
Agar	15.0 g

All components were dissolved in distilled water to have a final volume of 1L. pH was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

4. P Agar

Peptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
Glucose	1.0 g
Agar	15.0 g

All components were dissolved in distilled water to have a final volume of 1L. pH was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

5. Yeast Extract Agar, Van Niel's (NA)

Yeast extract	10.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Agar	20.0 g

All components were dissolved in distilled water to have a final volume of 1L. pH was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

6. Yeast Extract Agar

Peptone	9.5 g
Yeast extract	7.0 g
Beef extract	5.0 g
NaCl	5.0 g
Agar	15.0 g

All components were dissolved in distilled water to have a final volume of 1L. pH was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

7. Tryptic Soy Agar (TSA)

Formula per liter:

Tryptone (pancreatic digest of casein)	17 g
Soytone (papaic digest of soybean meal)	3 g
Dextrose	2.5 g
NaCl	5 g
K_2HPO_4	2.5 g

It was commercially available. 30.0 g of it and 15.0 g of agar was suspended in 1L of distilled water. pH was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

8. MS Agar

Peptone	1.0 g
Yeast extract	1.0 g
Glucose	1.0 g
Agar	15.0 g

All components were dissolved in distilled water to have a final volume of 1L. pH was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

9. M9 Minimal Agar

M9 salts	200 ml
20% glucose solution	20 ml
Agar	15.0 g

Composition per liter of M9 salts:

$Na_2HPO_4.7H_2O$	64.0 g
K_2HPO_4	15.0 g
NaCl	2.5 g
NH_4Cl	5.0 g

The salt solution was divided into 200 ml aliquots and sterilized by autoclaving for 20 minutes at 121 ºC.

Salt solution and filter sterilized (with a 0.22-micron filter) glucose solution was added to sterile distilled water (cooled to 50ºC or less) to have final volume of 1L. pH was adjusted to 7.0 with 1N NaOH.

10. Eosin Methylene Blue Agar (EMB)

Composition per liter:

Buffers	2.0 g
Eosin Y	0.4 g
Lactose	10.0 g
Methylene blue	0.065 g
Peptones	10.5 g
Agar	13.0 g

It was commercially available. 36 g of this compound was suspended in 1L of distilled water. ph was adjusted to 7.0 with 1N NaOH. Sterilized by autoclaving for 20 minutes at 121 ºC.

APPENDIX B

LONG STORAGE OF CULTURES

%90 glycerol stock solution was prepared with distilled water.

The bacteria growing on the surface of an agar plate was scraped into 2ml of PBS buffer in a sterile Falcon tube. Cells were resuspended in the PBS buffer gently by vortex. 1 ml glycerol was added from stock to have a final concentration 30% of glycerol. It was gently mixed in an inverted position. Aliquots of the glycerinated culture were dispensed into the sterile Eppendorfe tubes and transferred to -70ºC.

To recover the bacteria, the frozen surface of the culture was scraped with a sterile inoculating needle, and then the bacteria that adhere to the needle were immediately streaked onto the surface of an appropriate agar plate. The remaining frozen culture was returned to storage at -70ºC.

Phosphate-Buffered Saline (PBS)

Composition per liter:

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g

All components were dissolved in 800 ml of distilled water. pH was adjusted to 7.4 with HCl and it was completed to 1L with distilled water. Then the solution was dispensed into aliquots and sterilized for 20 min at 121 ºC by autoclaving. Aliquots were stored at room temperature.

APPENDIX C

BUFFERS FOR PLASMID ISOLATION

1. TE Buffer (ph 8.0)

10 mM Tris.Cl (ph 8.0)
1 mM EDTA (ph 8.0)

2. Solution I

50 mM glucose
25 mM Tris.Cl (ph 8.0)
10 mM EDTA (ph 8.0)

Autoclaved for 20 minutes at 121 ºC and stored at 4 ºC.

3. Solution II

0,2 N NaOH (freshly diluted from a 10 N stock)
1% SDS

4. Solution III

5 M Potassium acetate	60 ml
Glacial acetic acid	11,5 ml
H_2O	28,5 ml

APPENDIX D

BUFFERS OF AGAROSE GEL

1. TAE Electrophoresis Buffer

Working solution (1X)	Concentrated stock solution (per liter) (50X)
0.04 M Tris-acetate	242 g Tris base
0.001 M EDTA	57.1 ml glacial acetic acid
	100 ml 0.5 M EDTA (pH 8.0)

Solution was autoclaved for 20 minutes at 121 ºC and stored at room temperature to prevent precipitation.

2. Gel-Loading Buffer

6X Buffer	Storage temperature
0.25% bromophenol blue	4 ºC
40% (w/v) sucrose in water	

3. Stock Solution of Ethidium Bromide (10 mg/ml)

0.1 g ethidium bromide was dissolved in 10 ml distilled water. Solution was stirred on magnetic stirrer for several hours to ensure that dye was completely dissolved. As this solution is light sensitive, the bottle was covered with aluminum foil and stored at room temperature.