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Characterization of a Staphylococcal Trimethoprim Resistance Gene

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entitled

Characterization of a Staphylococcal Trimethoprim Resistance Gene

has been approved by his committee as satisfactory completion of

he degree of Master of Science.

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27 August 1986
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Characterization of a Staphylococcal
Trimethoprim Resistance Gene

A thesis submitted in partial fulfillment of the requirements for
the degree Master of Science at Virginia Commonwealth University.

By

Jerald Preston Coughter, B.S.
Clemson University, 1982

Director: Gordon L. Archer, Professor
            Departments of Microbiology
            & Immunology and Medicine

Virginia Commonwealth University
Richmond, Virginia
August, 1986

Dedication

For Peter, Ryan, and Pierce.

Acknowledgements

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Characterization of a Staphylococcal Trimethoprim Resistance Gene

Abstract

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Trimethoprim resistance(Tp^r) is encoded by conjugative plasmids in clinically significant staphylococcal isolates. Two genetically and physically similar plasmids from S. aureus, pGO1 and pGO5, have Tp^r genes that map in different locations on these plasmids. In order to study the relatedness of the Tp^r genes and their products to other known Tp^r genes, a 1.2 kb fragment of pGO1 and a 4.2 kb fragment of pGO5 were cloned in E. coli and used as probes for in situ filter hybridization experiments.

A 500 base pair subclone of the original 1.2 kb fragment containing only the staphylococcal Tp^r structural gene, showed no homology with genes from E. coli encoding a dihydrofolate reductase(DHFR) with an altered Tp^r binding affinity or the B. subtilis gene for DHFR. Positive hybridization signals were seen with restriction fragments from pGO1, pGO5, and plasmid DNA from five other Tp^r staphylococci. A 700 bp portion of the original fragment showed homology with several different restriction

fragments of EcoRI-digested pGO1 and pGO5, suggesting the presence of repeated sequences on both plasmids. These sequences corresponded to areas of the plasmids known to be involoved in deletions which occur during viral transductions.

Lysates of bacteria containing the cloned and native Tp^r genes were assayed spectrophotometrically for DHFR activity and compared with activity of E. coli containing genes for DHFR type I and type II. In addition, the Tp IC_{50}(the concentration of Tp required to reduce DHFR activity by 50%) was determined. Tp^r staphylococci containing the plasmid-encoded Tp^r gene had twenty times higher specific activity than Tp sensitive staphylococci. E. coli containing the cloned staphylococcal gene had DHFR activity equal to that of staphylococcal strains from which the clones were derived and 300 times higher activity than Tp sensitive E. coli. Determination of the Tp IC_{50} showed the staphylococcal protein to be 7000 times more resistant to Tp than the normal cellular DHFR, but four times less resistant to Tp than the DHFR type I and 450 times less resistant than DHFR type II. The staphylococcal Tp^r gene product is a protein with DHFR activity that is resistant to Tp inhibition. The gene is expressed in E. coli, but is dissimilar to several previously characterized E. coli Tp^r genes.

INTRODUCTION

Numerous studies have shown that resistance to most antibiotics occurs by mechanisms involving inactivation, detoxification, altered transport, or altered binding of drugs to cellular targets. Resistance to inhibitors of dihydrofolate reductase(DHFR), such as trimethoprim, arises from a variety of mechanisms involving enzyme alteration, cellular impermeability, enzyme overproduction, inhibitor modification, and loss of binding protein[12]. The mechanism of greatest clinical importance is the production of novel, resistant, plasmid-encoded DHFRs.

In 1972, Fleming et al.[16] reported the discovery of R-plasmids which conferred on Escherichia coli and Klebsiella species a high level resistance to trimethoprim. An R-plasmid, R388 was later shown by Aymes and Smith[5] to increase the resistance of E. coli to trimethoprim by about 10,000 fold and to mediate the synthesis of a DHFR which was about 20,000 times less suseptible to inhibition by trimethoprim than the native chromosomal enzyme. These kinds of enzymes are now known as type I. This mechanism of resistance was of particular interest as it was the first example of an R-factor-conferred resistance where the cellular target and not the antimicrobial agent was modified to manifest the resistant phenotype. Skold and Widh[42] further characterized this novel type of DHFR, showing that R-plasmid R483 produced a type I enzyme that differed from the normal, cellular enzyme by being more heat-sensitive and more

resistant to trimethoprim inhibition. More recently, Aymes and Smith[6] reported that among its other properties, the DHFR mediated by R-plasmid 388 had a molecular weight approximately twice that of the sensitive chromosomal DHFR and exhibited an insensitivity to methotrexate. Pattishall et al.[34] reported in 1977 a second type of R-plasmid DHFR, now known as type II. This enzyme was completely resistant to methotrexate and trimethoprim, yet it retained the ability to bind dihydrofolate tightly. The complete nucleotide sequences of the genes encoding DHFR types I and II are now known[17, 11]. There is no significant homology between them, though the type I sequence shows some homology with areas of the E. coli chromosome that encode amino acids associated with substrate, cofactor, and inhibitor binding. The existence of a third type of plasmid-encoded DHFR in E. coli was reported by Fling et al.[18] in 1982. This enzyme differed in size, immunologic specificity, and binding characteristics as compared to type I and type II.

Grey et al.[21] studied the mechanism of trimethoprim resistance in 36 strains of E. coli and P. mirabilis that did not contain R-plasmids. The main mechanism of resistance in the majority of E. coli studied was the production of altered DHFR with a decreased suseptibility to trimethoprim. There was a correlation between minimum inhibitory concentration(MIC) of trimethoprim and IC_{50} of trimethoprim(that is, the concentration of the drug required to reduce enzyme activity by 50 percent) for the DHFR of these strains. In some cases however, the IC_{50} was

in excess of that expected. A suggested explanation for this discrepancy was that some strains, in becoming resistant to trimethoprim, also became permeable to the drug resulting in a disproportionate increase in IC_{50} of the DHFR. It is known that some bacteria have a permeability barrier to antifolate drugs[47], so that the sensitivity of the isolated enzyme is far greater than suggested by the MIC.

Other strains tested by Grey et al. appeared to have different mechanisms of resistance. Among these were strains producing an enzyme with a higher specific activity than the normal cellular DHFR, some strains with decreased permeability for trimethoprim, and possibly some strains that produced a second, more resistant form of DHFR. In strains with an increased specific activity, it was not clear whether the increase was due to more enzyme being produced or an increase in the activity of the enzyme due to an alteration in structure. Impermeability has been implicated as a mechanism of resistance to trimethoprim in strains of Streptococcus faecalis[20] which showed no changes in either levels or sensitivity of the DHFR. Jackson and Harrap[26] showed in 1973 that only 5% of the normal DHFR activity was necessary for the functioning of some mammalian cell lines; the same may apply to bacteria. Thus, while a second DHFR was not detected, it could have been present in small amounts. The presence of a second enzyme more resistant than the normal sensitive DHFR was shown in an aminopterin-resistant mutant of Streptococcus faecalis[32] as well as in strains containing R-

plasmids[5,42,6,34].

Baccanari et al.[7] have shown that sequential passage of E. coli into increasingly higher concentrations of trimethoprim results in the appearance of colonies that grow in the presence of >500ug/ml of trimethoprim. These cells show a 500-1000 fold increase in DHFR levels. It is doubtful that such strains are selected in humans, because such resistance is rapidly lost unless the cells are grown in concentrations of trimethoprim that would be difficult to reach in vivo. However, despite the occasional occurrence in bacteria of other resistance mechanisms discussed above, it is clear that trimethoprim-resistant DHFRs encoded by R-plasmids are the major cause of trimethoprim resistance among clinical isolates[12].

Because of their ubiquity and ability to move freely from plasmid to plasmid, transposons are thought to have played a large role in in the rapid evolutionary spread of bacterial drug resistance during the last two decades. A transposon is a specific DNA sequence carrying a recognizable genetic determinant, such as drug resistance, that moves from one replicon to another. Transposition is a nonreciprocal event that occurs at the same frequency in the presence or absence of the requirements for normal recombination, that is, a functional recA gene and regions of extensive homology between the participating DNA sequences[27]. Since the original recognition of a transposon carrying an ampicillin resistance determinant(Tn1) from plasmid RP4[23], many others have been recognized. Barth et

al.[9] reported in 1976 the transposition of a DNA sequence encoding resistance to trimethoprim and streptomycin from R-plasmid R483 to other replicons. Originally designated TnC, this transposon is now called Tn7. Barth and Datta[8] went on to show that Tn7 exists in nature on E. coli plasmids of different incompatibility groups as well as the E. coli chromosome. The significance of this finding was demonstrated in 1979 and 1980 with the emergence of clinical isolates of E. coli with non-transferable high-level trimethoprim resistance(>1024ug/ml). Such high level resistance had previously been associated with the presence of a trimethoprim resistance plasmid and it was considered possible that the observed resistance was due to a plasmid that was incapable of transferring to the standard E. coli K12 recipient strain used. However, in 1981, Towner[44] showed that in these isolates trimethoprim resistance was encoded by a chromosomally-located transposon which could not be distinguished from Tn7. Earlier, Shapiro and Sporn[40] found that the trimethoprim resistance determinant of plasmid R751 transposed to bacteriophage lambda. The world wide spread of resistance to broad-spectrum penicillins in plasmids of many kinds of bacteria of many genera has been attributed, at least partly, to the spread of transposon Tn1. It seems, therefore, that circumstances are favorable for the spread of resistance to trimethoprim, which, like ampicillin, is widely used in hospitals.

Richardson[38] reported in 1983 that while 16% of clinically

significant S. epidermidis isolates from England were highly trimethoprim resistant(MIC >500ug/ml), only 4% of epidemiologically independent isolates from northern Europe and North America were trimethoprim resistant. The latter figure is in contrast to the 17% trimethoprim resistance frequency reported in American S. epidermidis isolates by Archer et al. in 1986[1]. Archer et al. also reported a higher frequency of resistance among S. aureus(10%) isolates than was earlier reported[3]. Thus, high-level trimethoprim resistance among both S. epidermidis and S. aureus seems to be increasing in this country.

Conjugative transfer of aminoglycoside resistance plasmids among staphylococci was first reported in 1983 by Forbes and Schaberg[19] and independently confirmed by McDonnell et al.[31]. Archer and Johnston[2] identified a group of related self-transmissible plasmids found in both S. epidermidis and S. aureus isolates that encoded resistance to aminoglycosides. This group exhibited five different restriction endonuclease digestion patterns. Archer et al.[1] also showed that a methicillin-resistant S. aureus isolate resistant to trimethoprim transfered that resistance serially by filter mating to suitable S. aureus and S. epidermidis recipients. Resistance to gentamicin and beta-lactamase production were cotransferred. All three resistance determinants were encoded on a single 55 kilobase plasmid(pGO5)(Figure 1). A similar trimethoprim-resistant, gentamicin-resistant conjugative plasmid was also found in clinical isolates of methicillin-suseptible S. aureus and

methicillin-resistant S. epidermidis. In situ filter hybridization showed that there was homology between a cloned 4.2 kilobase EcoRI fragment of pGO5 containing the trimethoprim resistance determinant and other plasmid-associated trimethoprim resistance genes from staphylococci, but not with gram-negative plasmid-encoded trimethoprim resistance genes. Plasmid-mediated trimethoprim resistance has not been previously reported among staphylococcal isolates from the United States. While such resistance has recently been reported among methicillin-resistant S. aureus isolates from Australia[45], the nature of this determinant has not been characterized in any detail. Plasmid- mediated trimethoprim resistance has not been reported previously in coagulase-negative staphylococci.

This thesis is a report of the first characterization of the staphylococcal trimethoprim resistance gene and its protein product. The gene was mapped using restriction endonuclease analysis. The gene was cloned in E. coli and its relatedness to other known trimethoprim resistance genes was determined. The gene product was analyzed biochemically and compared to other known trimethoprim resistance gene products. These data were used to determine the mechanism of resistance. The results of in situ filter hybridization experiments have suggested that a repeated sequence of DNA may mediate both intermolecular and intramolecular rearrangements that might account for the presence of the trimethoprim resistance determinant in different locations on otherwise similar replicons. This is the first report of possible insertion sequence-like elements in staphylococci.

MATERIALS AND METHODS

A.   Chemicals, Media, and Antibiotics

     Restriction enzymes and reaction buffers were purchased from International Biotechnologies, Incorporated(IBI, New Haven, CT). T_4-DNA ligase and DNA nick translation kits were bought from New England Nuclear(NEN, Boston, MA). Agarose and acrylamide were obtained from IBI. Technical grade cesium chloride was bought from Kawecki, Berlyce Industries, Incorporated(KBI, Reading, PA). Trimethoprim, ampicillin, tetracycline, RNase, and various compounds such as boric acid, lysozyme, lysostaphin, calcium chloride, tris, folic acid, NADPH, and ethidium bromide were purchased from Sigma Chemical Company(Sigma, St. Louis, MO). Mueller-Hinton agar and broth were from BBL Microbiology Systems(BBL, Cockeysville, MD). Antibiotic media #5 and brain heart infusion broth were obtained from Difco Laboratories(Difco, Detroit, MI). L-broth was from Gibco Laboratories(Gibco, Madison, WI). Solvents such as chloroform and hydrochloric acid were purchased from J. T. Baker Chemical Company(Phillipsburg, NJ). Phenol was obtained through Scientific Products(S/P, McGaw Park, IL).

B.   <u>Bacterial Strains</u>

<u>strain/phenotype</u> <u>E. coli</u>	<u>plasmid/phenotype</u>	<u>remarks/reference</u>
SK1592/<u>hsd</u>R4 Gal$^-$		restriction deficient, transformation recipient (Kushner[28])
D1204/<u>laci</u>^{q}O$^+$z$^-$	pOP203(A$_2$$^+$)/Tet^{r}A$_2$$^+$	<u>lac</u> repressed host strain for plasmid pOP203(A$_2$+) (Winter and Gold[46])
P17	pFE506/Tmp^r	colE1::Tn7, DHFR type 1 (Fling[17])
P113	pFE364/Tmp^r	DHFR type 2 (Fling[17])
P1242	pFE1242/Tmp^r	DHFR type 3 (Fling[18])
<u>S. aureus</u>		
RN450		<u>S. aureus</u> 8325-4 (Novick[33])
RN4220		RN450 derivative, restriction deficient (Novick[33])
G1	pGO1/Tmp^r	wild isolate, Virginia (Archer[1])
G5	pGO5/Tmp^r	wild isolate, Pennsylvania (Archer[1])
WG525	pWG53/Tmp^r	wild isolate, Australia (Grubb[45])

C.   <u>Stock Solutions</u>

E. coli mini-lysate buffer   50mM tris, 15% sucrose, 50mM EDTA

DHFR assay buffer   50mM tris, pH7.5, 150mM KCl,
                                    1mM EDTA, 10mM mercaptoethanol

gel denaturant   1.5M NaCl, 0.5M NaOH

gel neutralizer   3M NaCl, 0.5M tris, pH7

SSC   1X = 0.15M NaCl, 0.015M NaCitrate

50X Denhardt's Reagent   0.02% ficoll, 0.02% BSA,
                                    0.02% polyvinylpyrollidone

SSPE   0.18M NaCl, 10mM $NaPO_4$,
                                    1mM EDTA, pH7

Prehybridization Solution   5X Denhardt's Reagent, 5X SSPE,
                                    200ul denatured salmon
                                    sperm DNA, 50% formamide

Hybridization Solution   1X Denhardt's Reagent, 1X SSPE,
                                    200ul denatured salmon
                                    sperm DNA, 50% formamide

Probe Denaturant   50% formamide, 10mM tris, pH7,
                                    0.1mM EDTA

TE Buffer   50mM tris, 5mM EDTA, pH8

TES buffer   50mM tris, 5mM EDTA, 500mM NaCl, pH8

TBE buffer   0.089M tris-borate, 0.089M Boric
                                    acid, 0.002M EDTA

SDS   sodium dodecyl sulfate in TE

Brij   5% Brij 58, 1% deoxycholate,
                                    0.05M EDTA, 0.05M tris, pH8

Tracking Dye   0.07% bromophenol-blue,
                                    7% SDS, 33% glycerol

ethidium bromide solution   ethidium bromide 10mg/ml in TBE

low salt buffer   0.1M NaCl, 0.05M EDTA, pH6.9

D.   <u>Isolation of Plasmid DNA from E. coli</u>

When a colony displayed a desired phenotype, it was lysed by a "mini-lysate" procedure[14] to examine its plasmid DNA. In this procedure, an overnight culture of the colony was lysed by the addition of lysozyme, SDS, and KAc. To remove any cellular RNA, RNAse was added. Soluble proteins were extracted by treatment with phenol resulting in an aqueous layer which contained the plasmid DNA. This DNA was used for restriction digestion and electrophoresis as described below(section F).

E.   Purification of Plasmid DNA by Equilibrium
<u>Centrifugation</u> in Cesium Chloride-Ethidium Bromide Gradients

If the minilysate procedure indicated that the <u>E. coli</u> cells lysed contained a desired DNA construct, cultures of the corresponding colony were lysed and the procedure of Clewell and Helinski[43] was followed to obtain plasmid DNA. Isolation of staphylococcal plasmid DNA was by a procedure reported by Archer <u>et al.</u>[4], in which lysostaphin is used to prepare osmotically fragile cell forms. In each case the resulting DNA solution contained covalently closed, circular plasmid DNA as well as some broken, linear molecules of chromosomal DNA which were too small to be separated with the other cellular debris. Plasmid DNA was further purified by the procedure of Radloff <u>et al.</u>[37]. In this procedure, the DNA solutions from above were mixed with solutions of cesium chloride and ethidium bromide, and the mixture was centrifuged for 48 hours at 40,000 RPM in a Sorvall

70Ti rotor at room temperature. Because the two bacteria have different GC content, the concentration of cesium chloride used was different for E. coli and staphylococcal plasmid DNA purifications(for E. coli, 8 g of CsCl/7.7 ml of DNA solution; for staphlococci, 5.9 g CsCl/6.4 ml DNA solution). Following ultracentrifugation, the presence of ethidium bromide allowed the visualization of a plasmid DNA band using an ultraviolet light. A fraction containing the band was collected and treated with isopropanol to extract the ethidium from the DNA. The solution was the dialysed overnight to remove the cesium chloride.

F.   Restriction Endonuclease Digestion and Ligation Reactions

Restriction endonuclease digestion of DNA was performed according to the manufacturer's specifications. Reactions were generally carried out in a total volume of 15ul(12.5ul DNA, 1.5ul 10X reaction buffer, 1ul restriction enzyme). Tubes were placed in a 37°C multiblock heater and digestion allowed to proceed for 90 minutes. Reactions were stopped by the addition of tracking dye if the DNA was to be electrophoresed or by ethanol precipitation and phenol extraction if the DNA was to be used in a ligation. DNA ligations were performed using T_4-ligase, usually in a total volume of 25ul(21.5ul DNA, 2.5ul 10X ligation buffer, 1ul ligase). Incubation was either overnight at 4°C or for one hour at 25°.

G.   Transformation of Competent Cells

     A suitable recipient strain(SK1592) of E. coli was
transformed, according to the method of Davis[15], with purified
plasmid DNA.   Transformed cells were selected phenotypically by
plating on media containing antibiotics.   Selective media was
prepared by adding one or more antimicrobial agents(trimethoprim
@ 25ug/ml, tetracycline @ 10ug/ml, ampicillin @ 20ug/ml) to
Mueller-Hinton agar before casting into petri plates.

H.   Agarose Gel Electrophoresis

     DNA solutions were electrophoresed through 0.7 or 0.9%
agarose in TBE buffer according to a method adapted from
Maniatis[30]. Gels were run in TBE buffer either vertically(14cm
x 5.0 x 0.2cm) or horizontally(7.5cm x 5.0cm x 0.2cm).
Electrophoresis was performed by applying a current of 100 volts.
When the dye front reached the end of the gel(approximately 120
minutes for vertical, 90 minutes for horizontal), gels were
removed and stained for ten minutes with an ethidium bromide
solution.   Gels were destained for five minutes under cold,
running tap water and placed on an ultraviolet
transilluminator(UVP Inc., San Gabriel, CA) to visualize the DNA.
Photographs of gels were taken with a Polaroid camera(S/P).   DNA
size markers were purchased from BRL and the size of DNA
restriction fragments was determined relative to those markers by
linear regression analysis.

I.   Polyacrylamide Gel Electrophoresis

Polyacrylamide gels[30] were run in the same vertical apparatus as agarose gels. Polyacrylamide was prepared by mixing 4g acrylamide, 0.13g bis-acrylamide, 40mg ammonium-persulfate, and 80ml TBE buffer in a 250ml erlenmeyer flask.   Immediately before pouring the gel, 80ul TEMED(N,N,N',N',-tetramethylethylene diamine) was added to begin the polymerization reaction. The gel was allowed to polymerize for one hour before the addition of DNA. A current of 150 volts was applied to drive the DNA through the polyacrylamide.   When the dye front reached the end of the gel(approximately one hour), the gel was removed, stained with an ethidium bromide solution for 20-30 minutes, and destained under cold, running tap water for 20-30 minutes.   DNA was visualized and photographs taken in the same manner as described for agarose gels.

J.   In situ Filter Hybridization

To   assess   DNA-DNA   homology,   <u>in   situ</u>   filter hybridization("Southern blotting") was performed by the method of Southern[38]. Following agarose gel electrophoresis, the gel was stained and photographed as usual. The gel was exposed to ultraviolet light for 2-5 minutes in order to introduce single strand breaks into the DNA.   DNA was denatured by soaking the gel in gel denaturant.   This was followed by soaking in gel neutralizer.

Nitrocellulose paper(Schleicher and Schuell, Keene, NH) was

prepared as described by Maniatis[30]. Gloves were worn throughout the procedure. The filter was placed on top of the gel in such a manner that the DNA would transfer from the gel to the nitrocellulose as 20X SSC passed through the gel and the filter paper. Following incubation overnight, the gel was peeled off the nitrocellulose and restained with ethidium bromide to determine the efficiency of transfer. The filter was placed between two sheets of 3MM paper, clamped between two pieces of glass, and dried under a vacuum at 80^OC for two hours. After drying, the filter was stored in a vacuum dessicator at room temperature.

In order to determine if there was homology between the DNA which was blotted to the nitrocellulose paper(target) and the cloned staphylococcal plasmid DNA in question(probe), DNA was radiolabelled with ^{32}P by in vitro nick translation. Nick translation was performed according to the instructions provided in the NEN nick translation kit. The stringency of hybridization between the probe and the target is determined by the ionic strength of the solutions and the T_m, that is, the temperature at which a DNA duplex is 50% denatured. The T_m for a given experiment is dependent upon the concentration of formamide used and the G + C ratio of the DNA. Each increase of 1% in the formamide concentration lowers the T_m of a DNA duplex by 0.7^OC[30]. For 80% stringency, the filter was prehybridized at 42^OC for one hour in prehybridization solution(50% formamide),

the correct volume of which is determined as $100ul/cm^2$ of filter.
During this incubation, the probe was denatured by the addition
of 300ul of formamide and 200ul of probe denaturant and heating
at $65^{o}C$ for five minutes. The filter was then soaked overnight
at $42^{o}C$ in hybridization solution plus the probe. After
incubation overnight, the filter was twice washed for 15 minutes
in a solution of 2X SSPE and 0.1% SDS, and twice in a solution of
0.1X SSPE and 0.1% SDS. After the filter was allowed to air dry
at room temperature, it was placed in a Dupont X-Ray
cassette(S/P) with a piece of Kodak X-Ray film and exposed 24-
72 hours at $-70^{o}C$.

K.   Gene Product Analysis

DHFR was isolated as described by Sheldon and Brenner[41].
Overnight cultures were harvested by centrifugation and lysed in
DHFR assay buffer. Staphylococci were lysed by treatment with
5mg/ml lysostaphin. E. coli strains were lysed by sonication
with a microprobe on a Fisher sonicator. DHFR is labile to
prolonged sonication and care was taken to use short bursts of
sonication while keeping the cells on ice.

DHFR activity was measured using a Beckman Model 25
spectrophotometer by the method described by Poe et al.[36]
involving the decrease in absorbance that occurs at 340nm when
NADPH and dihydrofolate(FH_2) are reacted to form $NADP^+$ and
tetrahydrofolate(FH_4), respectively. FH_2 was prepared by the
method of Blakely[10]. Assays were performed at room

temperature.   Standard conditions included, in addition to assay buffer, 100uM NADPH, and 0.01mM FH_2, and an appropriate amount of cell extract to achieve a final volume of 1.16ml.   Baseline values for all strains were determined by measuring the absorbance before the addition of FH_2.   After each strain was examined for DHFR activity, assays were repeated with the addition of increasing amounts of trimethoprim(10^{-7} to 10^{-2}M) to determine the IC_{50}(the concentration of trimethoprim at which 50% of DHFR activity was inhibited).

L.   Disk-plate Bioassay for Trimethoprim

     To determine if trimethoprim was being altered or destroyed by bacterial cultures a disk-plate bioassay was performed[29]. Absorbant paper disks were inoculated with broth cultures of trimethoprim resistant or trimethoprim sensitive bacteria.   The disks were placed on a surface of agar containing a dispersion of indicator organism.   Antibiotic diffused from the disk and inhibited growth of the organism.   After a suitable incubation period, the zone of inhibition around each disk was measured. The sizes of the inhibition zones produced by known concentrations of antibiotic were plotted against the concentrations to form a standard curve.   Zones of inhibition were measured using a Fisher-Lilly antibiotic zone reader(Fisher) and the concentration of trimethoprim in the sample was determined from the standard curve.   This value was compared to the known concentration of trimethoprim in the sample prior to incubation to determine if the trimethoprim was destroyed.

Two trimethoprim resistance plasmids, pGO1(figure 1) and pGO5(figure 2), were shown by restriction endonuclease mapping to be genetically similar. pGO1 is from a clinically significant methicillin-susceptible S. aureus isolated at the Medical College of Virgina's newborn intensive care unit, while pGO5 is from an epidemic methicillin-resistant S. aureus isolated at the Philadelphia Veterans Hospital. Resistance markers(gentamicin, quarternary ammonium-ethidium bromide, and trimethoprim) are in different locations, relative to each other, on these plasmids.

```
        A               C     E    F       B      D   G   H
/                   /     /    /      /         /      /  / /
0                   ********************         *******50Kb
¦-->¦           <--¦--¦   ----------
Tmp             Gm Qam    Tra
```
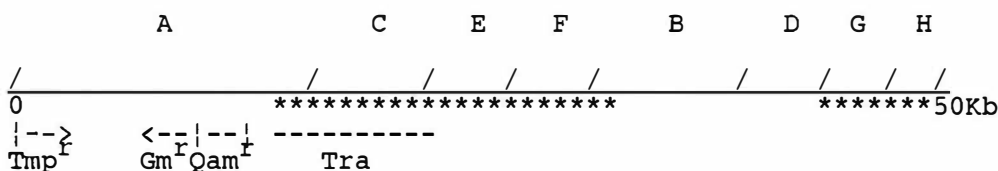
Figure 1. EcoRI restriction digest map of pGO1. / indicates restriction site, capital letters indicating descending order of migration in agarose gels, **** indicates area known to delete during transduction experiments.

Previously, EcoRI digests of pGO5 and a deletion derivative of pGO1 in which the 15 Kb EcoRI A fragment was reduced to 8.7 Kb (pGO1-5A) were cloned into pOP203(A_2^+), transformed into restriction deficient E. coli recipient SK1592[28], and selected

on nutrient agar containing trimethoprim and tetracycline. Engineered by Winter and Gold[46], pOP203(A_2^+) is a vector that allows for positive selection of clones because it contains the A_2 gene of Q-<u>beta</u> phage. The A_2 gene encodes a protein that kills the cell unless the gene is interrupted by inserted(cloned) DNA. All trimethoprim-resistant <u>E. coli</u> transformants contained either the 4.2 Kb <u>Eco</u>RI F fragment of pGO5 or the 8.7 Kb <u>Eco</u>RI A fragment from pGO1-5A and were resistant to trimethoprim to the same degree as were the staphylococci from which the genes were obtained(MIC >1000ug/ml).

```
          A            E    G    F    C       B       D   H
/                       /   /    /    /       /       /   / /
0                                                            55
   <-|---|          --------    |-->       |----|
   Gm Qam              Tra      Tmp         Bla
```

Figure 2. <u>Eco</u>RI restriction digest map of pGO5. / indicates restriction site, capital letters indicate descending order of migration on agarose gels.

It was noted that some clones containing the appropriate fragments did not express trimethoprim resistance. Restriction endonuclease mapping revealed that in these clones the fragments were inserted in the opposite orientation of those expressing resistance. Because the A_2 gene in the cloning vector is under the control of the <u>lac</u> promotor/operator, it was possible that

the cloned fragment did not include the normal staphylococcal promotor and that expression of trimethoprim resistance was under control of the lac promotor/operator. To investigate this possibility, pGO12, the clone containing the 4.2 Kb fragment from pGO5, was transformed into D1204, an E. coli strain containing an i^q mutation on an Flac plasmid so that it hyperproduces lac repressor[46]. Trimethoprim resistance was expressed at the same high level in these transformants as it was in SK1592 in which the promotor was fully induced. This result suggested the cloned fragments included their own staphylococcal promotor for the trimethoprim resistance gene and that this promotor was functional in E. coli hosts. The conclusion that the clone included the staphylococcal promotor was further supported when the 4.2 Kb fragment from pGO5 was subcloned onto the E. coli vector pBR322(designated pGO16) and full expression of trimethoprim resistance was retained.

Restriction endonuclease mapping of pGO11, the 8.7 Kb fragment cloned on pOP203(A$_2$$^+$), revealed the existence of a BglII restriction site 1.2 Kb from the end of the EcoRI fragment. This site was exploited in defining the limits of the trimethoprim resistance gene because the vector contained a single BglII site downstream of the promotor. Following digestion with BglII and religation, the remaining 1.2Kb EcoRI-BglII fragment of pGO1-5A was found to be sufficient to encode trimethoprim resistance. This clone, pGO15(Figure 3), was analysed for restriction sites in order to better define the

structural limits of the gene and to obtain probes for <u>in situ</u> filter hybridization studies. In addition, pGO15 was also transformed into D1204, the strain hyperproducing <u>lac</u> repressor. These transformants retained full expression of trimethoprim resistance, suggesting that this clone(pGO15) also included the staphylococcal promotor.

Cells containing the pGO15 clone exhibited MIC values similar to those of the native staphylococcal strain. Neither

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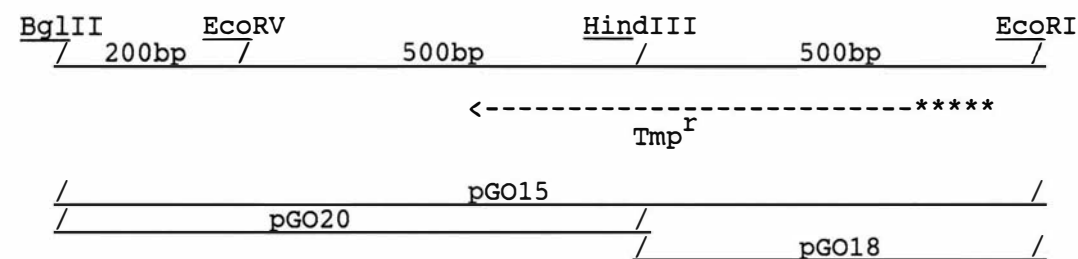
Figure 3. Structural Map of Trimethoprim Resistance Gene showing approximate distances between restriction sites(/). The precise locations of the 5' and 3' termini of the gene are not known.

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the 500bp <u>EcoRI-Hind</u>III nor the 700bp <u>HindIII-Bgl</u>II fragment mediated trimethoprim resistance when subcloned on pBR322(pGO18 and pGO20, respectively) indicating that the single <u>Hind</u>III site fell within the structural gene. A 2.2 Kb <u>Bgl</u>II-<u>Eco</u>RV fragment of pGO1 subloned on pBR322 encoded trimethoprim resistance, indicating that the <u>Eco</u>RV site was outside the gene.

The entire trimethoprim resistance structural gene was contained in pGO15 while only portions of it were carried by pGO18 and pGO20. These three clones were employed as probes for _in situ_ filter hybridization studies to determine the relatedness of the gene to other known trimethoprim resistance genes. Table 1 summarizes the results of these experiments. No homology was seen between any of the probes and the _E. coli_ DHFR genes. pGO15 did show homology with chromosomal DNA from some resistant staphylococcal strains and plasmid DNA from both resistant and sensitive strains. Homology was also seen with a resistance plasmid from an Australian, methicillin-resistant isolate. No homology was seen with the cloned genes from _B. subtillis_ encoding DHFR and thymidine kinase.

pGO18 showed homology only with plasmid DNA that encoded trimethoprim resistance and with chromosomal DNA from resistant strains. When the target DNA was digested with restriction enzyme _EcoRI_, hybridization was seen only with fragments known to include the trimethoprim resistance determinant.

pGO20 showed homology with plasmid DNA that encoded trimethoprim resistance and with trimethoprim-sensitive plasmids similar to pGO1 and pGO5. When pGO1 target DNA was digested with restriction enzyme _EcoRI_, hybridization of the pGO20 probe was exhibited with multiple fragments. Figure 4 shows a linearized _EcoRI_ restriction map of pGO1 indicating areas that gave

Table 1. Summary of results of in situ
filter hybridization experiments

| Target DNA | | ^{32}P-labeled probe | | |
plasmid	Tmp suscept.[a]	pGO15	pGO18	pGO20
pGO15	r	+	+	+
pGO18	s	+	+	-
pGO20	s	+	-	+
pGO16	r	+	+	+
pGO1	r	+	+	+
pGO5	r	+	+	+
pGO2	s	+	-	+
pGO3	s	+	-	+
pGO4	s	+	-	+
pGO7	s	+	-	+
pGO71[b]	s	+	-	+
pGO72[b]	s	+	-	+
pGO73[b]	s	+	-	+
pGO74[b]	s	+	-	+
pGO75[b]	s	+	-	+
S. epi. chromosome	r	+	+	+
ColE1::Tn7	r	-	-	-
pFE364	r	-	-	-
pWG53	r	+	nd	nd
pER1	s	-	nd	nd

a. resistant(r) = growth in broth culture with 20 ug/ml Tmp
   sensitive(s) = no growth in broth culture with <10 ug/ml Tmp
b. Conjugative plasmids from Gm^{r}Tmp^s isolates

positive hybridization signals with pGO20 and areas where

deletions sometimes occured during transduction experiments.   It

can be seen that these areas overlap.   Another area that showed

homology with pGO20 is the area to which the insertion site of a

beta-lactamase transposon has been localized.   Such homology was

seen also between pGO20 and trimethoprim-sensitive plasmids
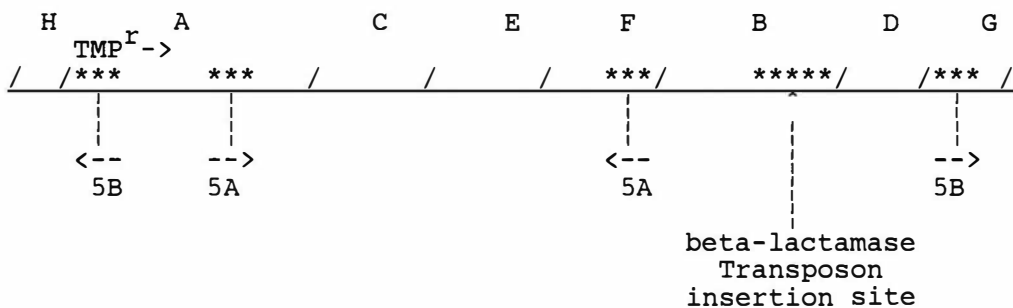
similar to pGO1 and pGO5.

```
 H         A           C       E       F        B        D      G
   TMP^r->
/   /***      ***    /     /     /  ***/    *****/    /*** /
    ¦         ¦                   ¦         ¦         ¦
    ¦         ¦                   ¦         ¦         ¦
   <--      -->                  <--       ¦        -->
    5B        5A                  5A        ¦         5B
                                            ¦
                                            ¦
                                   beta-lactamase
                                       Transposon
                                   insertion site
```

Figure 4.   Linearized restriction map of pGO1.
       key: ***** = areas which show homology with pGO20.
                ¦   = sites where deletions occur during
                      transductions.
                /   = EcoRI restriction sites
             Capital letters indicate descending order of
             migration of EcoRI fragments in agarose gels

Two possible mechanisms of resistance were investigated.

First, an assay was designed to measure whether or not

trimethoprim is destroyed or altered by resistant bacteria.   As a

negative control, one sample contained a solution of trimethoprim

and no bacteria.   An organism known to destroy trimethoprim was

not available for use as a positive control, however SK1592
transformed with pFE364(encoding DHFR type II) is known to be
extremely resistant to trimethoprim[17]. The strains of
interest in this assay were a wild staphylococcal isolate
containing the whole pGO1 plasmid(661) and SK1592 transformed
with pGO15, the cloned trimethoprim resistance gene from pGO1.
Measurements of the amount of trimethoprim remaining in the
cultures after incubation periods showed no evidence of
destruction of the drug(Table 2).

Table 2. Disk-Plate Bioassay for Trimethoprim Destruction

Strain	Trimethoprim in Culture(ug/ml)							
	0hr	1hr	2hr	3hr	4hr	5hr	6hr	24hr
none	3.3	4.2	3.7	3.3	2.7	3.0	2.9	2.9
661	3.1	2.8	3.1	3.1	3.6	2.3	3.1	a
SK1592/pGO15	4.2	4.3	3.4	3.1	3.4	3.0	3.1	2.9
SK1592/pFE364	3.9	4.2	3.9	6.9	3.8	2.9	2.9	2.0

a. culture died

Second, an assay was developed to determine if the
trimethoprim resistance gene product had any DHFR-like activity,
the mechanism described for plasmid-mediated trimethoprim
resistance in E. coli. The data is summarized in Table 3. Five
E. coli strains and four S. aureus strains were assayed.
Negative controls were a transformant of SK1592 containing
pBR322, and two staphlococcal strains, RN450 and RN4220.

Positive controls were transformants of SK1592 containing ColE1::Tn7 encoding _E. coli_ DHFR type I and pFE364 encoding DHFR type II. The clones assayed were transformants of SK1592 containing pGO16(the trimethoprim resistance gene from pGO5 cloned on pBR322) and pGO15 (the trimethoprim resistance gene from pGO1 cloned on pOP203(A_2^+)). Also assayed was RN450 transformed with pGO1-5A, the trimethoprim-resistant deletion derivative of pGO1, and RN4220 transformed with pGO5. Trimethoprim resistant staphylococci expressed a protein with a DHFR activity twenty times greater than that of trimethoprim sensitive staphylococci. _E. coli_ clones of the staphylococcal determinant exhibited specific activities equal to the staphylococcal isolates and 300 times greater than trimethoprim sensitive _E. coli_. The specific activities of the staphylococcal protein expressed in _E. coli_ clones were not significantly different from those of _E. coli_ DHFR type I and type II. The similarities in specific activity demonstrated by these transformants indicated that the staphylococcal trimethoprim resistance gene product was a DHFR-like protein. The high levels obtained for MIC's showed the resistance was equally well expressed in _E. coli_ as in the staphylococcal strains. However, the staphyloccal gene product was four times less resistant than _E. coli_ DHFR type I and 450 times less resistant than type II. These results suggested the proteins were dissimilar.

Table 3.   Characterization of DHFR produced by
           transformed E. coli and S. aureus strains

Strain(plasmid)	DHFR		
	Spec. Act.[a]	IC_{50} [b]	MIC[c]
SK1592(pBR322)	0.2	0.01	2.5
SK1592(pGO16)	5.8	7.7	>1000
SK1592(pGO15)	18	5.6	>1000
SK1592(ColE1::Tn7)	7.1	29	>1000
SK1592(pFE364)	5.3	3600	>1000
RN450	1.2	0.01	10
RN450(pGO1-5A)	21	6.9	>1000
RN4220	4.8	0.01	10
RN4220(pGO5)	14	1.0	>1000

a.   specific activity is measured as nM of folate reduced/min/mg
     total cellular protein.
b. IC_{50} is the uM concentration of trimethoprim required to
     reduce DHFR activity by 50%.
c.   MIC is the minimum amount of trimethoprim (expressed in ug/ml)
     neccesary to inhibit growth in a broth culture.

DISCUSSION

Trimethoprim resistant staphylococci express a DHFR-like protein with a specific activity twenty times greater than that of trimethoprim sensitive isolates. The trimethoprim resistance determinant is encoded on large conjugative plasmids and in the chromosomes of some isolates. The plasmid-encoded determinant has been cloned in E. coli on a 1.2 kilobase DNA fragment inserted into the vector pOP203(A_2^+) and on a 4.2 kilobase fragment inserted into the vector pBR322. These clones expressed trimethoprim resistance in E. coli at levels as high as those seen in staphylococci and equal to that of the previously characterized E. coli DHFR genes. These genes are expressed equally well on low copy number plasmids in staphylococci and high copy number vectors in E. coli. Trimethoprim resistance can be used, therefore, as a marker on shuttle plasmids.

Restriction endonuclease mapping of the E. coli clones of staphylococcal DNA has shown the gene to be encoded on a one kilobase EcoRI-EcoRV fragment. A HindIII site located 500 base pairs from the EcoRI site has been shown to inactivate the gene. No other sites for common restriction endonucleases have been found within the gene.

Gene expression in one orientation and not the other may suggest the direction of transcription. When transcription is in the same direction as that of the lac promotor or when the lac promotor is turned off, trimethoprim resistance is expressed.

When the direction of transcription of the staphylococcal promotor is in opposition to that of the <u>lac</u> promotor, trimethoprim resistance is not expressed, perhaps because the staphylococcal promotor has a lower affinity for <u>E. coli</u> RNA polymerase than the <u>E. coli</u> <u>lac</u> promotor.

Enzyme assays have shown that the gene product of the trimethoprim resistance determinant is a DHFR-like protein. The MIC's for both trimethoprim resistant staphylococci and <u>E. coli</u> clones containing the staphylococcal gene were equal to those of transformants containing the <u>E. coli</u> DHFR genes (MIC > 1000ug/ml). Likewise, the specific activities of the enzymes produced by the various transformants were very similar. However, the staphylococcal enzyme was four times less resistant to inhibition by trimethoprim(measured as the IC_{50}) than <u>E. coli</u> DHFR type I and 450 times less resistant than type II. These results suggested that while the trimethoprim-resistance gene product is a DHFR-like protein and over-production of it may be the cause of resistance, the enzyme is different than those found in <u>E. coli</u>.

This conclusion was further supported by the results of <u>in situ</u> filter hybridization experiments. The 1.2 Kb <u>EcoRI</u>-<u>BglII</u> fragment inserted into pOP203(A_2^+) was used as a probe. Target DNA included <u>Eco</u>RI digests of plasmids containing the staphylococcal trimethoprim resistance gene, plasmids encoding <u>E. coli</u> DHFR type I and type II, and a cloned <u>B. subtilis</u> DHFR gene. No homology was seen with the <u>E. coli</u> or <u>B. subtilis</u> genes. The

trimethoprim resistance gene in staphylococci is not likely, therefore, to be closely related to those from these species.

Bioassays of the level of trimethoprim in cultures showed no change after incubations of various lengths of time. This was true of both staphylococcal and E. coli strains harboring the resistance determinant. That there was no change in the concentration of trimethoprim in the cultures shows that destruction of the drug was not occuring and further supports an altered target enzyme as the mechanism of resistance.

Positive hybridization signals were seen between the pGO20 clone(the region downstream of the structural gene) and several EcoRI restriction fragments of pGO1 and pGO5, suggesting the presence of a repeated sequence. These areas of homology coincided with areas of pGO1 and pGO5 known to be involved in deletions and transposon insertions. The pGO20 probe also gave positive hybridization signals with restriction digest fragments of a variety of plasmids similar to pGO1 and pGO5, but which do not encode resistance to trimethoprim and which did not hybridize with pGO18(the structural gene probe). This repeated sequence may represent an insertion sequence that mediates rearrangements such as those responsible for the differences between pGO1 and pGO5. The origin of this sequence is, of course, open to speculation, but if further characterization shows it to be a defective transposon or insertion sequence, it may be possible to employ this element as a genetic tool in the characterization of staphylococcal determinants by methods involving recombination or

insertional inactivation.

IS elements are discrete, mobile genetic entities able to insert into new sites on the same or another replicon[24]. The repeated sequence cloned from pGO1 appears at least five times on pGO1 and at least four times on pGO5. It appears multiple times on a variety of other plasmids and in the chromosomes of some isolates. Most IS elements studied are between 0.7 and 1.8 kilobases in size, though there is considerable variation. The cloned area of pGO1 containing the repeated sequence is 0.7 kilobases. Approximately 100 bases at the 5' end are part of the trimethoprim resistance gene. It is also possible that the repeated sequence extends beyond the BglII site that marks the 3' end of the clone.

All such elements sequenced share the structural feature of carrying inverted terminal repeats of about 10-40 base pairs. Alterations within these inverted repeats often affect transposition activity[39]. Therefore, it is possible that mutations in the nucleotide sequence could lead to a reduction in the efficiency with which an IS element transposes. The sequence of pGO20(as well as the structural gene) remains to be determined.

IS elements can mediate DNA rearrangements apart from transposition events[22]. IS elements also provide homologous DNA for general recombination systems. IS-mediated DNA rearrangements, together with rec-dependent recombination between IS elements and excission of the elements, may lead to gene

duplication and amplification.   Also possible is the generation or decay of IS-flanked transposons, as well as other DNA restructuring. Deletions could be explained by a process in which an IS element transposed into a replicon that already carried a copy of the same IS element[35].   Depending upon the orientation of the transposed IS element relative to the preexisting copy, reciprocal homologous recombination would result in   either inversion or deletion.   IS-mediated deletions have been shown to remove one of the flanking repeats[13], resulting in an inability to further transpose.   The efficiency of these recombinational processes depends on the length of the IS element, the presence of particular sequences on or near the IS element[25], and probably the spatial proximity of the two interacting elements. These kinds of IS mediated DNA rearrangements may also occur in rec independent cells[24].   Such IS-mediated rearrangements may help expand a prokaryotic organism's ability to adapt to new environments and provide a selective advantage to the population of organisms harboring mobile genetic elements.   This process could explain construction of multiply resistant plasmids, such as pGO1 and pGO5, and their lack of transposons.

        The association of IS elements carrying functional genes with transmissible plasmids facilitates the horizontal spread of bacterial genes to distantly related organisms. Microorganisms take advantage of these elements for adaptation to environmental conditions. Transposition of IS elements may occur more readily in resting cells(and probably under stress conditions) than in

exponentially growing cells[25]. Controlled conditions in the laboratory probably limit the number of IS-mediated rearrangements likely to be observed to much less than their actual frequency in nature. The low transformation frequencies generally obtained with staphylococci may preclude observing transposition events that occur rarely.

pWG53, the trimethoprim resistance plasmid isolated in Australia, also encodes resistance to quaternary-ammonium and ethidium bromide compounds at a determinant which maps close to the trimethoprim resistance gene. This is similar to pGO1 and pGO5 and further suggests a common ancestry for these determinants and the plasmids harboring them. Plasmid pWG53 also contains a transposon encoding resistance to gentamicin. This gentamicin resistance gene is homologous to the resistance gene on pGO1, pGO5, and plasmids related to them. In the American isolates gentamicin resistance is not transposable. The transposability, or lack thereof, of the gentamicin resistance determinant may be another example of IS-like elements mediating intramolecular rearrangements. Determination of the nucleotide sequence of pGO20 will be required to further investigate this phenomenon.

Characterization of the trimethoprim resistance determinant and its evolutionary history continue. Three areas of further study will be pursued in the immediate future. The most enlightening of these likely will be nucleotide sequencing of the gene and the putative insertion sequence(s) nearby. This should

yield information as to regulatory regions and functional or defective genes. Recombinational experiments will help to determine if intramolecular recombinations are in fact mediated by the repeated sequence seen on pGO1 and pGO5, and if these rearrangements occur in rec deficient hosts. Purification of the gene product will tell more about the mechanism of resistance, as well as lead to further experiments to characterize the gene and its background. Purified DHFR can be injected into rabbits for the purpose of obtaining antibodies to the protein. These can then be used to examine the antigenic relationship of this DHFR to other known DHFRs.

Plasmid-encoded trimethoprim resistance has not been previously described among staphylococcal isolates in this country and has only recently been reported in Australia[45]. That these plasmids are conjugative implies that resistance to trimethoprim is likely to spread, particularly in those hospitals where these plasmids have been identified. Definition of a new mechanism of antibiotic resistance may suggest new modes of clinical treatment to avoid development of new resistant strains and the spread of existing ones, as well as expand our knowledge of resistance in virulent pathogenic bacteria. Of particular interest is how new resistance genes arise and how the determinants encoding them move among replicons. Insertion sequence-like elements have not previously been reported in staphylococci, but may play a role in the development of multiply-resistant plasmids.

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