GROWTH AND PLASMID CHARACTERISTICS OF A PORCINE-DERIVED SALMONELLA TYPHIMURIUM RESISTANT TO CHLORTETRACYCLINE

A Senior Thesis

By

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Group: Biology

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Submitted to the Office of Honors Programs and Academic Scholarships Texas A&M University in partial fulfillment of the requirements for

1997-1998 UNIVERSITY UNDERGRADUATE RESEARCH FELLOWS PROGRAM

April 16, 1998

Approved as to style and content by:

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USDA, ARS, FAPRL

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Honors Programs and Academic Scholarships

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Abstract

Growth And Plasmid Characteristics Of A Porcine-Derived Salmonella Typhimurium Resistant To Chlortetracycline

J. Mason Shiflett, (Dr. Michael Hume) University Undergraduate Fellow, 1997-1998, Texas A&M University, Department of Biology

This study was a preliminary investigation in which porcine derived Salmonella typhimurium was selected for resistance to the equivalent of 200 and 400 grams of chlortetracycline per ton of swine feed. These resistant strains were cultured at 25° C and 37° C, serially diluted, plated, counted, and compared to the non-resistant control. Strain viable cell counts were lower in the resistant strains than in the control. However, viable cell counts in the resistant groups was about the same at both temperatures as were the viable cell counts of the control. Another preliminary study that intended to show the abilities of the resistant strains to transfer resistance to the non-resistant strain gave unexpected results. Plasmids were isolated for the strains (i.e., control, 200 g/ton CT-resistant, and 400 g/ton CT-resistant) and the two resistant that had been cultured in continuous-flow cultures of porcine cecal bacteria. The plasmids were electrophoresed on agarose gels. While all five strains had plasmids, one of the strains cultured in the continuous-flow culture had additional plasmids when not detected in the preparations.

SUMMARY

This study was a preliminary investigation in which porcine derived Salmonella typhimurium was selected for resistance to the equivalent of 200 and 400 grams of chlortetracycline per ton of swine feed. These resistant strains were cultured at 25° C and 37° C, serially diluted, plated, counted, and compared to the non-resistant control. Strain viable cell counts were lower in the resistant strains than in the control. However, viable cell counts in the resistant groups was about the same at both temperatures as were the viable cell counts of the control. Another preliminary study that intended to show the abilities of the resistant strains to transfer resistance to the non-resistant strain gave unexpected results. Plasmids were isolated for the strains (i.e., control, 200 g/ton CT-resistant, and 400 g/ton CT-resistant) and the two resistant that had been cultured in continuous-flow cultures of porcine cecal bacteria. The plasmids were electrophoresed on agarose gels. While all five strains had plasmids, one of the strains cultured in the continuous-flow culture had additional plasmids when not detected in the preparations.

INTRODUCTION

Salmonella typhimurium infections are like many other bacterial infections in that S. typhimurium adheres to the intestinal epithelium (Tekeuchi 1967; Heffernan et al. 1987; Finlay et al. 1988) and penetrates the epithelium at the Payer's patches. Multiplication of the bacterium takes place in local lymph nodes and the Payer's patches (Hackett et al. 1986; Heffernan et al. 1987; Finlay et al. 1988). Unlike other bacterial infections, once S. typhimurium is in the blood and is taken up by macrophages, the

bacterium not only survives, but also undergoes division inside of the macrophage until the load becomes too great. This results in fulminant septicemia in the infected organism (Fields et al. 1986; Hackett et al. 1986; Heffernan et al. 1987). Virulent serovars of Salmonella have in common a large non-conjugative plasmid, which is essential for intracellular growth (Terakado et al. 1983; Helmuth et al. 1985; Nakamure et al. 1985; Fields et al. 1986; Pardon et al. 1986; Gulig and Curtiss 1987; Hovi et al. 1988).

The reduction in Salmonella typhimurium colonization in agricultural meat products is of major concern to producers of agricultural products and consumers. For this reason, the U.S. Department of Agriculture, Agricultural Research Service, Food Animal Protection Research Laboratory at Texas A&M University has been developing competitive exclusion (CE) cultures derived from the cecal material of broiler chickens to control S. typhimurium colonization in broiler chicks (Nisbet et al. 1992; Hume et al. 1995; Nisbet et al. 1996). Twenty-nine strains of chicken cecal bacteria representing 10 genera, were isolated and identified to make a characterized continuous-flow (CCF) culture that performs in the same manner as the CE culture but with the added advantage of knowing precisely with which organisms the chicks were being inoculated (Corrier et al. 1995; Corrier et al. 1995; Hume et al. 1995). Characterization of ribosomal DNA from 457 different strains of S. typhimurium, suggests that the main sources of human S. typhimurium infection are pig and cattle (Nastasi et al. 1992). Work is underway on a CCF culture for swine similar to the one developed for broiler chicks. The antibiotic chlortetracycline has been used frequently in livestock feed. Antibiotic feed additives are used as growth promoters (Medical Encyclopedia, Inc. 1955) and therapeutic treatments for bacterial infections (Gutzmann et al. 1975). The question has been raised about the

characteristics that might be encountered with a strain of S. typhimurium that was resistant to chlortetracycline, and how would the plasmid profile of chlortetracycline-resistant S. typhimurium cultured in a CE culture appear. The objective of this study to was to determine some of the characteristics, such as temperature sensitivity and transference of resistance, found in a chlortetracycline-resistant strain of S. typhimurium. Plasmid analysis of chlortetracycline-resistant strains cultured in CE cultures and in typical broth media were also explored.

MATERIALS AND METHODS

Development of chlortetracycline-resistant S. typhimurium. Laboratory stock S. typhimurium (pST), originally a swine isolate, was used to select mutant S. typhimurium resistant to chlortetracycline (CT). The stock bacteria were cultured in increasing concentrations of chlortetracycline dissolved in tryptic soy broth (TSB) until a level equivalent to 400 g of chlortetracycline per one ton of feed was reached. After the level of 400 g/ton was reached, only the 200 g/ton, 400 g/ton and the original stock solutions were used for further investigation. Chlortetracycline at 200 and 400 g/ton of feed are subtherapeutic levels often encountered in the swine producer industry. For the plasmid analysis, two additional strains of pST were cultured in continuous-flow cultures of pig cecal contents. Porcine continuous-flow culture 1 (pCF1) was isolated from the cecal material of a pig that had not had CT in its feed, and porcine continuous-flow culture 4 (pCF4) was isolated from cecal material of a pig that was receiving the equivalent of 200

g/ton CT in its feed. Both pCF1 and pCF4 were cultured in the presence of CT at a level equivalent to 400 g/ton; thus both cultures were resistant to CT at the 400 g/ton level. The Salmonella obtained from the two continuous-flow cultures were originally added to the culture to determine the anti-Salmonella effects over time.

Thermosensitivity. Each strain was cultured overnight at 37° C in TSB. The control was cultured again in TSB, the 200 g/ton resistant strain cultured in 200g/ton CT, and the 400 g/ton resistant strain cultured in 400 g/ton CT for another 24 hours. All three strains were then cultured at 25° C and 37° C overnight. Cultures were then serially diluted, plated on brilliant green agar (BGA), and the colonies were counted following incubation at 37° C for 24 hours. The BGA contained nalidixic acid and novobiocin to select for these strains of Salmonella in mixed cultures of bacteria.

Transference. BGA plates were prepared to contain the equivalent of 200 g/ton and 400 g/ton of CT. The 200 g/ton plates were streaked down the middle with the S. typhimurium resistant to 200 g/ton CT. Likewise, the 400 g/ton plates were streaked with 400 g/ton CT-resistant pST. All plates were incubated overnight at 37° C. The streaked plates were then overlaid with 4 ml of BGA with the respective concentration of CT. Non-resistant S. typhimurium was then spread over the whole plate and were incubated overnight at 37° C. Cultures were grown in triplicate in three replicate experiments.

Plasmid analysis. Plasmids were isolated from Salmonella strains using a rapid extraction method as described ~~by Bennet~~ et al. (1990). Tris-borate (0.089 M Tris-borate, 0.089 M

boric acid, 0.002 M EDTA) was used as a running buffer (Fritsch et al. 1982) and sucrose (0.25% bromophenol blue, 40% sucrose in H_2O) as a loading buffer (Fritsch et al. 1982). Two sets of preparations were run on a horizontal, 0.6% agarose gel at 50V for one hour and 40V for 3 hours. One set of preparations contained plasmids nicked by topoisomerase I, while the other set of preparations was not treated with topoisomerase I. Each set contained Salmonella plasmid preparations obtained from the control strain, 200 g/ton CT resistant strain, 400 g/ton CT resistant strain, and the Salmonella obtained from pCF1 and pCF4. Ethidium bromide (0.5 μg/ml) was used as the stain.

RESULTS

Development of chlortetracycline-resistant S. typhimurium. The stock pST was successfully trained to be resistant to CT at the levels equivalent to 200 g/ton of feed and 400 g/ton of feed. The resistant strains showed slower growth than the non-resistant strains when cultured in the absence of the antibiotic. Overnight growth at 37° C in TSB resulted in an average log colony forming units (CFU) of 8.84 for the non-resistant pST, 7.77 for 200 g/ton CT-resistant pST, and 7.13 for 400 g/ton CT-resistant pST.

Thermosensitivity. This experiment was run three times with each strain cultured and plated in triplicate. In the first run (Fig. 1), there was a significant drop in cell count for both the 200 and 400 g/ton resistant strains when cultured in TSB at 25° C as opposed to their growth at 37° C ($p = 0.01$ for both). When cultured in the presence of CT, only the 400 g/ton resistant strain showed a significantly lower cell count when incubated at 25° C

as opposed to 37° C ($p = 0.00$). In the second run (Fig. 2), only the 200 g/ton resistant strain cultured in TSB showed a significant drop in cell count when incubated at 25°C ($p = 0.01$). In the third trial (Fig. 3), there was no significant decrease in the cell count between the resistant strains grown at either 25° C or 37° C. On all trials, the control showed no significantly lower cell count at either temperature.

Transference. Originally this experiment involved streaking non-resistant pST directly over and at 90° angles to streaks of CT resistant pST on plates with CT levels equivalent to 200g/ton and 400g/ton. However, this technique gave inconclusive results since growth was seen over every streak, whereas it was expected only over streak of resistant pST and the junctions of the resistant and non-resistant pST streaks. Thus, the overlay technique was developed. Growth over the entire top surface of the plate was observed for the overlay technique. The colonies were tested to insure that there was no contamination. In the first run, there was contamination from an unknown source by an unknown bacterium apparently resistant to CT. This contamination was partly the result of the absence of nalidixic acid and novobiocin in the BGA plates. Nalidixic acid and novobiocin were not thought to have been needed since the inoculum source was a monoculture of pST. For the second and third run, nalidixic acid and novobiocin were added into the plates and successfully selected only pST. Although there was no more contamination, the non-resistant pST still grew over the entire upper surface of the plate.

Plasmid analysis. Plasmids were found for all five strains isolated (non-resistant pST, 200 g/ton CT-resistant, 400 g/ton CT-resistant, pCF1, and pCF4). Each of the five strains expressed the same plasmid profile with the exception of the pCF1 strain, which had additional bands of smaller plasmids. The large plasmid seen in all the tested strains is larger than 8.5 kbp and is 0.7 mm from the origin. The pCF1 strain had multiple bands between the 0.8 and 1.0 mm marks as well as between the 1.5 and 1.9 mm marks.

DISCUSSION

Development of chlortetracycline-resistant S. typhimurium. The slowness of growth in the resistant strains of pST even in the absence of CT was expected. This is mostly likely the result of the extra metabolic processes that the resistant strains perform enabling them to survive in the presence of CT. The non-resistant strain did not have to expend this energy and could thus direct more of its metabolism directly to growth.

Thermosensitivity. The CT-resistant strains had a lower cell count in the earlier trials, but as the trials progressed, the difference between the cell count of the two strains incubated at 25° C and at 37° C lessened. This was mostly likely due to the strains becoming more adapted to the change in temperature as the more viable cells were selected for in the earlier experiments. As expected, the resistant cells had a lower cell count when grown in the presence of CT than when grown in the absence, and also as expected the non-resistant strain had larger cell counts at 25° C and 37° C than the resistant strains. Clearly, the metabolic activity that is required to grow in the presence of CT is a hindering factor when in the absence of CT.

Transference. The hypothesis of this experiment was based on the belief that if the CT-resistant strains were transferring their resistance to the non-resistant strain, growth of the non-resistant strain would happen only along the overlaid streak of CT-resistant bacteria. If the resistant strains were not transferring resistance, then no growth would appear. If growth along the overlaid streak had occurred, it could have been assumed that the

resistant strains were producing a diffusible product, which disabled the CT. Growth over the entire surface of a plate did not fit into either scenario proposed in the hypothesis.

Plasmid analysis. The results of this experiment indicate that there were plasmids in all five strains. Only the pCF1 strain showed any difference in that it had additional plasmids of lesser size as compared to the other four strains. It is probable that the pCF1 strain obtained the extra plasmids from other bacteria in the continuous-flow culture. From these results, it cannot be assumed that resistance to CT is plasmid derived. Plasmid fingerprints may reveal dissimilarities between the plasmids of the different strains, and certainly gene mapping would uncover any discrepancies between the plasmids, but time did not permit such studies. It is interesting to note that during the isolation of the 200 g/ton and 400 g/ton CT-resistant strains, the cells remained flocculent after being treated with lysozyme and centrifuged for 10 min. at 8,000 g. This may indicate a rearrangement of the outer covering of the cell wall. The pST, CF1, and CF4 strains did not demonstrate this characteristic as they were pelleted after being treated with lysozyme and centrifuged.

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Figure 1. Three porcine derived S. typhimurium (pST) strains grown in tryptic soy broth (TSB) at 25° C and at 37° C. The values seen on the left are the log of the colony forming units (CFUs) per ml of media. The control is pST that is non-resistant to chlortetracycline (CT). The 200R and 400R strains refer to pST resistant to the equivalent of 200 g and 400 g of CT per ton of pig feed, respectively. "Con" refers to those strains grown in the absence of CT, and "Trt" refers to those strains grown in presence of CT (200 g/ton CT equivalent for 200R and 400g/ton CT equivalent for 400R).

Fig. 1

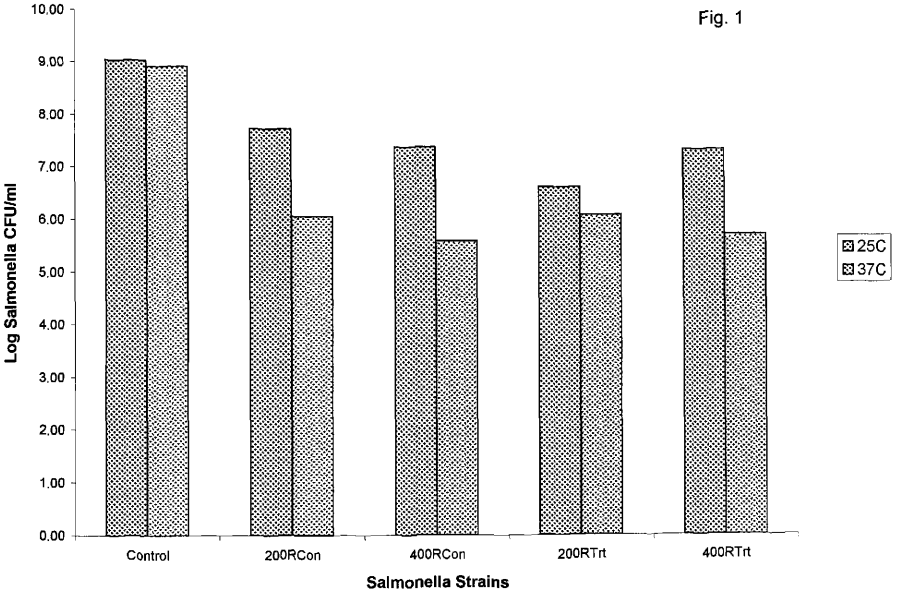
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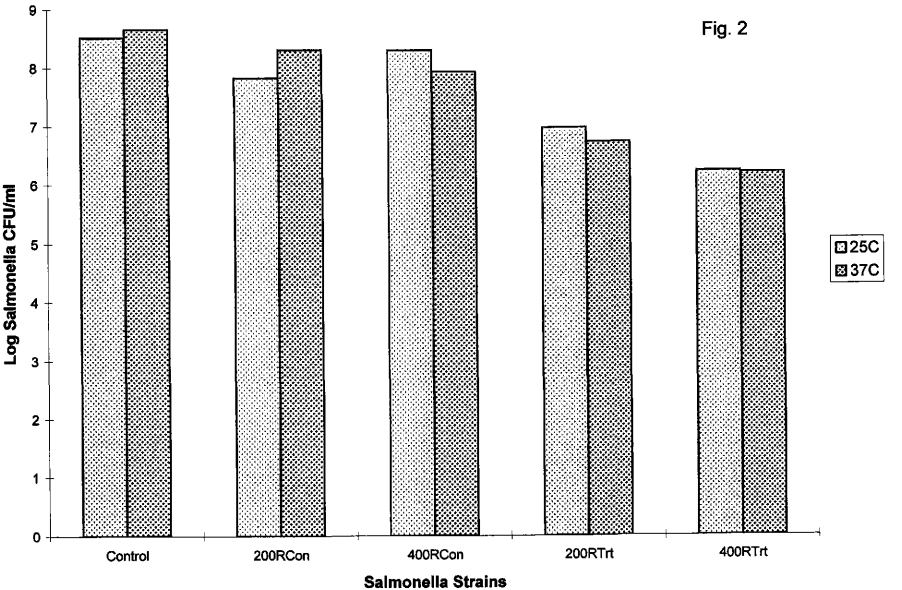
Figure 2. Three porcine derived S. typhimurium (pST) strains grown in tryptic soy broth (TSB) at 25° C and at 37° C. The values seen on the left are the log of the colony forming units (CFUs) per ml of media. The control is pST that is non-resistant to chlortetracycline (CT). The 200R and 400R strains refer to pST resistant to the equivalent of 200 g and 400 g of CT per ton of pig feed, respectively. "Con" refers to those strains grown in the absence of CT, and "Trt" refers to those strains grown in presence of CT (200 g/ton CT equivalent for 200R and 400g/ton CT equivalent for 400R).

Fig. 2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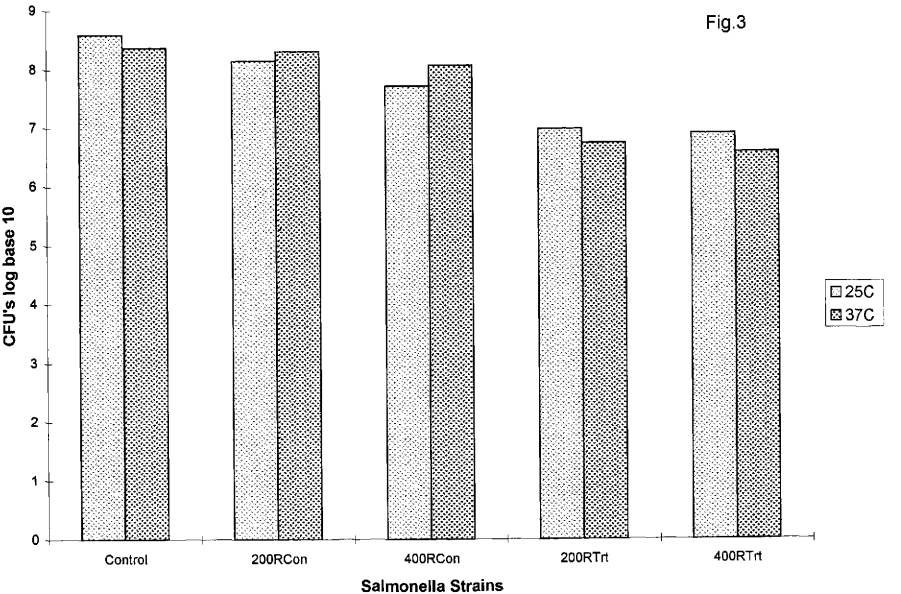
Salmonella Strains
25C
37C

Figure 3. Three porcine derived S. typhimurium (pST) strains grown in tryptic soy broth (TSB) at 25° C and at 37° C. The values seen on the left are the log of the colony forming units (CFUs) per ml of media. The control is pST that is non-resistant to chlortetracycline (CT). The 200R and 400R strains refer to pST resistant to the equivalent of 200 g and 400 g of CT per ton of pig feed, respectively. "Con" refers to those strains grown in the absence of CT, and "Trt" refers to those strains grown in presence of CT (200 g/ton CT equivalent for 200R and 400g/ton CT equivalent for 400R).

Fig.3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