

# Comparison of Antibiotic Susceptibility Patterns between *Serratia marcescens* Strain Isolated in 1920 versus 2008

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**Abstract:** *Serratia marcescens* is a non-pathogenic, saprophytic, Gram-negative bacterium classified as a member of *Enterobacteriaceae*. It has recently been classified as a nosocomial pathogen, causing infections such as urinary tract infections, lower and upper respiratory tract infections, and septicemia. These nosocomial infections have mostly been detected in intensive care units (ICUs). Though there is a standard treatment for *S. marcescens* infections, the bacterium has recently exhibited resistance to several antibiotic treatments. This study was conducted to determine the difference between two strains of *S. marcescens* (1920 and 2008 strains) using antibiotic resistance as the main criterion. In this study, antibiotic susceptibility tests using three classes of antibiotics (beta-lactams, aminoglycosides, and sulfonamides), genomic DNA isolation, restriction fragment length polymorphism (RFLP), and polymerase chain reaction (PCR) were used to evaluate the differences between the two strains. PCR was performed using primers for the integron classes 1, 2, and 3, and the methicillin-resistance gene *mecA*. Genomic DNA digestions were carried out using EcoRI-HF and Hind III. Both the strains were found to be resistant to the beta-lactams, but susceptible to the aminoglycoside and sulfonamides and both the strains lacked integron elements. However, RFLP of genomic DNA showed differences, suggesting possible variation within the genomic DNA sequences of 1920 versus 2008 strains. ©2014 Oklahoma Academy of Science

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## Introduction

In recent years, some hospitalized patients and recent outpatients have acquired infections from hospitals and healthcare facilities. These infections have come to be known as nosocomial infections, with most of them occurring within intensive care units (Ivanova 2008). Most nosocomial infections are caused by antibiotic resistant bacteria, making them very difficult to treat. The emergence of antibiotic resistant bacteria causing nosocomial infections was marked by the wide and rapid spread of methicillin-resistant *Staphylococcus aureus* (MRSA) (Chen 2003) in hospitals of developed

countries such as the United States and the United Kingdom. The emergence of MRSA marked the beginning of an exponential increase in the number of multi-drug-resistant bacteria; however, this increase has somewhat abated over the years (Ellner 1987). Nevertheless, due to the recent rampant use of antibiotics in both human medicine and animal husbandry, intense selective pressures have created resistance to several classes of antibiotics such as beta-lactams, sulfonamides, aminoglycosides, tetracyclines, and many others (Rowe-Magnus 2002). Such resistance-conferring elements can be transferred

between members of the same species or of different species. Antibiotic resistance-conferring elements transfer between bacteria mainly by vertical transfer of genetic material, via reproductive processes and by horizontal transfer of genetic material such as that seen in the transfer of the shiga toxin gene from *Shigella* to *Escherichia coli* (Rowe-Magnus and Mazel 2002). This transfer is usually achieved using the R plasmid as a major vector (Okuda 1984).

*Serratia marcescens* is a non-pathogenic, saprophytic, Gram-negative, and ubiquitous water organism classified as a member of *Enterobacteriaceae* (Sleigh 1983, Bagattini 2004.). Previously, most cultured *S. marcescens* exhibited a red or orange coloration that was caused by the production of a pigment known as prodigiosin (Sleigh 1983). However, *S. marcescens* colonies not exhibiting this distinct coloration have often been seen in bacteriology and hospital laboratories (Wilfert 1970); these strains are suspected to be pathogenic (Sleigh 1983). Because of the ubiquitous nature of bacteria, *S. marcescens* reservoirs can be located in diverse places including homes, offices, hospitals and other healthcare facilities. Pathogenic bacterial infections are usually treated with a single antibiotic or a combination of antibiotics that have different chemical properties and modes of action. There has been a global surge of antibiotic resistance organisms.

Antibiotic resistance-conferring elements can be transferred via mobile genetic elements known as integrons. These integrons have several general features such as the presence of the integrase gene *intI*, a gene cassette recombination site *attI*, and a promoter region  $P_{ant}$  for gene expression (Bennet 1999); all of these features can be used for identification. These integrons can not only used to transfer antibiotic resistance genes from one bacterium to another, but they can also be used to transfer genes that code for different characteristics such as toxins (Collis 1993). Such elements (both antibiotic resistance and other characteristics) are carried on gene cassettes inserted into the integrons. Gene cassettes, sequences of genes with a recombination site *attC* and no promoter

(Waites 2000), cannot be expressed independently. Without insertion into an integron, these gene cassettes cannot be replicated (Bennet 1999) and thus cannot be propagated. Therefore, the needed insertion is done via site-specific recombination at the recombination site *attI* of the integron, mediated by an integrase protein (Collis 1993). Five different classes of integrons have been discovered: classes 1, 2, 3, 9, and an unnumbered class (Collis 2002), with classes 1, 2, and 3 being the most studied. These three classes of integrons are differentiated using specific markers unique to each class. Class 1 integrons, usually found on the transposon *Tn402* (Collis 2002) have an integrase gene *IntI*, a recombination site *attI*, a promoter region  $P_{ant}$  at its 5' conserved region, and a variable 3' conserved region (Waites 2000). In contrast, class 2 integrons have the integrase gene *IntI* at the 3' conserved region (Waites 2000). Class 3 integrons were first identified in a *S. marcescens* strain isolated in Japan in 1993 (Arakawa et al., 1995). Although class 3 integrons have a structure comparable to class 2, their recombination between the 59-bp element and secondary sites occurs at lower frequencies. Moreover, in class 3 integrons the *IntI3* can recognize and integrate at different *attC* sites (Collis 2002).

The aim of the study was to determine if the *Serratia marcescens* 1920's strain and 2008 strain have mobile DNA elements known as integrons and to assess their resistance to antibiotics such as penicillin, oxacillin, trimethoprim-sulfamethoxazole (TMP-SMZ), and kanamycin.

## Methods

**Organisms used.** Three organisms were used in this study. ATCC 60 (designated I-20) ATCC 13880 (designated I-08) and ATCC 33592 (designated MRSA) were purchased from American Type Culture Collection (Manassas, VA). The I-20 strain was deposited by Army Medical School, Washington, DC in 1925, the ATCC 33592 strain was deposited by Schaeffler S in 2006 and the ATCC 13880 strain (designated I-08) was deposited in 2008 by M Koccur (Verslypea 2011). All cultures were maintained on nutrient agar slants and cultured in TSB at 25°C for 18 h.

**Antibiotic Susceptibility Test.** In preparation for the antibiotic susceptibility test, cultures of the samples were grown in Tryptic Soy Broth (TSB) and incubated on an Incubating Mini Shaker (VWR, Arlington Heights, IL) at 25°C for 18 hours. After incubation, the strains were standardized to obtain a 0.5 McFarland Standard (absorbance of 0.1-0.15 at 540 nm) using UV-Vis Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Standardized samples were incubated on Mueller-Hinton agar at 37°C for 18 hours with penicillin, oxacillin, trimethoprim-sulfamethoxazole, and kanamycin disks (BD-BBL, Franklin Lakes, NJ). Results were documented using GelDoc XR+ System (Bio-Rad Laboratories, Hercules, CA) and zones of inhibition were measured.

**Polymerase Chain Reaction.** Genomic DNA was extracted from pure cultures of all three strains using a modified protocol for Gram-positive and Gram-negative bacteria using the PureLink Genomic DNA Isolation Kit (Invitrogen, Carlsbad, CA.). Primer sets for the *mecA*, *Igr1*, *Igr2*, *Int13* and *Sa16s* genes (see below for sequences) were used to amplify respective genes using the genomic DNA from each strain. The PCR cycling conditions for *mecA* primers were as follows: one step of denaturation at 94°C for 4 min, followed by 30 cycles of 94°C for 0.5 min, 53°C for 0.5 min, 72° for 1 min, and a final extension cycle at 72°C for 4 min. For the *Igr1* and *Igr2* primers, the reaction conditions were: one step of denaturation at 95°C for 4 min, followed by 30 cycles of 94°C for 0.75 min, 64°C for 0.75 min, and a final extension cycle at 72°C for 1 min. The *Igr1* and *Igr2* reactions conditions were used for the *Int13* primers with a modification of the annealing temperature to 50°C. For the *Sa16s* primers, the reaction conditions were as follows: one denaturation step at 95°C for 3 min, followed by 40 cycles of 95°C for 0.5 min, 50°C for 0.45 min, 72°C for 2 min, and a final extension cycle at 72°C for 7 min. Amplicons were resolved by electrophoresis on a 1.2% agarose-Tris-Acetate-EDTA gels at 60V for 6 hours. Visualization was done using EZ-Vision dye (Amresco, Solon, OH) and documentation was carried out using the

GelDoc XR+ System (Bio-Rad Laboratories, Hercules, CA).

**Primer set sequences:**

Primer	Sequence (5' to 3')
Igr1	GCT CTA GAC CGA AAC CTT GCG CTC, and GGA ATT CAT GAT ATA TCT CCC AAT TTG T
Igr2	GCT CTA GAT AAT GTG CAT CGT GCA AGC, and GCG TTA TCT AGT TCG ACA TAG TCT
Int13	AGT GGG TCG CGA ATG AGT G and TGT TCT TGT ATC GGC AGG TG
MecA	CTC AGG TAC TGC TAT CCA CC and CAC TTG GTA TAT CTT CAC C
Sa16	GAA AGC CAC GGC TAA CTA CG and CAT TTC ACC GCT ACA CAT GG

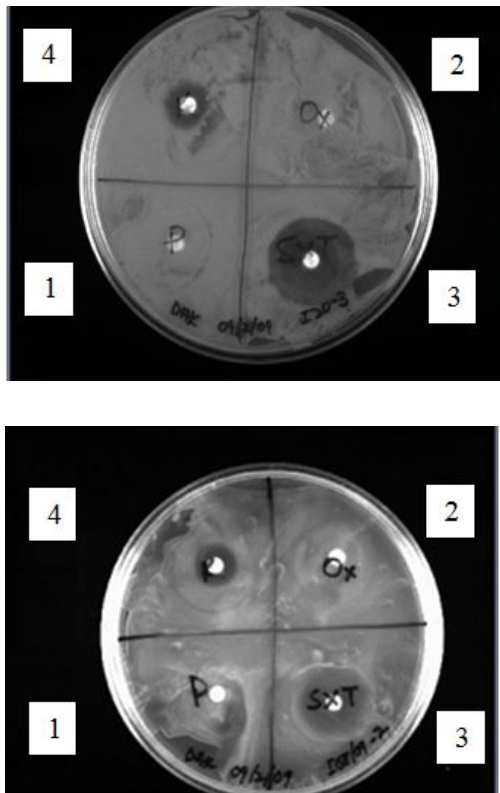
**Restriction Fragment Length Polymorphism.** Genomic DNA (2µg) from the I-20 and I-08 strains was digested using 20 Units EcoRI-HF and 20 Units HindIII-HF (New England Biolabs, Ipswich, MA). Single and double digestions were performed as per the manufacturer's instructions. Briefly, 2 µg of genomic DNA was digested using 20 U of restriction enzyme in a total volume of 50 µl at 37° C for 12 hours. Digests were resolved on 1.2% agarose-Tris-Acetate-EDTA gels at 50-95V for 5 hours and 30 min, visualized using EZ-Vision (Amresco, Solon, OH) and documented using the GelDoc XR+ System (Bio-Rad Laboratories, Hercules, CA).

**DNA Sequencing.** Genomic DNA was extracted from I-20 and I-08 strains using the PureLink Genomic DNA Isolation Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. DNA sequencing was performed to ascertain the presence of integron elements. Sequencing was done at Oklahoma Medical Research Foundation core facility (Oklahoma City, Oklahoma).

## Results

### Antibiotic susceptibility test

The antibiotic susceptibility test showed resistance to both beta-lactams used (penicillin and oxacillin) in both strains (Table 1). In contrast, both strains were susceptible to the sulfonamide TMP-SMZ and the aminoglycoside kanamycin, albeit to varying degrees (Table 1 and Figure 1).



**Figure 1. Plates showing zones of inhibition to penicillin (1), oxacillin (2), TMP-SMZ (3), and kanamycin(4) for the 1920 strain (I-20) (upper) and the 2008 strain (I-08) (lower).**

### PCR for integrin mobile elements

PCR was performed to determine the presence or absence of integrons, which have been shown to be responsible for the acquisition of resistance to antibiotics and other characteristics in *S. marcescens*. Neither strains showed evidence of containing the *mecA* gene (Figure 2, lanes 2 and 3), which is responsible for methicillin resistance in most bacteria, especially MRSA (De Lencastre 1994). Amplification using the

primer *Sal6s* served as an internal control for the PCR process (Figure 2; lanes 4, 5, and 6). The difference in intensity of the *Sal6s* bands might be due to loss of sample from the wells during the loading process for lanes 5 and 6. No amplicons were observed for either strains using *Igr1* (Figure 2, lanes 7 and 8), *Igr2* (Figure 2, lanes 9 and 10), and *Int13* (Figure 2, lanes 11 and 12) for classes 1, 2, and 3 integron sequences respectively.

### RFLP of genomic DNA

The strain I-08 showed no RFLP fragments in either single or double restriction digestions (Figure 3; lanes 2, 4, 6, and 8). Undigested genomic DNA was observed very close to the wells. However, the strains I-20 showed significant digestion in both the single (Figure 3, lanes 3 and 5) and double restriction digestions (Figure 3, lane 7). Few sizes were elucidated due to the close banding observed for the I-20 sample.

Observation of the genomic DNA of I-08 close to the wells, indicating high molecular weight DNA, prompted a resolution of the genomic DNA of both strains using 0.7% agarose-Tris-Acetate-EDTA gel electrophoresis. This showed a marked difference in the sizes of genomic DNA in both strains, though both samples were larger than 10 Kb (Figure 4)

### DNA sequencing

DNA sequencing of genomic DNA from both strains was attempted but was unsuccessful possibly due the large stretches of repeating adenine sequences within the genomic DNA (internal poly-A tails).

### Discussion

The *in vitro* bactericidal activity of antibiotics does not always correlate with their therapeutic effects in patients. However, their action on certain bacteria must be elucidated in order to advance to human testing. Kirby Bauer test was to qualitatively determine the efficacy of three different classes of antibiotics (beta-lactams, aminoglycosides, and sulfonamides) on *S. marcescens*. The results showing resistance to penicillin and oxacillin seem to indicate the presence of a beta-lactam resistance mechanism. Based on a

study conducted (Yang 1990), the main defense of *S. marcescens* against beta-lactam drugs is attributed to the production of the Richmond and Skye class I type of chromosomal beta-lactamase. Some strains of *S. marcescens* have also been known to produce plasmid-mediated beta-lactamases such as the TEM-1 enzyme, which confers resistance to penicillins and older cephalosporins (Yang 1990). These enzymes are responsible for the rupture of the beta-lactam rings found in drugs such as penicillin and oxacillin. These beta-lactam rings bind to the penicillin-binding proteins (PBPs) and inhibit the cross-linking of glycopeptide polymer units that form the cell wall mesh.

Another mechanism elucidated (Cozens 1986) suggests a phenotypically induced changes within the outer membrane reduces the drugs permeability across the outer and cytoplasmic membranes. One or both of these mechanisms might be responsible for the resistance to penicillin and oxacillin observed in both strains. This result is consistent with the findings that have shown that strains of *S. marcescens* have exhibited resistance to AZTREONAM, CEFOTAXIME, and even CEFTAZIDME (Bonnet 2000), all of which are advanced penicillins and cephalosporins. Since the ability to resist the action of beta-lactam rings can be conferred by exogenous genetic material (plasmids), it can be deduced that both strains have acquired genetic material that has conferred this ability. Alternatively, some studies have reported the acquisition of carbapenem (broad spectrum beta-lactam) resistance in some bacteria such as *Acinetobacter baumannii* after the loss of a 29-kD outer membrane protein (Mussi 2005) this might be a possibility in this study in light of genomic DNA size differences observed (Figure 4). The PCR results suggests that the antibiotic resistance is not via integrons since the three classes of integrons seem to be absent in both strains (Figure 2). Thus, it is appropriate to conclude that the production of beta-lactamase was conferred by some mechanism other than the insertion of resistance gene cassettes into integrons. Should integrons be considered for future studies, more specific primers should be used

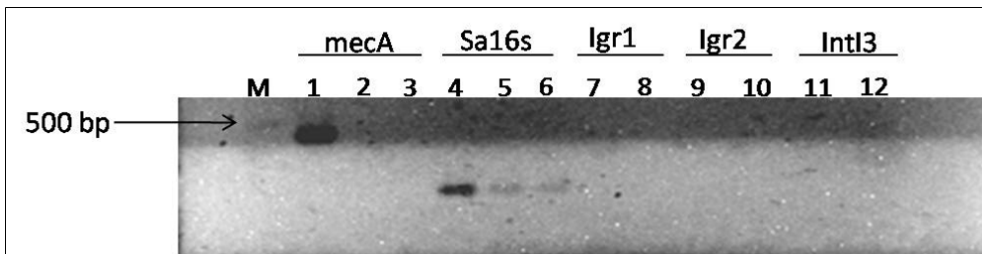
to target the first portion of the antibiotic resistance operon found in the other classes of integrons (class 9 and the unnumbered class), since all genes found in integrons coding for beta-lactamase have been found in the first position of the operon, with a single exception (Mussi 2005). Also, the 1920 strains showing resistance to penicillin suggests the ability of *S. marcescens* to obtain resistance elements from nature. Since the first therapeutic use of a penicillin drug was recorded in 1927, the 1920 strains must have obtained this resistance from contact with *Penicillium notatum* in its natural surroundings.

Aminoglycosides are naturally produced by some organisms such as *Streptomyces* spp and *Micromonospora* spp. These species have developed resistance to this class of antibiotics by methylation of the 16s rRNA found in prokaryotic 30s ribosomal subunits (Doi 2004). Another common mechanism for aminoglycoside resistance employed by different strains of *S. marcescens* is the modification of the drug by 'inactivating enzymes' that adenylate, acetylate, or phosphorylate the amino groups or hydroxyl group found in aminoglycosides (Hejazi and Falkner 1997). Though research has revealed that most Gram-negative bacilli such as *E. coli* O157:H7 have been shown to be resistant to kanamycin (Zhao 2001), these mechanisms seem to be absent as both strains in this study were susceptible to the aminoglycoside kanamycin. It was also observed that both strains were susceptible to the sulfonamide drug TMP-SMZ (Table 1). This observation is in agreement with the current use of TMP-SMZ in the treatment of nosocomial urinary tract infections caused by *S. marcescens* (Okuda 1984) in ICUs. Resistance to sulfonamides such as TMP-SMZ is usually the result of chromosomal mutations in the dihydropteroate synthase (DHPS) gene or the introduction of the *sulI* into integrons and/or *sul2* genes into plasmids (Skold 2000). Susceptibility of the two strains to TMP-SMZ would indicate that they lack any of the aforementioned mechanisms of resistance to sulfonamides.

**Table 1.** Antibiotic susceptibility test results showing zones of inhibition for both strains using four different antibiotics.

Sample Replicates	Antibiotics (mm)			
	Kanamycin (K-30)	Oxacillin (OX-1)	Penicillin (P-10)	TMP-SMZ (SXT1.25-23.75)
I-08 - 1	18	0	0	25
I-08 - 2	18	0	0	30
I-08 - 3	28	0	0	28
I-08 - 4	17	0	0	0.0
I-08 - 5	19	0	0	*
I-20 - 1	21	0	0	35
I-20 - 2	20	0	0	34
I-20 - 3	19	0	0	36
I-20 - 4	21	0	0	35
I-20 - 5	20	0	0	35

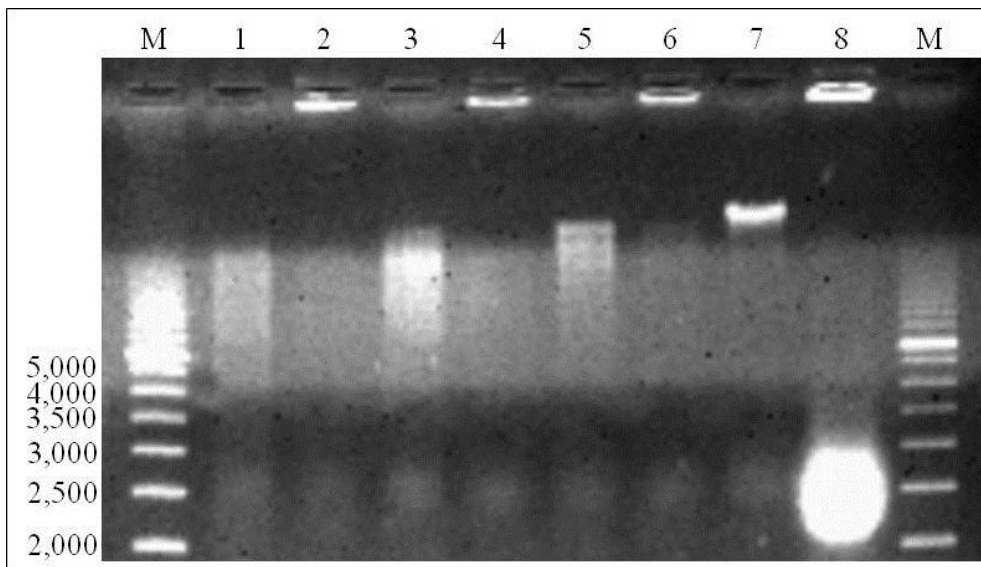
Interpretive standards in mm in the order of Resistant (R), Intermediate (I) and Sensitive (S) for: OX:  $\leq 10$ , 11-2,  $\geq 13$ ; K:  $\leq 13$ , 14-14,  $\geq 18$ ; P:  $\leq 28$ , -,  $\geq 29$ ; SXT:  $\leq 10$ , 11-15,  $\geq 16$ . \* - indicates undeterminable zone of inhibition



**Figure 2.** PCR amplification using *mecA*, *Sa16s*, *Igr1*, *Igr2*, and *IntI3* primers. The sizes of the molecular DNA marker shown on the left of gel. Lanes: M = molecular DNA marker EZ-Load (Bio-Rad Laboratories); 1 = MRSA with *mecA* primer, 2 = I-20 with *mecA* primer; 3 = I-08 with *mecA* primer, 4 = MRSA with *Sa16s* primer, 5 = I-20 with *Sa16s* primer, 6 = I-08 with *Sa16s* primer, 7 = I-20 with *Igr1* primer, 8 = I-08 with *Igr1* primer, 9 = I-20 with *Igr2* primer, 10 = I-08 with *Igr2* primer, 11 = I-20 with *IntI3* primer, 12 = I-08 with *IntI3* primer.

Chetoui et al. have used pulse-field gel electrophoresis of macrorestriction fragments with biotyping, esterase and ribo typing performed typing of nosocomial strains of *Serratia marcescens*. Both strains were comparable with respect to antibiotic sensitivity. We have used RFLP in this study to find any differences and/or similarities between these two strains at the genomic level. The efficiency of RFLP in determining similarities and differences in DNA sequences of similar species was demonstrated in a study by Debast et al. where RFLP analysis was

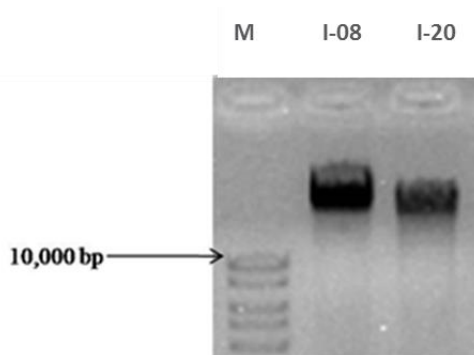
utilized to identify three genotypes of *S. marcescens* during a nosocomial outbreak in a hospital. In the present study, RFLP analysis indicated a pattern difference in the cleaved genomic DNA of the I-20 strains and the I-08 strains of *S. marcescens* (Figure 3). Treatment of the genomic DNA of both strains with Hind III and EcoRI-HF showed digestion of the DNA of the strains I-20 but did not do the same for the strain I-08. Based on the single band observed for the strains I-08, it was deduced that there were no cleavage sites for the two used restriction enzymes.



**Figure 3. RFLP analysis of genomic DNA isolated from MRSA, I-20, and I-08. DNA was digested with Hind III and EcoRI-HF. The sizes of the molecular DNA marker are shown on the left of the gel. Lanes are as follows: M – molecular DNA marker EZ-Load (Bio-Rad Laboratories), 1 = I-20 without RE, 2 = I-08 without RE, 3 = I-20 with Hind III, 4 = I-08 with Hind III, 5 = I-20 with EcoRI-HF, 6 = I-08 with EcoRI-HF, 7 = I-20 with Hind III and EcoRI-HF, 8 = I-08 with Hind III and EcoRI-HF.**

Since both strains are of the same species of organism, the absence of HindIII and EcoRI-HF cleavage sites in I-08 indicate a net insertion or deletion of genetic material at these sequences, eliminating the sites. Though either insertions or deletions are possible, the relatively large size of the genomic DNA of I-08 (Figure 4) would suggest a net insertion of genetic material over the 88-year period via class 9 or the unnumbered class integrons, plasmid insertion, or some other mechanism.

The results of the present study indicate that in addition to the acquisition of resistance elements due to selective pressures imposed by the use of antibiotics in modern medicine, *S. marcescens* can also obtain these elements from its natural environment. In the treatment of *S. marcescens* infections, however, the aminoglycoside kanamycin has been shown to be effective and holds potential as an alternative to the standard treatment using the sulfonamide TMP-SMZ.



**Figure 4. Agarose gel electrophoresis resolution of genomic DNA of the I-20 and the I-08 strain. The I-08 strain genomic DNA appears to show a difference in the size compared to the I-20 genomic DNA.**

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