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Agricultural Outlook Forum Presented: February 17, 2006

BY WAY OF EXAMPLE: BIOTECHNOLOGICAL DEFENSE AGAINST MASTITIS DISEASE

Bob Wall
Research Physiologist, USDA

Presented: Friday, February 17, 2006

BY WAY OF EXAMPLE:
BIOTECHNOLOGICAL DEFENSE AGAINST MASTITIS DISEASE

Robert J. Wall
Research Physiologist, Agricultural Research Service
U.S. Department of Agriculture

For 20 years we and others have been attempting to harness the power of transgenic livestock technology to enhance agricultural productivity. We came to believe the true power of transgenesis lies not in advancing the rate of genetic selection, but rather in endowing animals with traits that could not be achieved by standard breeding strategies. Many animal scientists started dreaming about altering the composition of milk to produce new dairy products. Ideas of transgenic cows producing low-fat milk, low-lactose milk and milk for the production of spreadable butter quickly captured the research community. That is, until the processors pointed out that they can already produce those products with cost efficiencies difficult to match by genetic engineers.

Biotechnology companies recognized the potential for producing bioactive compounds in the mammary glands of farm animals. There have been numerous examples of cost effective production of pharmaceuticals in milk of livestock. But, post harvest hurdles have limited product development. Nevertheless, several companies have mammary gland derived pharmaceuticals in the final stages of product development. The first nutraceutical produced in the milk of genetically engineered cattle to hit the market is likely to be lactoferrin. The academic community too has been exploring the potential of manipulating milk proteins to enhance milk's healthfulness, and nutritional and manufacturing properties. Instead, we have chosen to address mammary gland disease.

Our efforts have been focused on developing mastitis resistant dairy cattle for a variety of reasons. Mastitis is a disease of the mammary gland caused by pathogens that find their way into the lumen of the gland through the teat canal. Firstly, mastitis is a significant detriment to agricultural productivity worthy of the significant investment in resources needed to develop a transgenic cattle project. Mastitis is on every dairy farm and affects both the production and post harvest segments of the dairy industry. Estimated losses due to mastitis approach $2 billion annually in the United States alone, but it is clearly a global disease. Secondly, the incidence of mastitis has not declined appreciably since the implementation of the five-point control plan two decades ago. This plan, which includes extensive antibiotic therapy and culling of infected animals, did have a major beneficial effect, and some pathogens, such as *Streptococcus agalactiae* have been virtually eliminated in some countries. However, producers are still expending considerable effort in battling environmental pathogens such as *Escherichia coli* and *Streptococcus uberis*, and the contagious, often subclinical *Staphlococcus aureus.* Furthermore, because of the low heritability of mastitis resistance selective breeding has not been a useful tool for combating mastitis. Additionally, there is a strong desire to reduce the on-farm use of those antibiotics that are also used in human medicine, and certainly not least is a desire to improve the welfare of our dairy animals.

Staphylococcus aureus is one of the most prevalent bacteria that cause mastitis and is responsible for ~25-30% of all intramammary infections. Mastitis caused by *Staphylococcus aureus* is most often subclinical, however, a substantial incidence rate of clinical mastitis is associated with this pathogen. *Staphylococcus aureus* is regarded as a contagious mastitis pathogen because it is commonly spread from infected to non-infected cows at milking. Although the main reservoir of these bacteria is infected udders, *Staphylococcus aureus* have been recovered from surfaces all over the cow and can be readily disseminated from animal handlers who are carriers of this pathogen. Following penetration of the teat canal, these bacteria release a variety of toxins and products that are injurious to the milk producing cells of the mammary gland and impair the gland's immune defense mechanisms. The intramammary formation of abscesses around these bacteria and their capacity to reside intracellularly contribute to the ability of *Staphylococcus aureus* to establish a chronic infection that can persist for the life of the animal. The result of establishment of a subclinical, chronic intramammary infection is long-term decreased milk production that often goes undetected.

Effective strategies for the prevention and treatment of mastitis caused by *Staphylococcus aureus* remain elusive. Vaccines to prevent the establishment of intramammary infection by this pathogen have been around for decades, however, their efficacy has been limited. Their limited effectiveness may be due, in part, to improper immunization schedules, ineffective adjuvant formulation, and their inability to cross-protect against various strains. Since multiple strains can be present within any one herd or even within an individual cow, vaccines or other strategies with less restrictive strain specificity will be required to decrease the incidence of mastitis caused by *Staphylococcus aureus*.

Once established, *Staphylococcus aureus* infections are difficult to treat. These bacteria are able to reside intracellularly and are shed periodically into the milk. Their residence inside of the cells within the gland and the formation of abscesses around foci of infection restrict their contact with administered antibiotics. The percentage of *Staphylococcus aureus*-infected animals that can be cured during lactation with currently approved antibiotics is only between 10-30%. In a recent study of the efficacy of pirlymycin, a common antibiotic used in the treatment of these infections, only 13% of *Staphylococcus aureus* intramammary infections were cured following the recommended 2 day therapy. Extending the therapy to 5 consecutive days increased the cure rate to only 31%, a level which is below the break-even point at which costs incurred through treatment are balanced by increased production and the premium pay associated with milk containing lower numbers of milk somatic cells. Thus, treatment options for lactating cows infected with *Staphylococcus aureus* remain suboptimal.

Lysostaphin is a proteolytic enzyme produced by *Staphylococcus simulans* that cleaves specific bonds found in the peptidoglycan cell wall component of *Staphylococcus aureus*. Due to its potent lytic activity towards this pathogen, lysostaphin's ability to kill *Staphylococcus aureus* has been evaluated in several animal disease models . In a model of endocarditis in rabbits, lysostaphin was shown to be more effective than vancomycin at reducing valve vegetation bacterial counts. Further, lysostaphin has been reported to be effective in the treatment of eye infections caused by *Staphylococcus aureus*. With the advent of antibiotic-resistant bacteria, another key finding of lysostaphin's ability to kill *Staphylococcus aureus* is its equivalent lytic activity toward both methicillin-resistant and sensitive strains.

The efficacy of lysostaphin for the treatment of *Staphylococcus aureus*-induced mastitis has also been evaluated in a variety of animal models, including mice, goats, and cows. Infusion of lysostaphin into the *Staphylococcus aureus*-infected glands of mice was shown to significantly

reduce the number of viable bacteria. Lysostaphin has also been demonstrated to have a cure rate of ~20% when used to treat *Staphylococcus aureus* intramammary infections in lactating cows. Although this rate is comparable to commonly used antibiotics, lysostaphin's targeted specificity and low toxicity may make its use more advantageous. Further studies evaluating formulation, dosing, and treatment durations will be needed to determine whether its efficacy, when used as an intramammary infusate, can be enhanced.

In a recently published study we describe transgenic cattle, carrying a mammary-specific transgene encoding lysostaphin, that are resistant to infection by *Staphlococcus aureus*. Our first three lactating transgenic cows were infused with *Staphlococcus aureus* multiple times during their first lactations. Milk levels of lysostaphin varied between the transgenic animals and ranged from 0.9 to 14 µg/ml. Of the mammary glands of three transgenic cows that were infused with *Staphylococcus aureus*, only 14% became infected. In comparison, 71% of the quarters challenged in the control animals became infected. Even the transgenic cow expressing the least amount of lysostaphin (*i.e.*, 0.9 µg/ml) had an infection rate of only 33%. Of the three transgenic cows infused with *Staphylococcus aureus*, who expressed the highest concentration of lysostaphin in her milk (*i.e.*, 11 µg/ml), was completely resistant to infection. The level lysostaphin in milk of the three transgenic cows remained fairly consistent throughout lactation. The consistent level of expression conferred resistance throughout lactation.

We monitored cows for both a febrile response and for the induction of acute phase protein synthesis, the latter of which is a sensitive marker of infection. In addition to these systemic indicators, individual quarters were monitored for changes in milk somatic cell counts, which during mastitis are primarily composed of white blood cells that play a role in combating infection. As expected, control cows that developed established *Staphylococcus aureus* infections had increased body temperatures, elevated levels of circulating acute phase proteins, and increased milk somatic cells counts. In contrast, the transgenic animals demonstrated none of these signs of inflammation. These data suggest that lysostaphin prevents infection through its bactericidal properties and, thus, prevents the onset of inflammation.

We concluded from that limited study that the degree of protection afforded by the transgene was related to the concentration of lysostaphin in milk, as might be expected. We recently verified an animal from a fourth line, with an intermediate level of lysostaphin expression, is also protected against *Staphlococcus aureus* infection. *In vitro,* bacteria lysis assays confirmed milk from all of the animals kill *Staphlococcus aureus* in a dose dependent manner. But the lytic activity of the milk is less than would be predicted from lysostaphin concentration determined by ELISA.

The diminished specific activity of lysostaphin was not a total surprise. We observed the same phenomena in transgenic mice carrying a similar transgene. Cell culture experiments conducted prior to the mouse experiments revealed that native lysostaphin was being glycosylated, and completely inactivated by mammalian cells. Thus, we modified the glycosylation motifs within the lysostaphin coding sequence, to allow for production of bioactive lysostaphin. In doing so we may have inadvertently altered the protein's lytic potency. This modification appears to have the same consequence in cattle as in mice.

On the other hand, our transgenic mice were not a good predictor of transgene expression levels in cows. We may have influenced the level of expression by addition of enhancements to the transgene necessitated by our need for selectable markers to facilitate the somatic cell nuclear transfer process. A neomycin resistance gene, driven by a SV40 promoter was used to select for stable fibroblast integrants, while a second gene, a green fluorescent protein, driven by the

human elongation factor regulatory element, was used to confirm that cloned blastocysts expressed a functional transgene product before embryo transfer.

The benefits derived from these selectable markers may be counter balanced by their possible negative consequences. It is possible they are responsible for the relatively unimpressive levels of transgene expression observed in the cows. Both the neomycin resistance gene and the GFP gene are littered with CpG islands (stretches of 500 bases with 60% or greater CG content, and with CG dinucleotide content 65% greater than normally observed in the genome). Such sequences are well known for their propensity to become methylated and down regulate transgene expression.

The lysostaphin transgene clearly affords protection against *Staphlococcus aureus* mastitis. However, these animals have no special protection against other mastitis causing pathogens. To deal with other pathogens we are searching for additional antimicrobials based on an ever growing list of criteria. Clearly candidate genes must target an economically important pathogen, and cause no harm to the animal or the milk consumer. The protein must have antimicrobial activity in milk, a criterion that may eliminate a number of potential candidates, and, the new antimicrobial should not alter milk's physical, chemical or nutritional properties. Ideally the protein should be effective at low concentrations to avoid major changes in the milk protein profile. Finally, since cheese and yogurt production relies on bacterial cultures, the antimicrobial should not survive pasteurization, and if it does, should not inhibit growth of common starter culture organisms, or interfere with processing. Furthermore, it should be noted that this general strategy is based on the assumption that antimicrobial peptides will have no biological activity when taken orally. An assumption that has significant precedence, but of course, needs to be tested.

Creating mastitis resistant cows is obviously of value to the dairy industry. It still remains to be determined if this approach negatively impacts the downstream use and processing of milk as a food stuff. However, our recent publication leaves little doubt that a transgene can offer disease protection. Once the efficacy of this technology is proven, it is easy to imagine transgenic milk that not only protects against mastitis, but which contains agents that inhibit the growth of other unwanted microbes, effectively extending shelf life of milk and milk products. The scientific hurdles involved in finding a series of antimicrobials that meet the criterion described here are not insignificant, but are probably achievable in the next few years.

Novel Applications of Agricultural Biotechnology – State of the Science and Societal Acceptance to Transgenic Animals and Regulatory Issues

By Way of Example: Biotechnological Defense Against Mastitis Disease

2006 Agricultural Outlook Forum
Prospering in Rural America
February 17, 2006

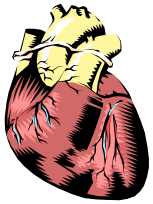
What has been done ?

Transgenic Livestock goals

1. Create new animal products

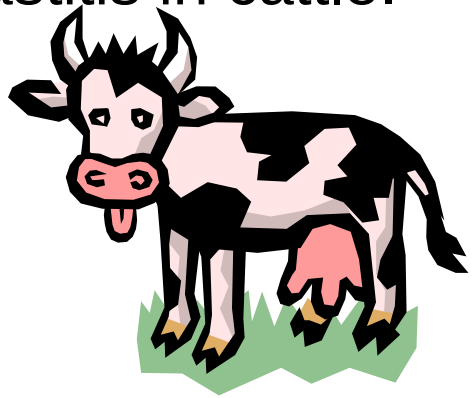
2. Improving animal well-being and food safety

3. Improving production efficiency & nutritional quality

4. Xenotransplantation 

The Mastitis Project

Goal: Reduce incidence and susceptibility to mastitis in cattle.

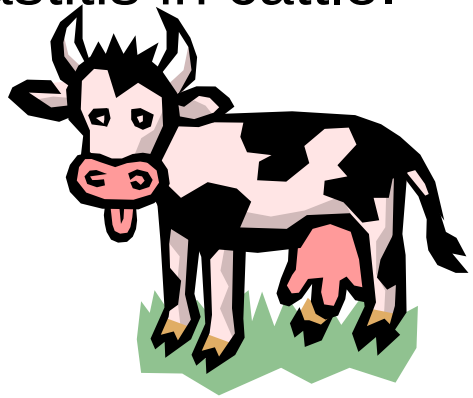


Facts about mastitis

- A third of dairy cows in the U.S. become infected
- Mastitis costs US producers ≈ $2 Billion / year
  - Treatment costs
  - Reduced milk yield, shelf-life, cheese yield
  - Animal well-being diminished, premature culling
- Vaccines, antibiotics and selective breeding are minimally effective

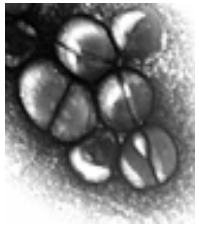
The Mastitis Project

Goal: Reduce incidence and susceptibility to mastitis in cattle.



Facts about mastitis

- **<u>Disease is on every dairy farm</u>**
- *Staphylococcus aureus* accounts for about a third of cases

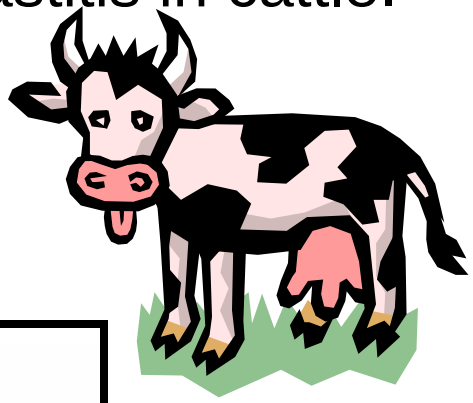


Staph. aureus

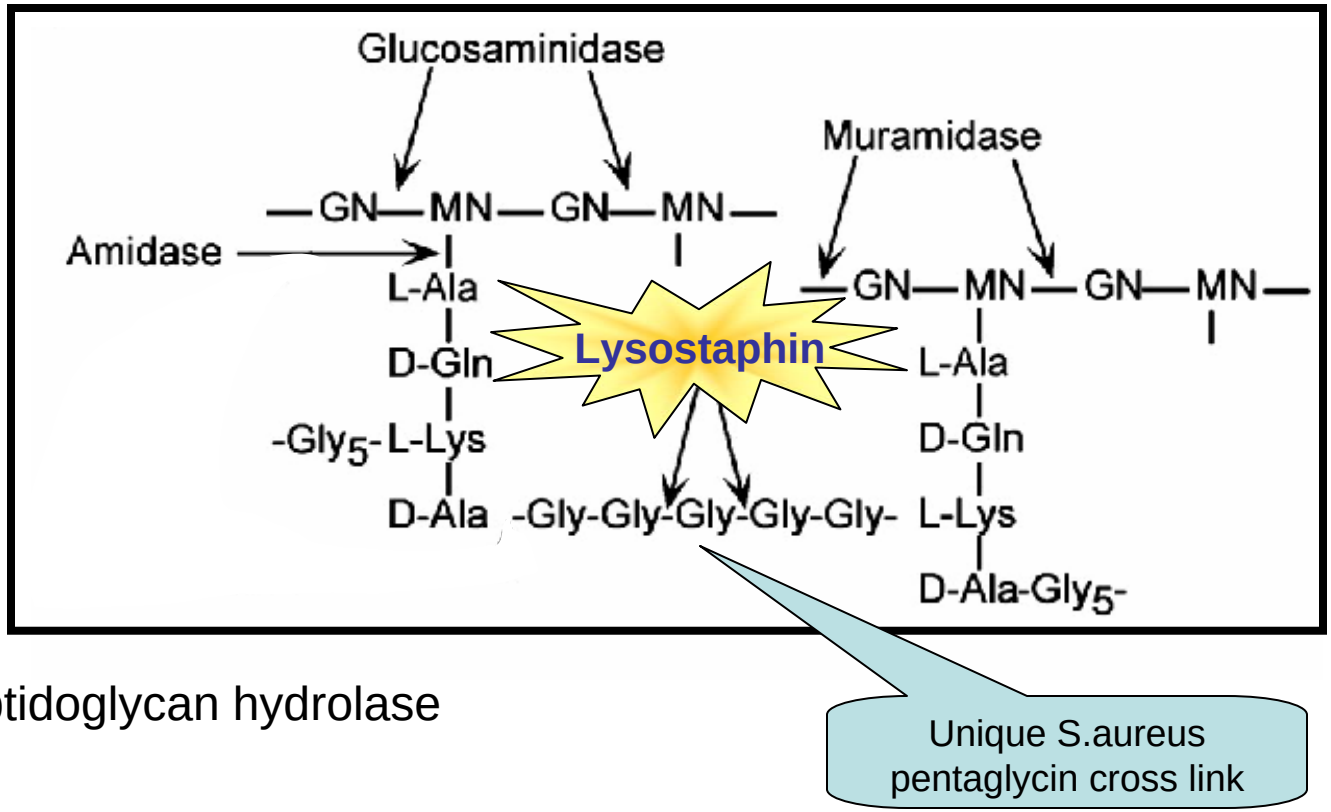
Culling is often the only solution

The Mastitis Project

Goal: Reduce incidence and susceptibility to mastitis in cattle.



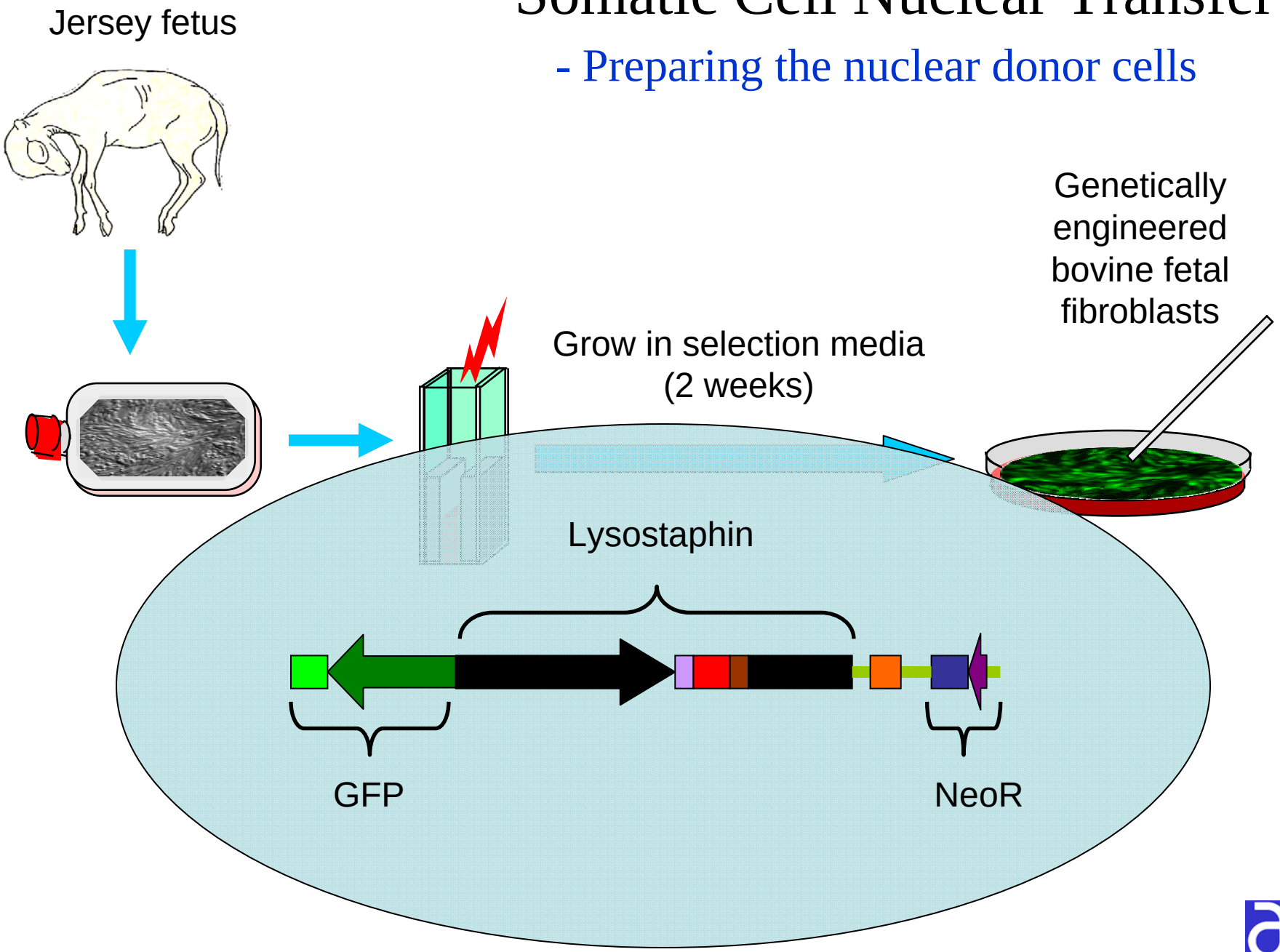
Gram-negative outer peptidoglycan layer



Peptidoglycan hydrolase

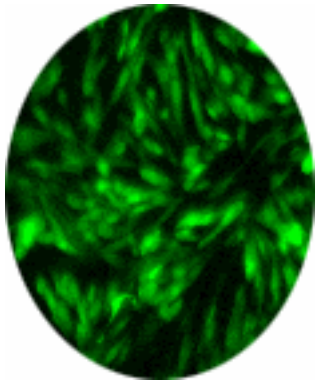
Somatic Cell Nuclear Transfer

- Preparing the nuclear donor cells

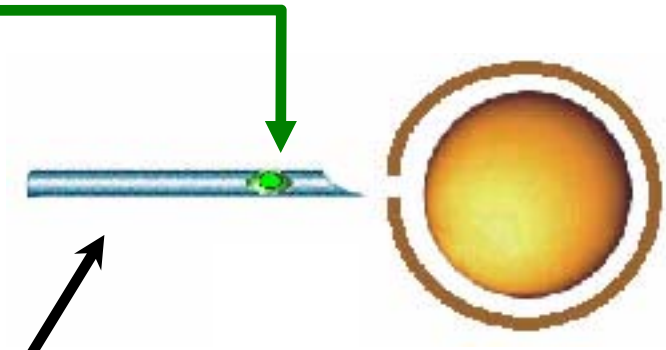


Somatic Cell Nuclear Transfer

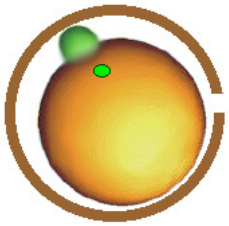
- Producing embryos



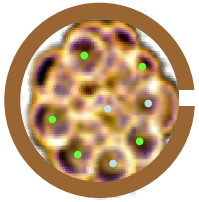
Genetically engineered donor cells



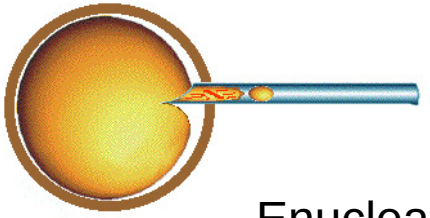
Fuse donor cell to empty egg



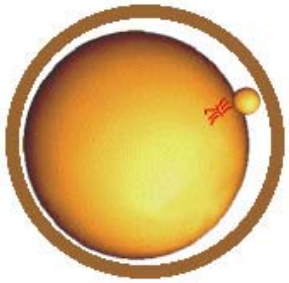
Simulate fertilization



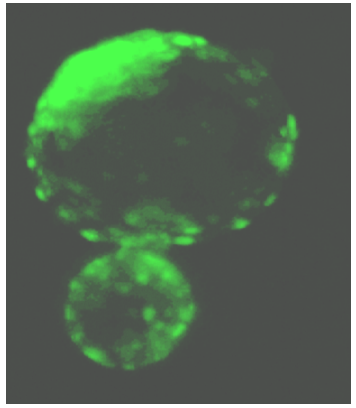
Culture for a week

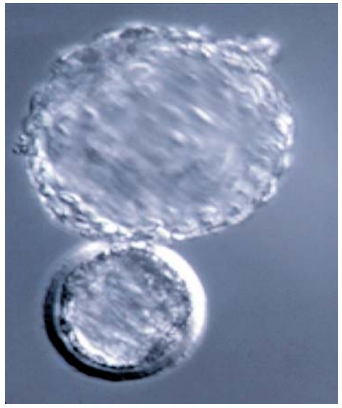


Enucleate unfertilized eggs



Slaughterhouse derived eggs



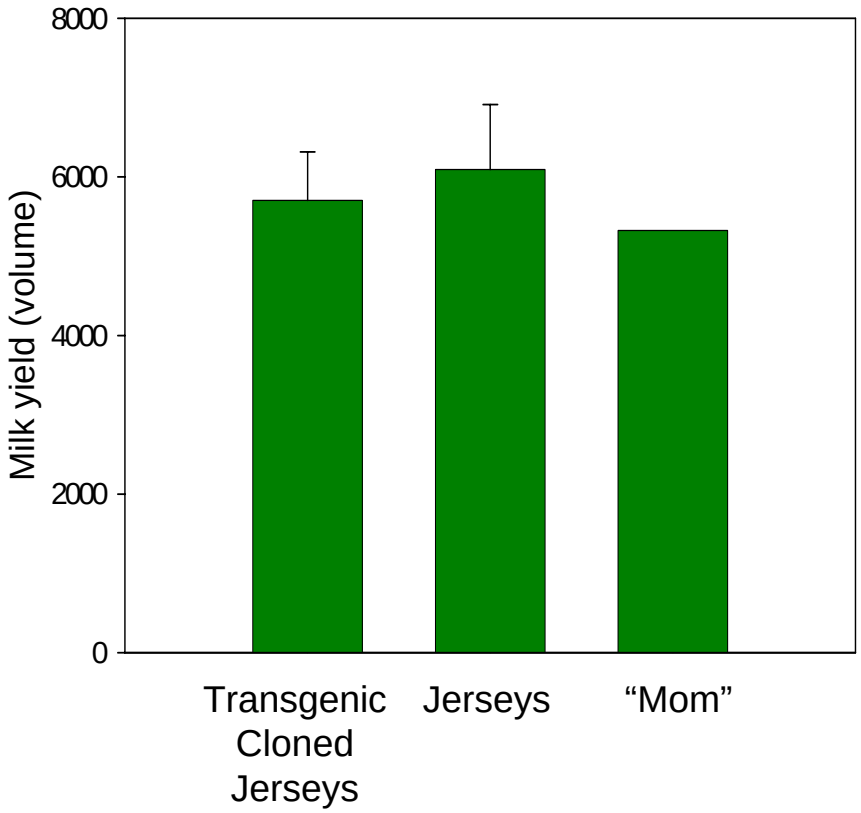


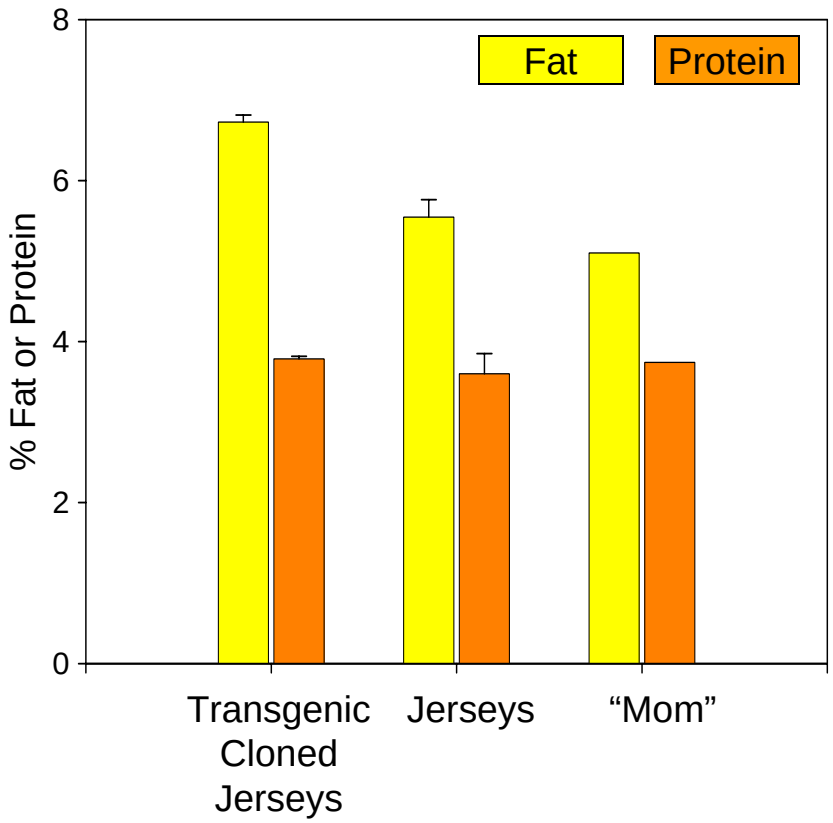
First genetically modified cows carrying a disease resistance gene

Annie

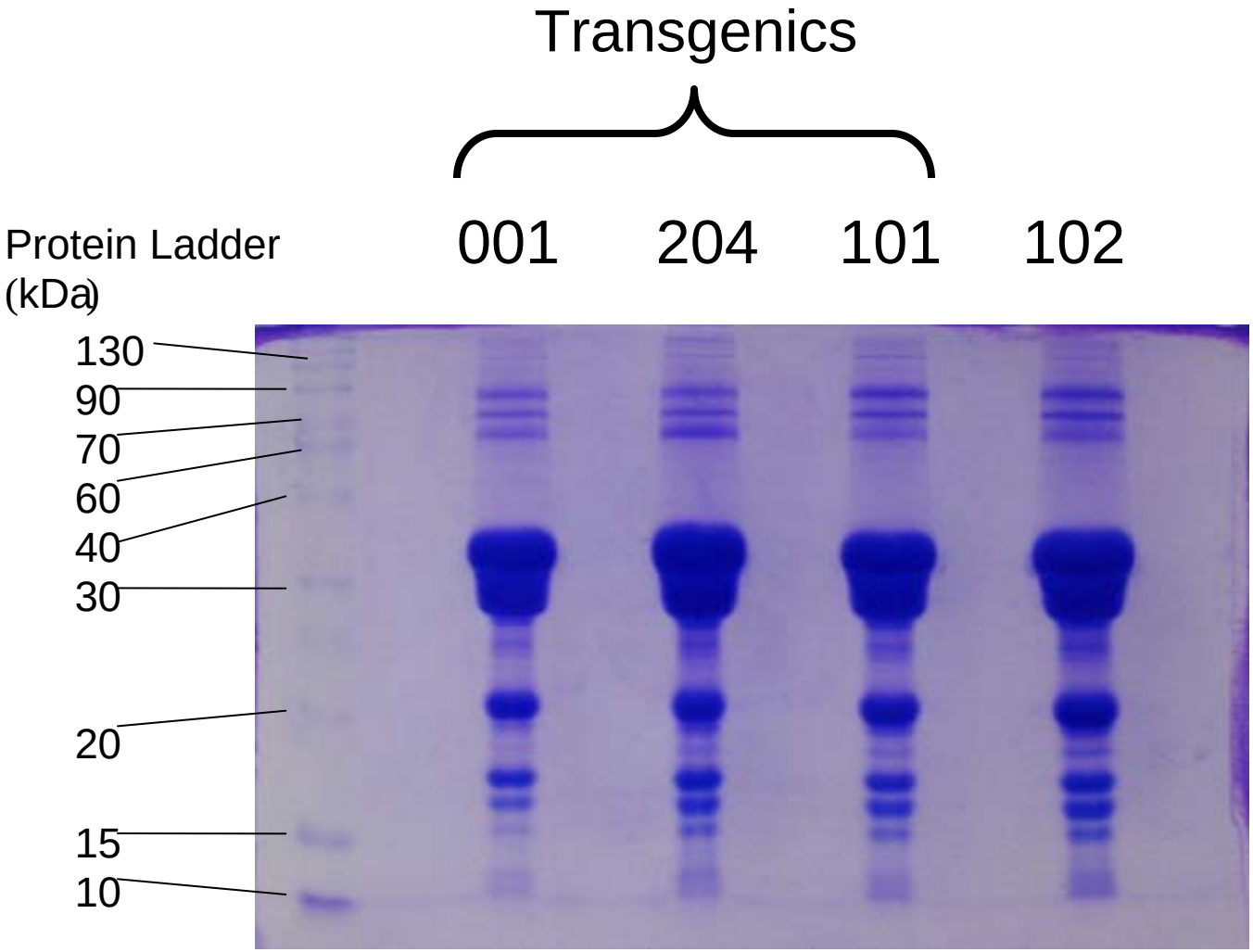
GEM

Estimated adult performance (305 Mature Equivalents) of Transgenic clones and their "Mother"

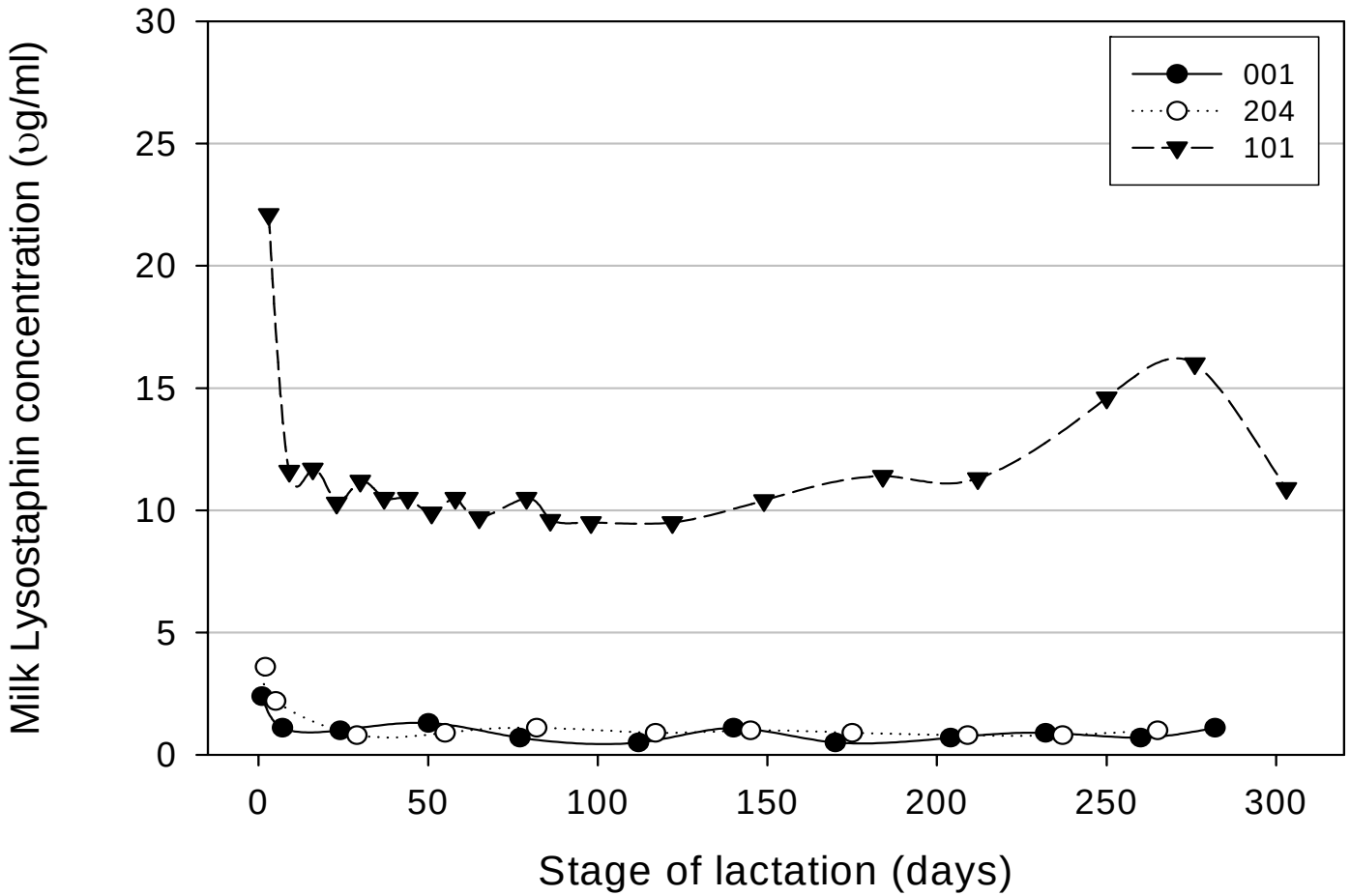




Milk Protein from Transgenic and Non-tg Jerseys

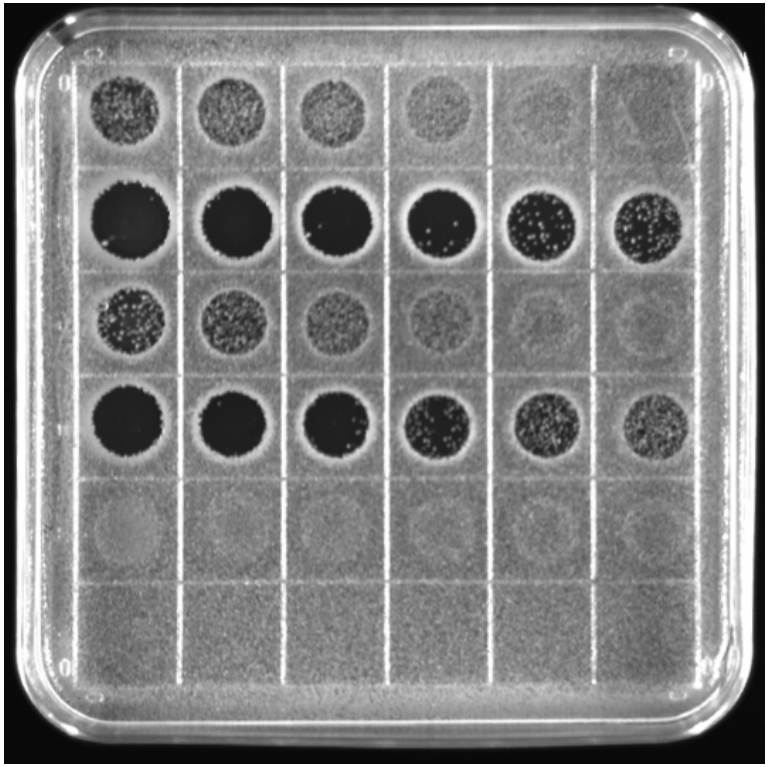


Lysostaphin in milk (1st lactations) of transgenic cloned Jerseys



Lysis of *S. aureus* by Milk from Transgenic Cows

Dilution of Milk 0 2 4 8 16 32



Tg 001

Tg 101

Tg 204

rLysostaphin

Non-tg 102

No sample

rLys conc. 1 0.5 .25 .13 .06 .03 μg/ml

Lawn ≈ 10^5 c.f.u.

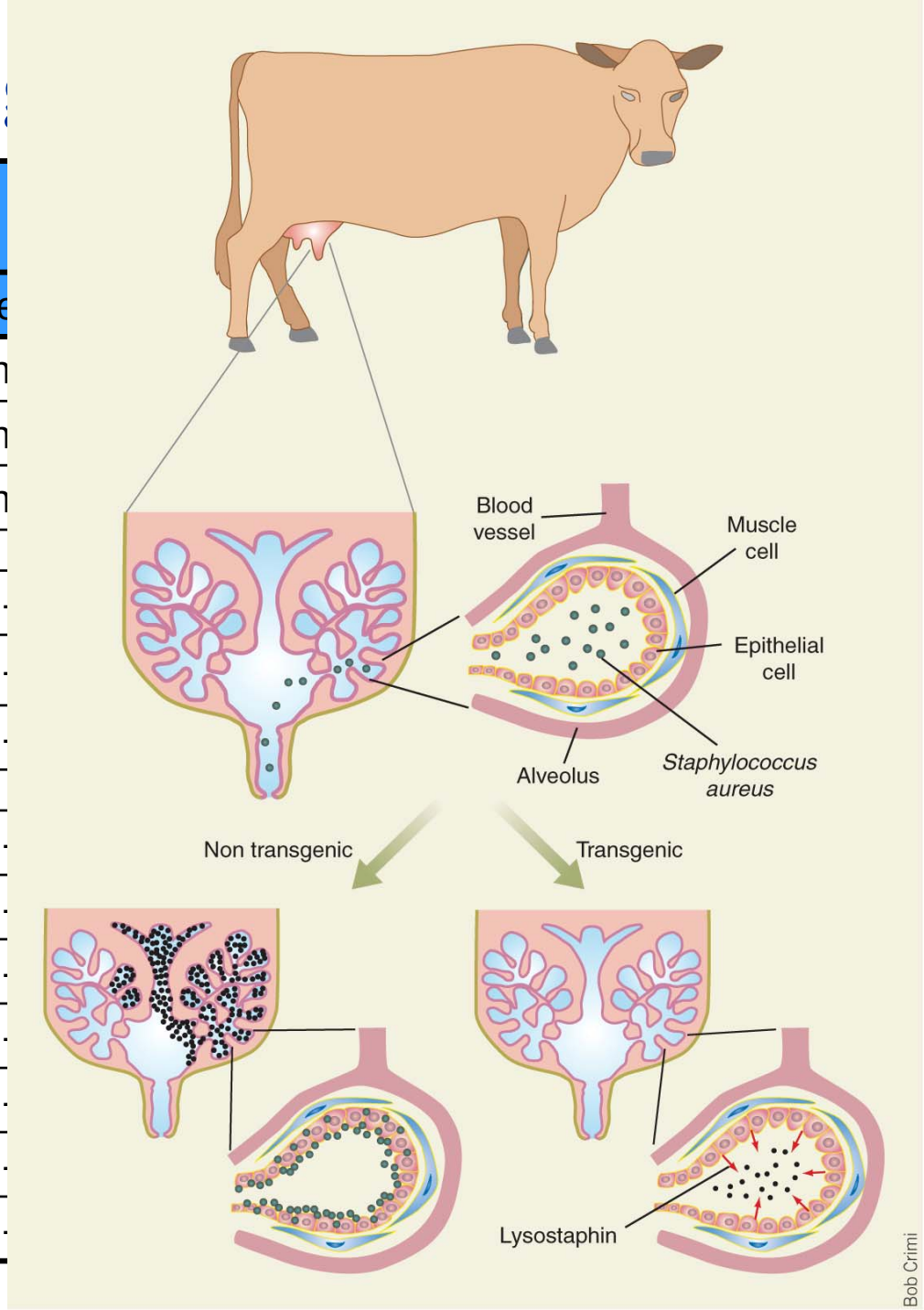
S. aureus Challenge – cow assignment schedule

		Stage of Lactation (days)			
Type	Cow	60	150	240	270
Transgenic Jersey	001	✓	✓		
Transgenic Jersey	101	✓	✓		✓
Transgenic Jersey	204	✓	✓		
Non-transgenic Jersey	102	✓	✓	✓	
Non-transgenic Jersey	207	✓			
Non-transgenic Jersey	208	✓			
Non-transgenic Holstein	2227	✓			
Non-transgenic Holstein	2234		✓		✓
Non-transgenic Holstein	2262	✓		✓	
Non-transgenic Holstein	2264	✓	✓		
Non-transgenic Holstein	2287	✓			
Non-transgenic Holstein	2288	✓			
Non-transgenic Holstein	2291	✓	✓		

3 *S. aureus* strains:
1 per gland
Saline in the 4th

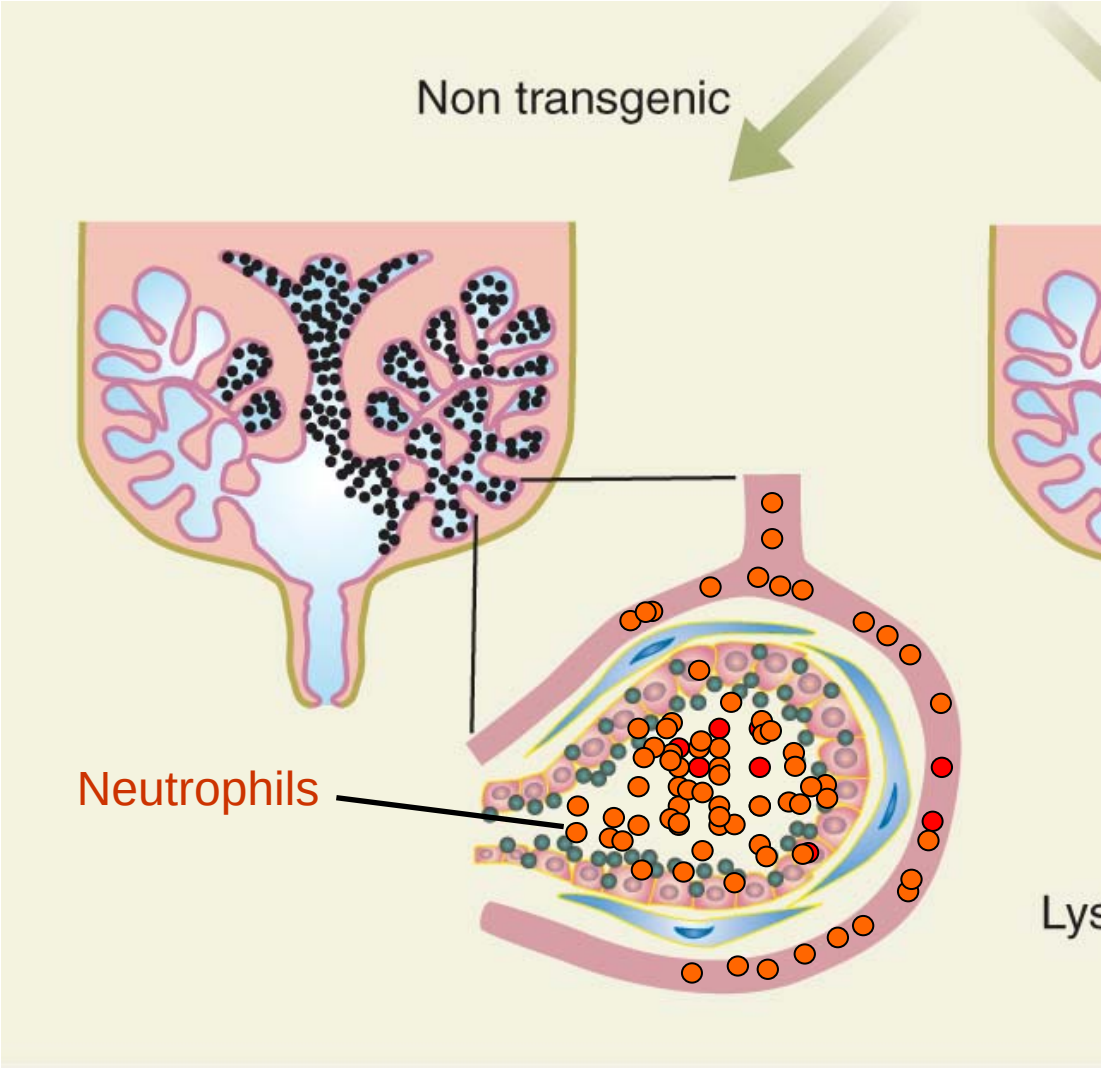
S. aureus Challen

Type	70
Tran	
Tran	
Tran	
Non-	
Non-	
Non-	
Non-	
Non-	
Non-	
Non-	
Non-	
Non-	
Non-	



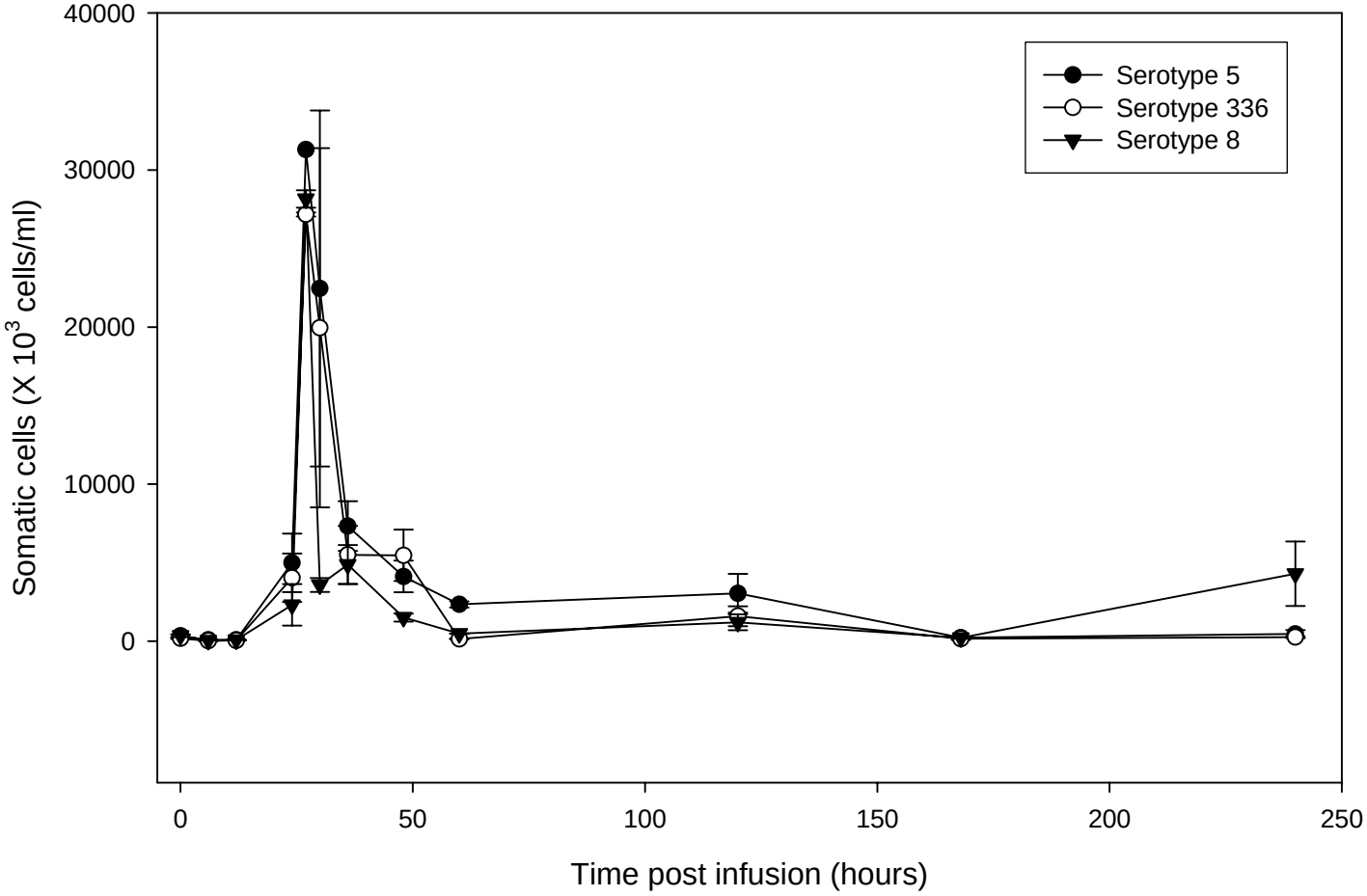
3 *S. aureus* strains:
1 per gland
Saline in the 4th

Milk “somatic cells” infiltrate gland in response to infection



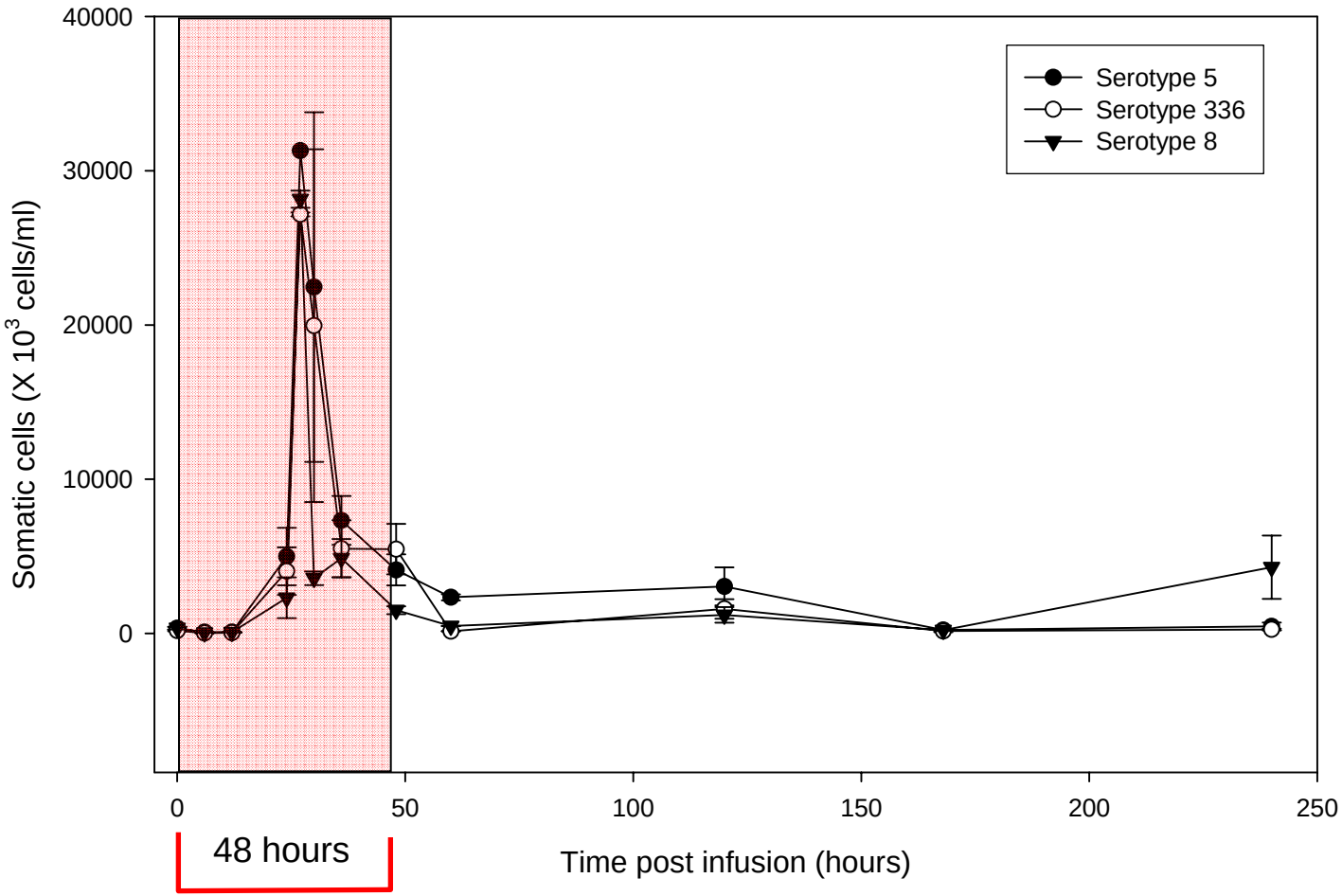
S. aureus Milk somatic cell response

10 Non-transgenic controls



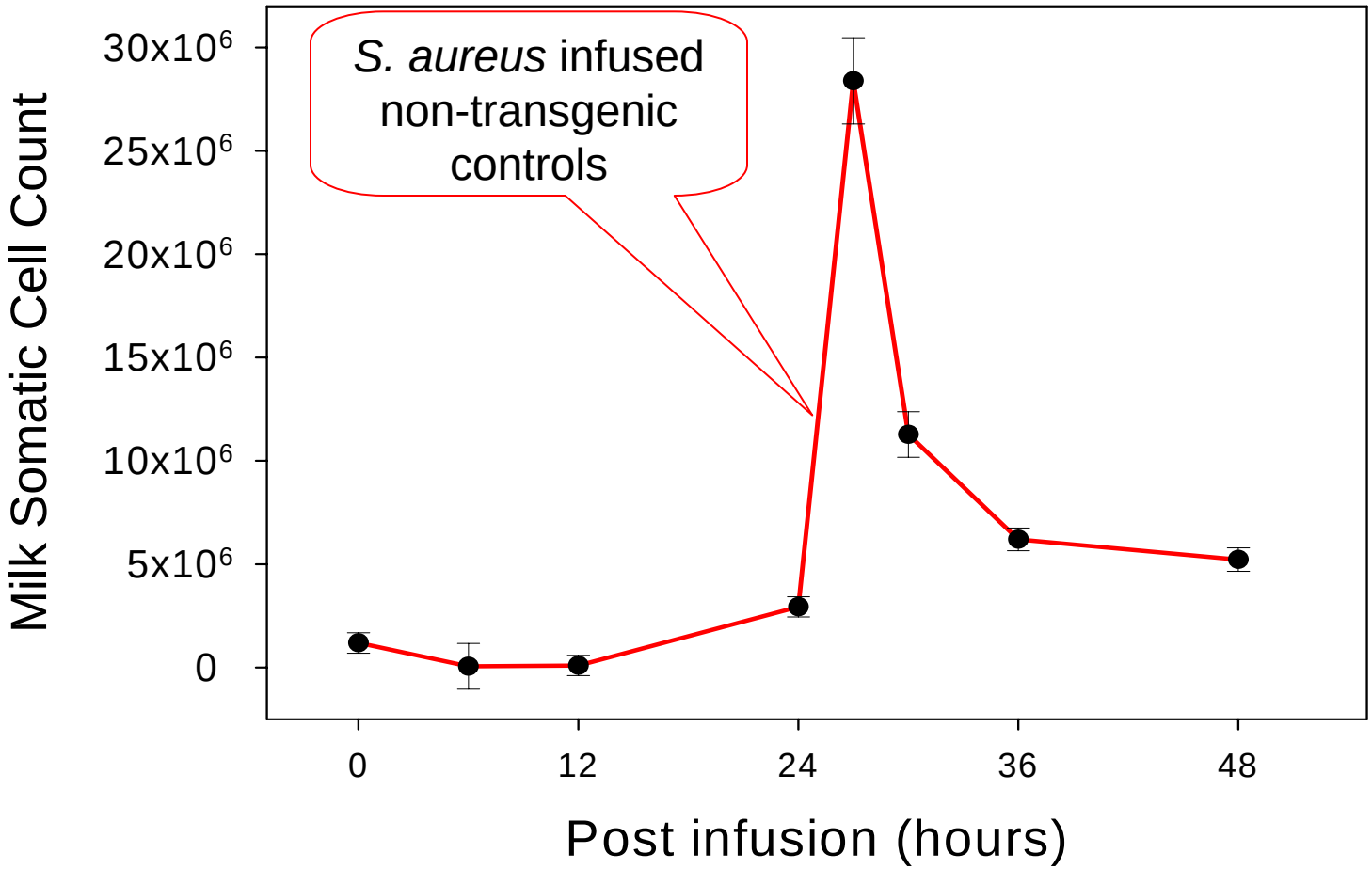
S. aureus Milk somatic cell response

10 Non-transgenic controls

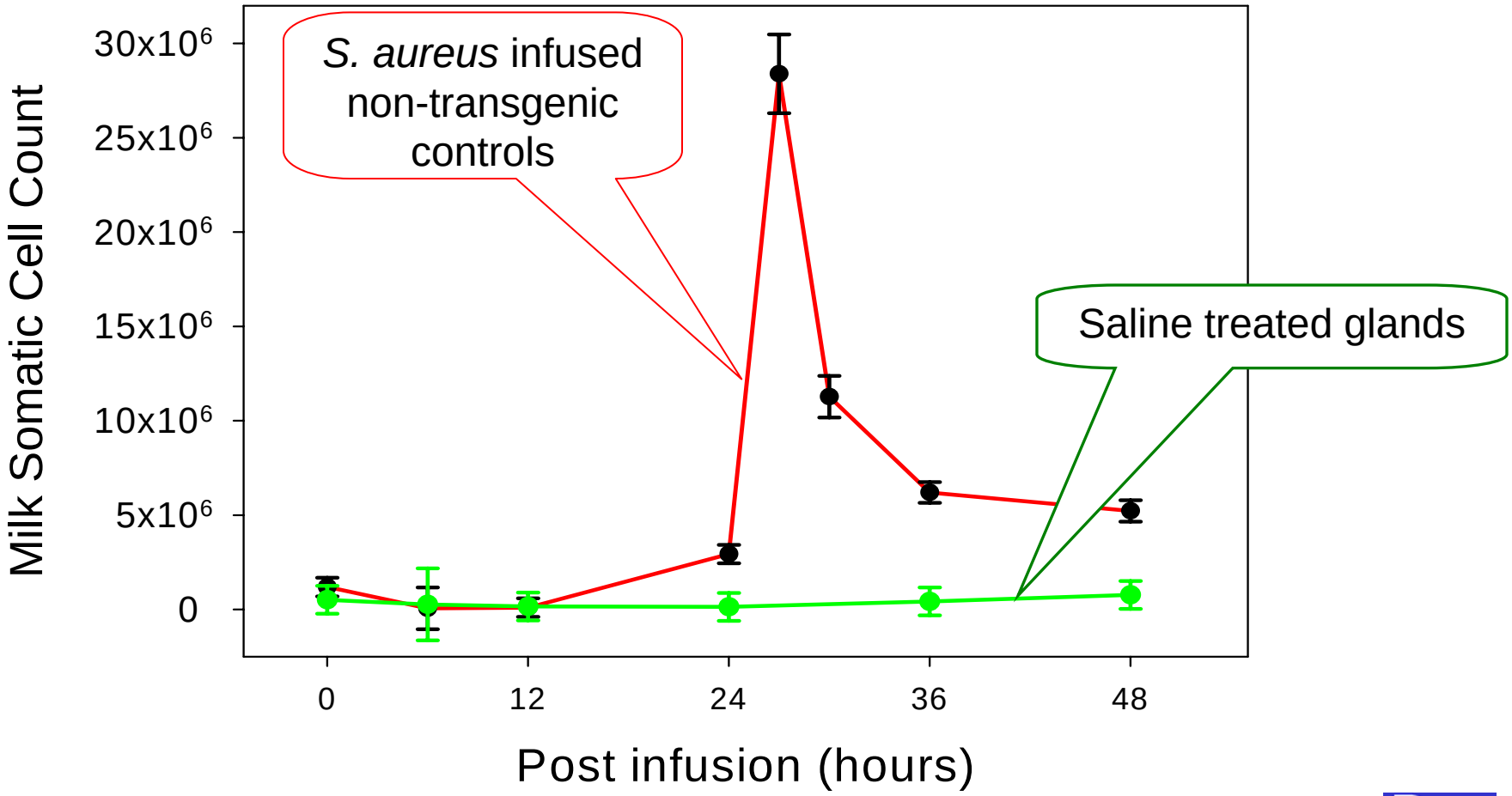


S. aureus Milk somatic cell response

10 Non-transgenic controls

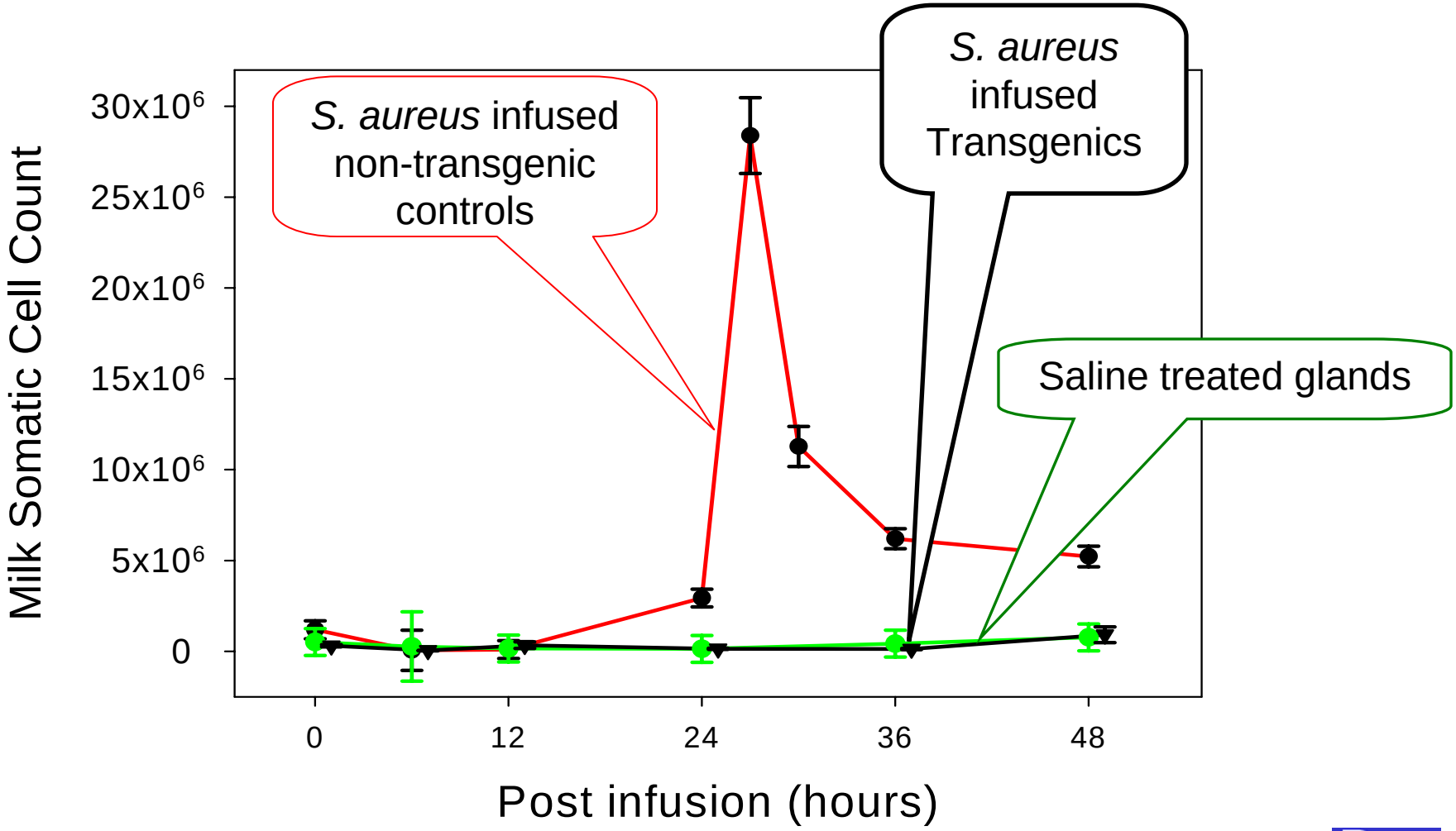


S. aureus Milk somatic cell response



S. aureus Milk somatic cell response

3 Transgenic, 10 Non-transgenic controls



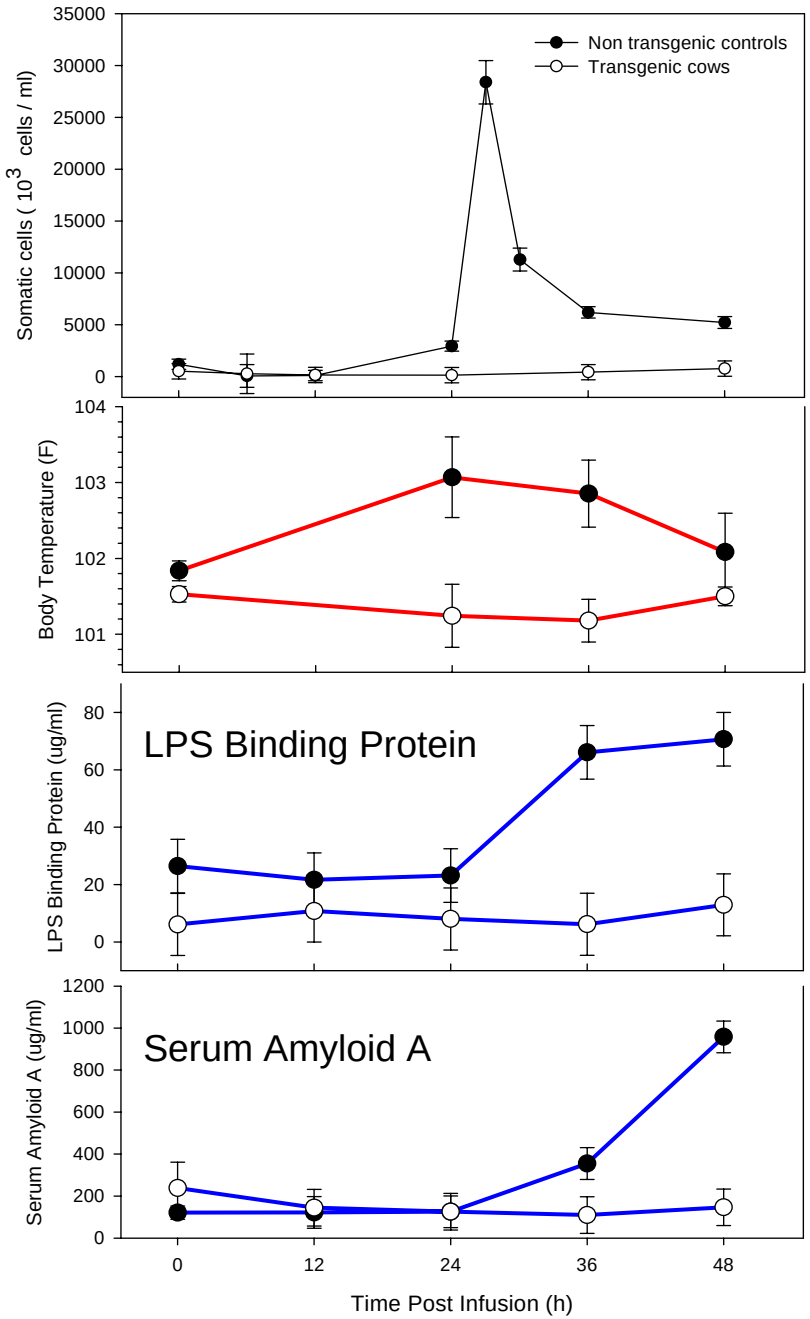
Immune Responses to *S. aureus*

Local response

Systemic response

Body temp

Acute phase proteins



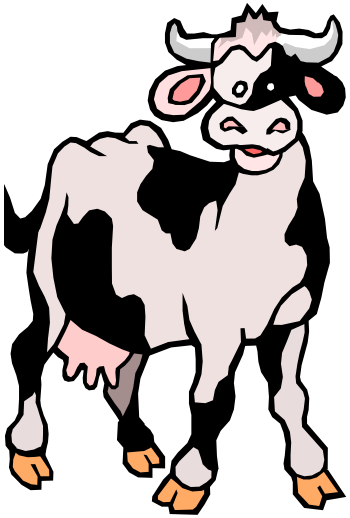
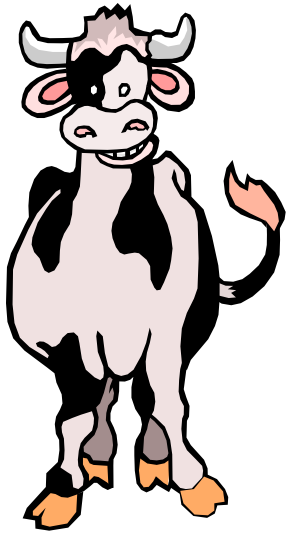
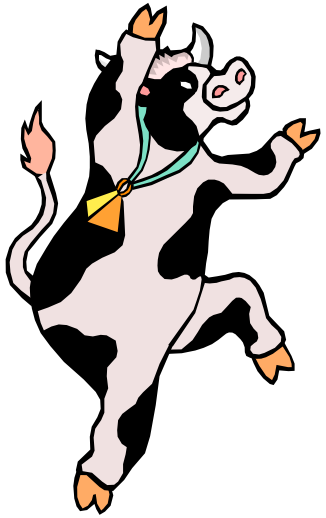
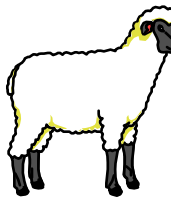
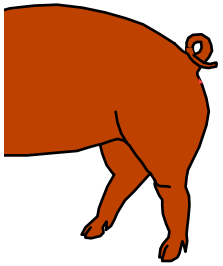
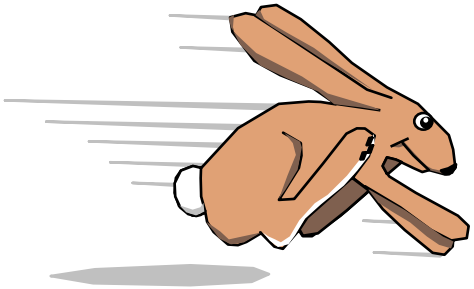
Infection rate (Infection = 2 consecutive milk samples with live *S. aureus*)

Cows		Glands	
Genotype	No.	Infused No.	Infected No. (%)
TG	3	21	3 (14%)
Non-TG	10	48	30 (63%)
Transgenic 001 **(0.7)**		6	2 (33%)
Transgenic 101 **(12)**		9	0
Transgenic 204 **(0.6)**		6	1 (17%)
Saline infused glands		23	1 (4%)

Summary

- Lysostaphin can protect against infection (3 µg/ml is probably enough)
- Lysostaphin seems to cause no harm

Future plans

- Immediate: Is the lysostaphin milk OK ?
- Identify additional anti-mastitis peptides
- Can we avoid resistant strains ?

Just the Beginning