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Staphylococcus simulans biovar *staphylolyticus* produces lysostaphin, an enzyme that lyses other staphylococci by hydrolyzing the polyglycine cross bridges in their cell wall peptidoglycans. *epr* confers lysostaphin resistance on staphylococci by specifying for the substitution of serines for some glycines in their peptidoglycan cross bridges. *epr* and *end* (the lysostaphin gene) both reside on plasmid pACK1 in *Staph. simulans* biovar *staphylolyticus* *epr* is similar in sequence to *zif*, the gene for resistance to the streptococcolytic enzyme zoocin A, which is produced by *Streptococcus zooepidemicus* 4881. The similarity of *epr* and *zif* suggests that these genes may have had a common origin. We are attempting to determine if *epr* also can function in streptococci to modify their peptidoglycan cross bridges to contain serines. One mechanism by which *epr* might be introduced into streptococci would be by sexual conjugation. If *end* and *epr* cannot be transferred to streptococci by conjugation, we will develop laboratory procedures that will allow us to introduce these genes into streptococci to determine if *epr* also can specify for modification of streptococcal cell walls.

Introduction

Staphylococcus simulans biovar *staphylolyticus* produces an extracellular glycyglycine endopeptidase (lysostaphin) that lyses other staphylococci by hydrolyzing the polyglycine cross bridges in the rigid peptidoglycan layer in their cell walls (Fig. 1) (1, 2). The lysostaphin endopeptidase gene (*end*) and the gene for lysostaphin endopeptidase resistance (*epr*) reside on a large plasmid called pACK1, which may be a conjugative plasmid (3, 4, unpublished data). *Streptococcus zooepidemicus* strain 4881 recently was found to produce a streptococcolytic enzyme called zoocin A. The nucleotide sequences of the genes for zoocin A (*zooA*) and for lysostaphin endopeptidase (*end*) have been determined and are very similar (6). In addition, the nucleotide sequences for the genes for resistance to lysostaphin endopeptidase (*epr*) and resistance to zoocin A (*zif*) also are very similar (7). The *end/epr* pair are located immediately adjacent to each other on plasmid pACK1 in *Staph. staphylolyticus*; the *zooA/zif* pair also are immediately adjacent to each other in *Strep. zooepidemicus* 4881 but are chromosomally encoded; both pairs of genes are transcribed in opposite directions (Fig. 2). *epr* confers resistance to lysostaphin endopeptidase by modifying the peptidoglycan cross bridges in staphylococci to contain serines in place of some glycines (8). The cell wall modification specified in streptococci by *zif* is not yet known. The sequence homology

between the enzyme/resistance gene pairs suggests that at some point in time one of these pairs was horizontally transferred from one group of organisms to the other and then was modified to function in its new location. In my project we are attempting to move the *end/epr* pair from *Staph. staphylolyticus* into *Streptococcus gordonii* DL1 by conjugation or by the use of recombinant DNA technology and determine if *epr* can function in streptococci to specify for the incorporation of serines in place of some of the alanines normally present in their peptidoglycan cross bridges (Fig. 3).

Materials and Methods

Bacterial Strains, plasmids, and growth conditions:

S. simulans biovar *staphylolyticus* (NRRL B-2628, originally obtained from Northern Regional Research Center, Peoria, IL), the lysostaphin-producing organism and harborer of the plasmid of interest (pACK1), *Staphylococcus aureus* FDA 209P (originally obtained from the stock culture collection of The Department of Botany and Microbiology, University of Oklahoma, Norman, Okla.), and *Staphylococcus aureus* RN4220 were grown in Brain Heart Infusion Broth (Difco Laboratories, Detroit, Mch.) at 37° C with shaking at 250 rpm on a Model G25 incubator shaker (New Brunswick Scientific, Inc., Edison, NJ). *S. gordonii* DL1 ATCC 10558 was grown aerobically and without agitation in Terrific Broth (Maniatis) plus

0.4 M sorbitol at 37° C and 5% CO₂ for high density cell growth 18 hours before electroporation. Then, one hour before electroporation, *S. gordonii* was grown in Terrific Broth plus 10% glycine and 0.4 M sorbitol.

Conjugation Experiments

In experiments to determine if pACK1 can be transferred to other bacteria by sexual conjugation, we are mixing cells of *Staph. simulans* biovar *staphylolyticus*, which are sensitive to the antibiotic streptomycin, with cells of a streptomycin-resistant strain of *Staph. aureus* RN4220 and then plating the mixture on agar plates containing streptomycin. If any of the *Staph. aureus* cells have acquired pACK1 by conjugation, they will produce colonies with clear areas around them due to lysis of the other *Staph. aureus* cells growing on the agar by the staphylolytic endopeptidase encoded on this plasmid. These putative transconjugants will be confirmed to be *Staph. aureus* and not spontaneous streptomycin-resistant mutants of *Staph. simulans* biovar *staphylolyticus* by testing for mannitol fermentation and for coagulase production, traits for which *Staph. aureus* is positive and *Staph. simulans* biovar *staphylolyticus* is negative.

Selection of a Streptomycin Resistant Strain

Duplicate Brain Heart Infusion Agar (BHIA) (Difco) plus streptomycin concentration plates were made for each antibiotic concentration ranging from 25 µg/ml to 130 µg/ml. For each antibiotic concentration, 0.1 ml of *S. aureus* RN4220 was plated on one plate and 0.1 ml of *S. simulans* was plated on the other. The antibiotic concentration that was selected prevented the growth of *S. simulans* while allowing for sparse growth of *S. aureus* RN4220. The colonies of RN4220 growing at this concentration were spontaneous mutants for streptomycin resistance and were selected to be recipient cells for the conjugation experiment.

Procedure

A 10 ml culture of RN4220 streptomycin-resistant (STr) was transferred to one 15 ml sterile centrifuge tube, and a 5 ml culture of the donor cells, *S. simulans*, was transferred to another 15 ml sterile centrifuge tube. Cells from each culture were harvested by centrifugation at 29° C on a IEC CR-6000 refrigerated centrifuge (Fisher Scientific, New Brunswick, NJ) for 20 minutes at 2000 rpm. After

centrifugation, the supernatant from the tube containing the *S. simulans* was aspirated without disturbing the pellet. 6 ml of the supernatant from the tube containing the recipient cells was then added to the tube of *S. simulans*. From the tube of RN4220 Str, 3 ml of the supernatant was aspirated, leaving just 1 ml in the tube. Both pellet were resuspended with a Fisher Vortex Genie 2. Following resuspension, the cells were allowed a 30 minute induction period (this period gave time for the donor cells to prepare for conjugation). When the induction period ended, .125 ml of *S. simulans* was mixed with 1 ml of RN4220 Str in a sterile, plastic culture dish. Using spread plate technique, 0.2 ml of the mixture was plated onto a BHIA + 115 µg/ml streptomycin concentration plate at 0 hour. Then, the mixture was plated out at 15 minute intervals for one hour. The plates remained upright for approximately 30 minutes until they were dry. These plates were incubated for 5 days at 37° C.

Plasmid Isolation and Analysis

Plasmids will be detected by growing the cells in liquid culture medium, lysing the cells, deproteinizing the lysates by extraction with phenol, precipitating the DNA with alcohol, and analyzing the DNA molecules present by agarose gel electrophoresis followed by staining with ethidium bromide. Plasmid DNA molecules will appear as easily recognizable bands distinct from the large band of chromosomal DNA in these types of analyses.

Small Scale Plasmid Prep. for Gram Positives

Cells of *S. simulans* for screening were harvested in a sterile microfuge tube by pelleting 1 ml of the culture in an Eppendorf 5414 Centrifuge for one minute. Subsequently, the sample was washed with 1 ml of TE buffer (Maniatis), resuspended with a vortex, and pelleted for one minute. The TE buffer was decanted following centrifugation, and the cells were resuspended in 1.0 ml ice-cold acetone (-20° C) with a sterile Pasteur pipet tip. In order to maximize the activity of the cells' autolysins, the sample was placed on ice for 15 minutes. The sample was then removed from the ice, pelleted, and the acetone was decanted. Using a hairdryer set on low heat, the pellet was dried by running the stream of air gently across the top of the tube. To further break down the cell walls of the lysed cells,

the pellet was resuspended in 250 μ l of a lysozyme solution (0.002 g lysozyme in 1 ml TE buffer) by running the microfuge tube across a microfuge tube holder. The sample was incubated for 45 minutes at 37° C. After incubation, 250 μ l of a sodium dodecyl sulfate solution (0.008 g SDS in 1 ml TE buffer) was added and mixed by rocking the tube to obtain plasmid DNA. In order to precipitate the protein, 500 μ l of a 5 M NaCl solution was added and mixed by inverting the tube gently. The tube was placed in a -20° C upright freezer for 24 hours.

Following the freezing period, the sample was allowed to thaw and return to room temperature, and then it was microfuged for 15 minutes in a cold room at 4° C. 650 μ l of the supernatant from the cool sample was removed without disturbing the protein pellet in the bottom of the tube and placed into a new, sterile microfuge tube. Using an equal volume of SEVAG (24 ml chloroform:1ml isoamyl alcohol), the mixture was extracted by inverting the tube 10 times and separated into two layers by microfuging for 5 minutes. 650 μ l of sample was removed from the top layer, the layer containing the DNA, without disturbing the protein interface and placed into a new, sterile microfuge tube. The DNA was precipitated with a 0.8 volume (390 μ l) of 2-propanol for ten minutes at room temperature. The precipitated DNA was collected by centrifugation for 15 minutes. In order to prevent the removal of DNA from the microfuge tube, the 2-propanol was aspirated while holding the tube hinge side up (DNA pellet located on the side of the tube with the hinge) and without disturbing the protein pellet. The DNA pellet was then washed with 70% ethanol, and tube was left to sit on its hinge for 2 minutes. The resuspended pellet was centrifuged for 2 minutes, and the supernatant was aspirated. The DNA sample was dried in a Temp-Blok Module Heater heating block at 65° C for approximately 10 minutes. Finally, the pellet was resuspended in 30 μ l of TE buffer by adding the solution down the back of the tube. The sample was microfuged again for one minute and was analyzed for pACK1 by 1% agarose gel electrophoresis. The rest of the DNA was placed into a -20° C upright freezer so that it could be used for electroporation later.

Electroporation

Strep. gordonii DL1 was grown up under the conditions for electroporation competence as described by (Buckely et als.) The strep. cells to be electroporated were harvested by microcentrifugation and then washed 4 times in a H₂O + 0.4 M sorbitol solution. After the final wash, the pellet was resuspended in 200 μ l of this H₂O/sorbitol solution. 40 μ l of this suspension was removed and placed into a new, sterile microfuge tube. Then, 12 μ l of the DNA sample containing pACK1 was mixed into the cell solution. To enhance the efficiency of transformation, the mixture was allowed a 30 minute pre-pulse incubation period as described by (Augrestin and Gotz). All 52 μ l of the cell-DNA mixture were placed into a sterile 2 mm gap Eppendorf Electroporation Cuvette and was pulsed at 1.45 V, 25 μ F capacitance, and 100 ohms resistance with a Bio Rad Gene Pulser. Immediately after electroporating, the cells were rescued in approximately 400 μ l of ice-cold Terrific Broth + 0.4 M sorbitol. The cells were recovered by incubation at 37° C at 5% CO₂ for 1 hour. After recovery, the cells were resuspended in 600 μ l of Columbia Broth (Difco) and allowed to incubate overnight.

Results and Discussion

If we are unable to move the *end/epr* pair into *Strep. gordonii* by conjugation or by electroporation of pACK1, then we will insert the *end/epr* pair into the streptococcal plasmid pVA 838 (9), which carries a gene for chloramphenicol resistance, and then introduce this recombinant plasmid into *Strep. gordonii* by electroporation. Electroporants will be identified based on their resistance to chloramphenicol and on their ability to produce lysostaphin endopeptidase. We then will purify the peptidoglycan from the cell wall of one or more of these electroporants and determine if *epr* can function to modify their peptidoglycan cross bridges to contain serines in place of some of the alanines.

Selection of Putative Transconjugates

After the 5 day incubation period, the concentration plates were viewed for colonies producing zones of cell lysis in the RN4220 Str lawn. Three colonies found on the 0 hour plate and three colonies found on the 30 minute plate were

producing lysostaphin. These colonies were removed from the plates with sterile toothpicks and streaked onto a Mannitol Salt Agar (Difco) plate and an indicator plate containing heat killed *S. aureus* FDA 209P cells. One colony from the 0 hour plate and two colonies from the 30 minute plate fermented mannitol and weakly produced the endopeptidase. Finally, these colonies were confirmed to be RN4220 Str transconjugates by subjecting them to a coagulase test. All putative transconjugates were able to produce coagulase.

Selection of Possible Electroporants

Using spread plate technique, the entire 1 ml culture of electroporated *Strep. gordonii* DL1 was plated out onto ten FDA 209P indicator plates (0.1 ml per plate). Colonies that produced zones of cell lysis on these plates were selected with sterile toothpicks and streaked out onto indicator plates. Isolated colonies producing zones of cell lysis on these plates were then analyzed for the presence of pACK1 by 1% agarose gel electrophoresis.

Electroporation of pACK1 into Streptococcal Cells

To date we have not been successful in detecting any streptococcal cells that have acquired pACK1 in the electroporation experiments. If we determine that pACK 1 is not able to replicate in *S. gordonii*, then we will insert the *end/epr* pair into the streptococcal plasmid pVA838, which carries a gene for erythromycin resistance (9), and then introduce this recombinant plasmid into *S. gordonii* and other streptococci by electroporation. Electroporants will be identified based on their resistance to erythromycin and on their ability to produce lysostaphin endopeptidase. We then will purify the peptidoglycan from the cell wall of one or more of these electroporants and determine if *epr* can function to modify their peptidoglycan cross bridges to contain serines in place of some of the alanines.

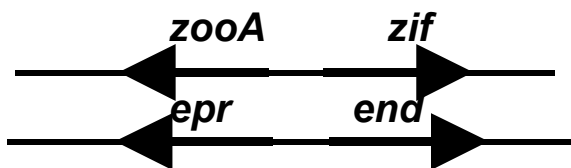
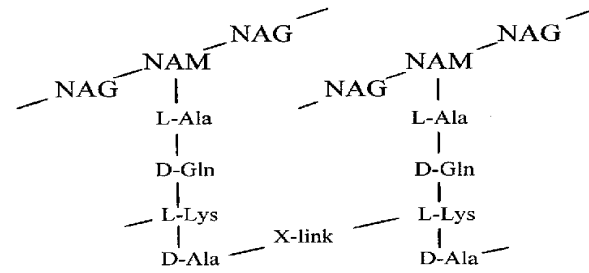


Figure 1. Gene pairs.



Endopeptidases - hydrolyze bonds in the peptide cross bridges

Glycosidases - hydrolyze bonds between the amino sugars in the PG backbone

NAM-L-Ala amidases - hydrolyze the bond between NAM residues and the first amino acid in the tetrapeptide chains

Figure 2. Peptidoglycan cross bridges.

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