© 2001 Nature Publishing Group http://biotech.nature.com

Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice

David E. Kerr[1]*, Karen Plaut[1], A. John Bramley[1,2], Christine M. Williamson[2,3], Alistair J. Lax[2,4], Karen Moore[5,6], Kevin D. Wells[5], and Robert J. Wall[5]

[1]*Department of Animal Sciences, University of Vermont, Burlington, VT 05405.* [2]*Institute for Animal Health, Compton Laboratory, Berks RG20 7NN, UK.* [3]*Current address: MRC Mammalian Genetics Unit, Harwell, Didcot, Oxfordshire, OX11 0RD, UK.* [4]*Current address: Oral Microbiology, King's College London, London SE1 9RT, UK.* [5]*Gene Evaluation and Mapping Laboratory, Agricultural Research Service, United States Department of Agriculture, Beltsville, MD 20705.* [6]*Current address: Department of Animal Sciences, University of Florida, Gainesville, FL 32611.* **Corresponding author (dkerr@zoo.uvm.edu).*

Received 6 September 2000; accepted 17 October 2000

Infection of the mammary gland, in addition to causing animal distress, is a major economic burden of the dairy industry. *Staphylococcus aureus* is the major contagious mastitis pathogen, accounting for approximately 15–30% of infections, and has proved difficult to control using standard management practices. As a first step toward enhancing mastitis resistance of dairy animals, we report the generation of transgenic mice that secrete a potent anti-staphylococcal protein into milk. The protein, lysostaphin, is a peptidoglycan hydrolase normally produced by *Staphylococcus simulans*. When the native form is secreted by transfected eukaryotic cells it becomes glycosylated and inactive. However, removal of two glycosylation motifs through engineering asparagine to glutamine codon substitutions enables secretion of Gln^{125,232}-lysostaphin, a bioactive variant. Three lines of transgenic mice, in which the 5′-flanking region of the ovine β-lactoglobulin gene directed the secretion of Gln^{125,232}-lysostaphin into milk, exhibit substantial resistance to an intramammary challenge of 10^4 colony-forming units (c.f.u.) of *S. aureus*, with the highest expressing line being completely resistant. Milk protein content and profiles of transgenic and nontransgenic mice are similar. These results clearly demonstrate the potential of genetic engineering to combat the most prevalent disease of dairy cattle.

Keywords: mastitis, animal biotechnology, antibacterial protein, *Staphylococcus aureus*

Mastitis is an inflammatory reaction of the mammary gland usually caused by a microbial infection, and it is the most prevalent disease of dairy cows[1,2]. This disease, in addition to causing animal distress, is estimated to cost dairy farmers approximately $200 per cow annually, corresponding to $1.7 billion annually for the US industry alone. Dairy processors also incur losses from detrimental changes in milk composition that accompany mammary gland inflammation. These changes reduce cheese yield and shelf-life of dairy products[3–6].

The incidence of contagious mastitis has greatly declined over the last 30 years with implementation of a five-point control plan[7]. However, *S. aureus,* which currently accounts for 15–30% of infections, has proved more difficult to control[8]. The cure rate for treatment of *S. aureus* mastitis with antibiotics is often <15%. This is attributed to incomplete penetration of the antibiotics throughout the gland and the potential survival of bacteria within host cells, leading to a recurrence of disease once treatment has ended[9,10]. Because *S. aureus* mastitis can be induced experimentally with <1,000 organisms[11,12], a few chronic infections within a herd can provide a persistent bacterial reservoir.

Lysostaphin is a potent peptidoglycan hydrolase secreted by *S. simulans*, having bactericidal activity against *S. aureus* at concentrations of <1 μg/ml in milk[11]. The enzyme's staphylolytic activity is mediated by hydrolyzing the polyglycine interpeptide bridges of the cell wall[13]. This specificity restricts its antibacterial activity to *Staphylococcus* sp., having little effect on other mastitis-causing organisms. However, the enzyme's specificity also makes it an ideal candidate as an antibacterial in milk because it does not seem to degrade milk proteins. The potential of bacterial lysostaphin for therapeutic or prophylactic control of staphylococcal mastitis has been demonstrated, initially in a mouse model[14] and subsequently in dairy cattle[11].

The use of transgenesis to direct expression of a foreign protein into mouse milk was first reported in 1987 by Gordon *et al*[15]. Lactation-specific transgenes have since been incorporated into pigs[16], sheep[17], goats[18], and cattle[19], primarily intended for pharmaceutical interests seeking to generate animal bioreactors. Mammary production of antibacterial proteins for enhanced mastitis resistance has been proposed as a prime agricultural application of this technology[14,20,21]. Antibacterial activity has been observed in milk from transgenic mice that were engineered to produce human lysozyme in milk[22].

In this report we demonstrate that secretion of a biologically active form of lysostaphin in milk protects transgenic mice against a substantial intramammary challenge of *S. aureus.* These results suggest that a genetic engineering approach will be an effective strategy for enhancing dairy animal welfare and combating the economic burden of mastitis.

Results

Modification of the bacterial lysostaphin gene for eukaryotic expression. Three lysostaphin-containing constructs were sequen-

© 2001 Nature Publishing Group http://biotech.nature.com

© 2001 Nature Publishing Group http://biotech.nature.com

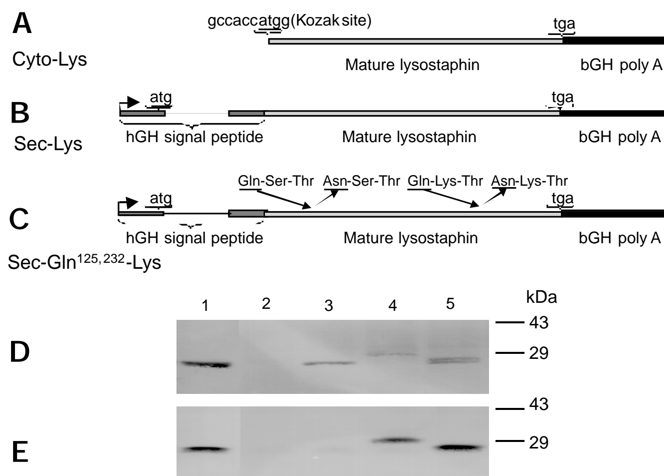

Figure 1. Western blot analysis of lysostaphin production by transfected COS-7 cells. (A) Construct map of cytosolically directed, native lysostaphin (Cyto-Lys). (B) Construct map of secreted, native lysostaphin (Sec-Lys). (C) Construct map of secreted, Gln^{125,232}-lysostaphin (Sec-Gln^{125,232} -Lys). Proteins in cell extracts (D) or medium (E) were probed with anti-lysostaphin antiserum. Lane 1, bacterial lysostaphin (100 ng/ml). Lanes 2–5: Cells were transfected with plasmids containing the CMV promoter driving expression of hGH (lane 2), Cyto-Lys (lane 3), Sec-Lys (lane 4), or Sec-Gln^{125,232}-Lys (lane 5).

tially assembled and evaluated following their transfection into COS-7 cells (Fig. 1). Eukaryotic production of cytosolically directed lysostaphin was achieved by addition of a Kozak site to the mature portion of the lysostaphin gene in a construct designated as Cyto-Lys (Fig. 1A). Transfected cells contained appropriately sized, immunoreactive lysostaphin with no apparent secretion of the protein (Fig. 1D, E, lane 3). Staphylolytic activity of the cell extract was confirmed with a bacterial plate assay in which the extract was applied to a freshly streaked lawn of *S. aureus* (Fig. 2A). The next construct, designated Sec-Lys, contained the human growth hormone (hGH) signal peptide coding region in place of the Kozak site (Fig. 1B). Transfected cells readily secreted immunoreactive lysostaphin, but western blot analysis indicated that it was ~3 kDa larger than the bacterial standard (Fig. 1E, lane 4). In addition, the protein lacked staphylolytic activity (Fig. 2A). Pretreatment of the sample with *N*-glycosidase-F followed by western blot analysis revealed a clear reduction in the apparent molecular mass of the expressed protein to that of the lysostaphin standard, indicating that glycosylation was responsible for the aberrant size and inactivity of eukaryotically produced lysostaphin. In an attempt to restore bioactivity, we assembled a new construct designated Sec-Gln^{125,232}-Lys, in which base-pair substitutions were generated to convert Asn125 and Asn232 to glutamine residues. COS-7 cells transfected with Sec-Gln^{125,232}-Lys secreted immunoreactive protein of the appropriate size (Fig. 1E, lane 5), and the medium contained staphylolytic activity (Fig. 2A).

Generation and characterization of transgenic mice expressing Gln^{125,232}-lysostaphin in milk. The Sec-Gln^{125,232}-Lys construct was inserted between the 4.2 kilobase 5′-flanking region and the 2.1 kilobase 3′-flanking region of the ovine β-lactoglobulin gene. The entire 7.7 kilobase BLG-lysostaphin fusion gene (BLG-Lys) was then used to generate transgenic mice. Eight potential founders carrying the BLG-Lys construct were identified by Southern blot analysis. Two of those animals did not transmit their transgene to progeny. Daughters of three other founders expressed lysostaphin in their milk at very low concentration (<0.020 mg/ml). The other three founders were used to establish lines for further studies. Milk obtained from offspring of one founder contained lysostaphin at 0.06 ± 0.01 mg/ml (mean ± s.e.; $n = 6$), and this was designated as the low-expressing line. Representative mice from a second line expressed 0.13 ± 0.03 ($n = 3$) mg/ml in milk, and this was designated the medium-expressing line. Two representative mice from the third line produced 1.3 mg/ml and 0.8 mg/ml in milk and is reported here as the high-expressing line. Northern blot analysis of RNA from various tissues of lactating transgenic mice from each of the lines revealed that lysostaphin expression was almost completely restricted to the mammary gland (data not shown).

Milk from these transgenic mice contains substantial staphylolytic activity (Fig. 2B). However, the eukaryotically produced Gln^{125,232}-lysostaphin appears to be 5- to 10-fold less active than bacterially derived lysostaphin. The reduced activity was also found in mammary tissue homogenates obtained from lactating transgenic mice. In eight homogenates obtained from four high-line mice the staphylolytic activity was 18 (±3)% of that expected from the concentration of immunoreactive lysostaphin present.

The total protein content of milk samples obtained from the three transgenic lines was quite variable, ranging from 35 to 97 mg/ml ($n = 11$), similar to the range of protein concentrations (35–99 mg/ml) in milk samples from control mice ($n = 6$). The general pattern of milk proteins visualized after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie blue staining was similar in all three lines of transgenic mice and in nontransgenic mice (Fig. 3). Western blot analysis of milk from BLG-Lys transgenic mice revealed an appropriately sized lysostaphin band (Fig. 4). Lysostaphin was not detected in milk from nontransgenic mice.

Intramammary challenge of lactating mice with *S. aureus*. Two abdominal mammary glands per mouse of lactating transgenic and nontransgenic mice (day 10 of lactation) were inoculated with *S. aureus* (10^4 c.f.u.). Mice were killed 24 h post infection, and viable *S. aureus* contained in mammary gland homogenates were enumerated. The dose of bacteria was sufficient to cause active intramammary infection in all infused glands of nontransgenic mice (Table 1). In contrast, glands of the high-line BLG-Lys transgenic

Table 1. Infection status and lysostaphin content of mouse mammary glands after *S. aureus* challenge

Line (expression level)	Glands infused[a]	Glands infected	Status of infected glands (c.f.u./gland)[b]			Lysostaphin in mammary homogenate (µg/mg protein) Mean ± s.e.
			Severe (>10^8)	Moderate (10^5–10^8)	Mild (10^2–10^5)	
Nontransgenic	22	22	17	4	1	0
BLG-Lys (low)	8	5	0	4	1	0.27 ± 0.02
BLG-Lys (medium)	6	3	0	3	0	0.86 ± 0.19
BLG-Lys (high)	8	0	0	0	0	12.2 ± 2.7

[a]Glands were infused on day 10 of lactation with *S. aureus* (10^4 c.f.u. /50 µl/gland).
[b]Infection status is based on number of *S. aureus* (c.f.u.) recovered from each gland 24 h post infusion. Glands containing fewer than 100 c.f.u. were considered as noninfected

© 2001 Nature Publishing Group http://biotech.nature.com

© 2001 Nature Publishing Group http://biotech.nature.com

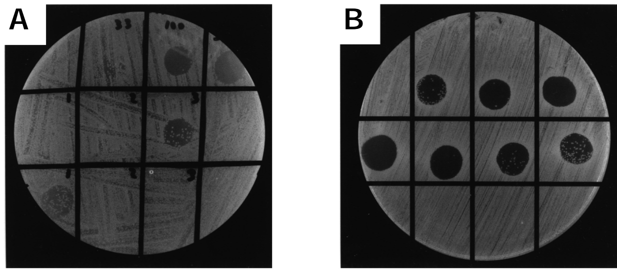

Figure 2. Bacterial plate assay for staphylolytic activity. Lytic zones developed on a lawn of *S. aureus* 24 h after sample application. (A) Samples derived from transfected COS-7 cells. Top row (left to right), bacterially derived lysostaphin (10, 33, 100, or 333 ng/ml). Middle row (left to right), conditioned medium from COS-7 cells transfected with Cyto-Lys, Sec-Lys, Sec-Gln^{125,232}-Lys, or hGH. Bottom row (left to right), cell extracts corresponding to the medium samples in the middle row. (B) Staphylolytic activity in mouse milk. Top row (left to right), bacterially derived lysostaphin (0, 62.5, 125, 250 ng/ml in PBS). Middle row (left to right), skim milk from a BLG-Lys transgenic mouse (medium-expressing line; 190 μg Gln^{125,232}-lysostaphin per milliliter of milk) diluted 1:50, 1:100, 1:200, or 1:400 in PBS. Bottom row (left to right), skim milk from a nontransgenic mouse diluted 1:50, 1:100, 1:200, or 1:400 in PBS.

mice were completely resistant to infection, as were ~40% of glands from the medium and low lines. Furthermore, 17 of 22 control glands were classified as severely infected, based on *S. aureus* recovery. Upon dissection these glands had obvious signs of trauma such as a friable texture and a bloody, viscous exudate. In marked contrast, the glands of transgenic mice consistently appeared normal, even in those eight glands from the low and medium lines that contained viable *S. aureus*. Degree of resistance to infection corresponded with the amount of lysostaphin in the glands (Table 1). Within lines, the amount of lysostaphin per milligram of homogenate protein was quite similar. Histological sections further confirmed the infection status (Fig. 5). Severely infected nontransgenic glands were characterized by infiltration of neutrophils and tissue disorganization. In contrast, histological sections of glands from bacterially challenged transgenic mice showed little cellular invasion and a well-preserved alveolar structure.

Discussion

Infection of the mammary gland is the most prevalent infectious disease of dairy cattle. Mastitis is characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils (PMNs), into milk, and by an increase in milk protease content[23]. Clinical infections are diagnosed by a red, swollen appearance of the gland and by protein flakes or clots in the milk. Milk from clinically infected cows is discarded. Subclinical infections, by definition, show no obvious signs of disease and although milk processing ensures product safety, milk quality is reduced in proportion to the amount of milk protein degradation.

Current therapies for mastitis rely heavily on the use of β-lactam antibiotics such as cepharin and penicillin derivatives. These nonprotein agents have had an enormously beneficial impact on dairy animal health and milk production, although there are drawbacks to their use. In particular, the stability of these compounds has necessitated a strict post-treatment time period during which milk must be discarded to prevent contamination of the milk supply. There is also concern that the use of antibiotics in animal agriculture is contributing to the development of drug-resistant bacteria. This concern is especially relevant for orally active antibiotics that are being used in human medicine. In contrast, protein-based antimicrobials such as lysostaphin will not likely be widely used in human or veterinary medicine because they would be digested and inactivated following oral administration, or run the risk of stimulating an immune response if used intravenously.

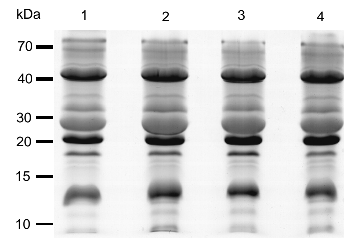

Figure 3. Proteins in milk collected from mice on day 10 of lactation. Proteins (50 μg/lane) were separated by 15% SDS–PAGE and visualized by Coomassie blue staining. Lane 1, Nontransgenic; lanes 2–4, BLG-Lys transgenic mice from high-, medium-, and low-expressing lines, respectively.

The current study has confirmed our hypothesis that transgenic production of lysostaphin by the lactating mammary gland will confer substantial resistance to staphylococcal mastitis[14,21]. Mice from the highest expressing line are completely resistant to experimental challenge, whereas mice from the medium- and low-expressing lines have substantial resistance. Following the bacterial challenge some glands from low- and medium-expressing transgenic mice did contain viable *S. aureus*. However, none of these glands were visibly altered, whereas most of the glands from nontransgenic mice showed obvious inflammation. The mouse mastitis model employed involves the intramammary infusion of 10,000 c.f.u. of *S. aureus*. This produces an acute infection not likely to occur naturally. With a much lower inoculum it is likely that even the low-expressing line of transgenic mice would have cleared the infection.

The Gln^{125,232}-lysostaphin variant demonstrates staphylolytic activity at concentrations of <1 μg/ml on a lawn of *S. aureus*. This level of foreign protein production by the transgenic mammary gland is easily obtainable. In the present study concentrations of >1 mg/ml were measured. During design of the transgene we chose the proven BLG expression cassette[24] in conjunction with the genomic portion of the hGH gene that encodes the hGH signal peptide. This portion of the hGH gene contributed an intron to the construct that may have enhanced its transgenic production[24,25]. The strategy to replace asparagine with glutamine in the glycosylation sites was based on the similarity of their side groups. This strategy has since been reported as being effective in maintaining the activity of proteins native to prokaryotes when produced by eukaryotic cells[26]. Perhaps other substitution strategies will result in a more active lysostaphin variant.

Production of lysostaphin by the BLG-Lys transgenic mice appears to have little effect on the physiology of the animal, the integrity of the mammary gland, or the milk that it produces. The transgenic animals

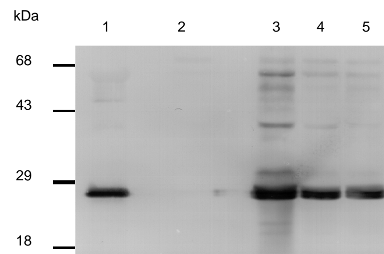

Figure 4. Western blot analysis of lysostaphin in milk collected from mice on day 10 of lactation. Lane 1, Positive control sample of bacterially derived recombinant lysostaphin (10 ng). Lane 2, Nontransgenic milk. Lanes 3–5, Milk from BLG-Lys transgenic mice from the high-, medium-, and low-expressing lines, respectively. Milk from these transgenic mice contained 75, 81, and 73 mg/ml of total protein, and 0.79, 0.12, and 0.06 mg/ml of lysostaphin; 0.1 μl milk was loaded per lane.

© 2001 Nature Publishing Group http://biotech.nature.com

© 2001 Nature Publishing Group http://biotech.nature.com

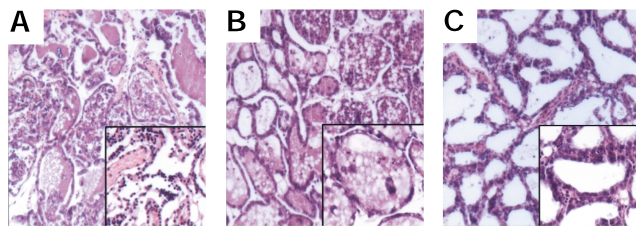

Figure 5. Hematoxylin and eosin stained sections of lactating mouse mammary tissue obtained 24 h after an intramammary infusion of *S. aureus* (10^4 c.f.u.). (A) Tissue from a severely infected nontransgenic mouse. Marked infiltration of PMNs (dark dots) in alveolar lumen and disorganized alveolar structure are apparent. (B) Tissue from a mildly infected BLG-lys transgenic mouse from the low expression line. Distinct milk fat droplets in lumen and some PMN infiltration are apparent. Structure of alveoli for the most part is intact. (C) Tissue from a noninfected BLG-lys transgenic mouse from the high expression line. Very few nonepithelial cells are visible, and alveolar integrity is normal.

© 2001 Nature Publishing Group http://biotech.nature.com

appear grossly similar to nontransgenic animals, are fertile, and are able to lactate successfully. The mammary glands appeared normal by histological examination, and milk protein profiles were similar to nontransgenic animals. We have not attempted to process the mouse milk into cheese or other fermented products. Lysostaphin activity is very specific for staphylococcal bacteria and should not have any affect on starter cultures used in the dairy food industry.

Bovine milk normally contains proteins that have antibacterial activity such as lactoferrin, lysozyme, and lactoperoxidase[27,28], but clearly these are insufficient to prevent mastitis. Efforts to produce antimicrobials and enhance disease resistance have resulted in the generation of transgenic mice and cattle that secrete human lysozyme[22], human lactoferrin[29], or bovine tracheal antimicrobial peptide (bTAP) in their milk[30]. Experiments *in vitro* demonstrated that transgenic mouse milk containing human lysozyme (0.38 mg/ml) had slightly enhanced bacteriostatic activity against *S. aureus*, but had no effect on *Escherichia coli*[31]. Challenge studies *in vivo* were not reported. The antimicrobial properties of the lactoferrin-supplemented milks have not been reported. However, the potency of lactoferrin in milk for mastitis-causing organisms is not very strong considering that its concentration in normal milk, which is 200–300 µg/ml, is unable to prevent infection, and its concentration in milk from infected cows often exceeds 1 mg/ml (ref. 32). The antibacterial activity of transgenic mouse milk containing bTAP (5 µg/ml) was not reported, nor were challenge studies done[30]. However, antibacterial activity of bTAP purified from transgenic milk by high-performance liquid chromatography was detected.

Lactating BLG-Lys transgenic mice have substantial resistance to *S. aureus* infection. However, they will not be protected against nonstaphylococcal infection, nor against infection in the nonlactating period, and bacterial development of lysostaphin resistance is a possibility. Solutions to these problems are being pursued. First, antibacterial proteins, with potent activities against other mastitis-causing organisms are known, and some will likely be amenable to a transgenic antimastitis strategy. Second, protection during the nonlactating period could potentially be achieved with a different 5′-regulatory region, possibly one triggered by lactogenesis or involution of the gland. Finally, the possibility of bacterial strains developing resistance to a transgenic strategy may be greatly reduced by the simultaneous production of two or more antibacterial proteins that have different bacteriostatic properties against the same species of pathogen.

Experimental protocol

Assembly of lysostaphin constructs. The coding region of mature lysostaphin was amplified from pCMLEM (ref. 21) and cloned into pcDNA3 (Invitrogen; Carlsbad, CA), creating pCMV-Lys. The expression plasmid pCyto-Lys (cytosolic-lysostaphin) was constructed by inserting a Kozak sequence and a start codon (ATG), 5′ to the mature lysostaphin sequence. The plasmid pSec-Lys (secreted lysostaphin) was constructed by inserting the hGH signal peptide-coding region into pCMV-Lys. The plasmid pSec-Gln^{125,232}-Lys encodes two N→Q mutations at positions 125 and 232 of mature lysostaphin. In both cases AAT to CAG codon changes were generated by PCR.

Generation of BLG-Lys transgenic mice. The 4.2 kb 5′-flanking region with the first 29 bases of the untranslated region and 2.1 kb of the 3′-flanking region of the ovine BLG gene were obtained from Dr. A.J. Clark (pBJ41; Roslin Institute, UK). A 1.4 kb, *Kpn*I–*Pvu*II fragment containing the hGH signal peptide region, the modified lysostaphin gene, and the bovine growth hormone (bGH) polyadenylation signal was excised from pSec-Gln^{125,232}-Lys and inserted into the *Eco*RV site of pBJ41, which forms the junction between the 5′ and 3′ components of the BLG gene. The entire 7.7 kb BLG-Gln^{125,232}-lysostaphin fusion gene (BLG-Lys) was then used to generate transgenic mice by standard techniques.

Sample collection. Conditioned media and cell extracts were obtained 48 h post transfection of COS-7 cells. Cells were transfected in six-well plates by the $CaPO_4$ technique. Each well was transfected with 31.5 µg of plasmid DNA encoding the various lysostaphin constructs or hGH as a negative control. The cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum without antibiotics. Conditioned medium was removed and cell extracts were prepared by three rounds of freeze-thaw lysis in 0.5 ml PBS. Both were clarified by centrifugation (5 min, 14,000 *g*) before storage (–20°C). The experiments were repeated three times.

Mouse milk samples were obtained at day 10 of lactation[22]. Skim milk was prepared by dilution (1:4 in PBS containing 33 mM EDTA), centrifugation (15 min, 14,000 *g*, 4°C), and then aspiration of milk infranatant for storage (–20°C)[33].

Intramammary challenge with *S. aureus*. Experimental mastitis was produced by intramammary inoculation essentially as described[14,34]. In the experimental model, two abdominal mammary glands of mice were inoculated with *S. aureus* (10^4 c.f.u./50 µl PBS) on day 10 of lactation. Pups were removed and inoculum was delivered to anesthetized mice by the teat canal with a 100 ml Hamilton syringe fitted with a 34-gauge blunt needle. Sterile India ink (1%), shown in preliminary experiments not to influence *S. aureus* growth, was added to the inoculum as a visual aid to confirm, at necropsy, that infusions were successful. Mice were maintained on an analgesic (buprenorphine) for the 24 h experimental period, then killed. Whole or bisected thoracic glands (L4 and R4) were homogenized in 2 or 1 ml of PBS, respectively. An aliquot was diluted 1:10 in PBS containing 20% glycerol for storage (–20°C) until subsequent bacterial enumeration. Viable *S. aureus* in the homogenate were determined by dilution plating on blood agar plates. Colony morphology and hemolytic clearing were used as indicators of *S. aureus*. Histological examination was preformed on bisected mammary gland tissue that had been formalin-fixed, paraffin-embedded, sectioned (5 µm), and stained with hematoxylin and eosin.

Detection of lysostaphin immunoreactivity (western and enzyme-linked immunosorbent assay, ELISA) and bioactivity. An antibody to purified bacterial lysostaphin (Sigma, St. Louis, MO) was custom developed in a rabbit (R. Sargent Inc., Ramona, CA). The antiserum was then affinity purified by chromatography over lysostaphin–agarose gel, and a portion was then conjugated to horseradish peroxidase (HRP; Bethyl, Montgomery, TX). Western blots were prepared from samples and standards separated on a 12% SDS–PAGE. Blots were developed with affinity-purified anti-lysostaphin antibody (1:4,000), then alkaline phosphatase-conjugated anti-rabbit IgG (Sigma), followed by BCIP/NBT substrate (Pierce, Rockford, IL). For ELISA, the affinity-purified anti-lysostaphin antibody served as the capture antibody, while the HRP-linked antibody (1:25,000) served for detection. The assay was developed with 3,3',5,5′-tetramethylbenzidine (TMB) substrate (Pierce), and standards were prepared from recombinant lysostaphin (Sigma). Lysostaphin bioactivity was detected in drops (15 µl) of standards, or samples, that were spotted onto culture plates that had just been streaked with a culture of *S. aureus* (bovine isolate, strain M60). Following an overnight incubation (37°C), bioactivity was observed as lytic zones in the bacterial lawn. In a second bioactivity assay the lysis of heat-killed *S. aureus* was followed spectrophotometrically (OD_{620}). Recombinant lysostaphin was used to construct a standard curve against which activity in dilutions of mammary homogenates was compared.

© 2001 Nature Publishing Group http://biotech.nature.com

Acknowledgments

We thank Juli Foster, Leah Schulman, Barbara Hughes, and Francis Kinghorn for excellent technical assistance. The work was funded in part by grants from The Northeast Dairy Foods Research Center, the Vermont Dairy Promotion Council, and the New England Dairy Promotion Board.

1. Kossaibati, M.A., Hovi, M. & Esslemont, R.J. Incidence of clinical mastitis in dairy herds in England. *Vet. Rec.* **143**, 649–653 (1998).
2. Myllys, V. *et al.* Bovine mastitis in Finland in 1988 and 1995—changes in prevalence and antimicrobial resistance. *Acta Vet. Scand.* **39**, 119–126 (1998).
3. Auldist, M.J., Coats, S., Rogers, G.L. & McDowell, G.H. Changes in the composition of milk from healthy and mastitic dairy cows during the lactation cycle. *Aust. J. Exp. Agric.* **35**, 427–436 (1995).
4. Auldist, M.J. *et al.* Effect of somatic cell count and stage of lactation on the quality and storage life of ultra high temperature milk. *J. Dairy Res.* **63**, 377–386 (1996).
5. Barbano, D.M., Rasmussen, R.R. & Lynch, J.M. Influence of milk somatic cell count and milk age on cheese yield. *J. Dairy Sci.* **74**, 369–388 (1991).
6. Klei, L. *et al.* Effects of milk somatic cell count on cottage cheese yield and quality. *J. Dairy Sci.* **81**, 1205–1213 (1998).
7. Bramley, A.J. & Dodd, F.H. Reviews of the progress of dairy science: mastitis control—progress and prospects. *J. Dairy Res.* **51**, 481–512 (1984).
8. Sutra, L. & Poutrel, B. Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. *J. Med. Microbiol.* **40**, 79–89 (1994).
9. Craven, N. & Anderson, J.C. Phagocytosis of *Staphylococcus aureus* by bovine mammary gland macrophages and intracellular protection from antibiotic action in vitro and in vivo. *J. Dairy Res.* **51**, 513–523 (1984).
10. Yancey, R.J., Sanchez, M.S. & Ford, C.W. Activity of antibiotics against *Staphylococcus aureus* within polymorphonuclear neutrophils. *Eur. J. Clin. Microbiol. Infect. Dis.* **10**, 107–113 (1991).
11. Oldham, E.R. & Daley, M.J. Lysostaphin: use of a recombinant bactericidal enzyme as a mastitis therapeutic. *J. Dairy Sci.* **74**, 4175–4182 (1991).
12. Watson, D.L. Vaccination against experimental staphylococcal mastitis in dairy heifers. *Res. Vet. Sci.* **53**, 346–353 (1992).
13. Schindler, C.A. & Schuhardt, V.T. Lysostaphin: a new bacteriolytic agent for the *Staphylococcus*. *Proc. Natl. Acad. Sci. USA* **51**, 414–421 (1964).
14. Bramley, A.J. & Foster, R. Effects of lysostaphin on *Staphylococcus aureus* infections of the mouse mammary gland. *Res. Vet. Sci.* **49**, 120–121 (1990).
15. Gordon, K. *et al.* Production of human tissue plasminogen activator in transgenic mouse milk. *Bio/technology* **5**, 1183–1187 (1987).
16. Wall, R. J. *et al.* High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. *Proc. Natl. Acad. Sci. USA* **88**, 1696–1700 (1991).
17. Wright, G. *et al.* High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. *Bio/Technology* **9**, 830–834 (1991).
18. Ebert, K. M. *et al.* Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Bio/Technology* **9**, 835–838 (1991).
19. Krimpenfort, P. *et al.* Generation of transgenic dairy cattle using ˙in vitro' embryo production. *Bio/Technology* **9**, 844–847 (1991).
20. Maga, E.A., Anderson, G.B., Huang, M.C. & Murray, J.D. Expression of human lysozyme mRNA in the mammary gland of transgenic mice. *Transgenic Res.* **3**, 36–42 (1994).
21. Williamson, C.M., Bramley, A.J. & Lax, A.J. Expression of the lysostaphin gene of *Staphylococcus simulans* in a eukaryotic system. *Appl. Environ. Microbiol.* **60**, 771–776 (1994).
22. Maga, E.A., Anderson, G.B. & Murray, J.D. The effect of mammary gland expression of human lysozyme on the properties of milk from transgenic mice. *J. Dairy Sci.* **78**, 2645–2652 (1995).
23. Verdi, R.J., Barbano, D.M., Dellavalle, M.E. & Senyk, G.F. Variability in true protein, casein, nonprotein nitrogen, and proteolysis in high and low somatic cell milks. *J. Dairy Sci.* **70**, 230–242 (1987).
24. Whitelaw, C.B. *et al.* Targeting expression to the mammary gland: intronic sequences can enhance the efficiency of gene expression in transgenic mice. *Transgenic Res.* **1**, 3–13 (1991).
25. Wall, R.J. & Seidel, G. Jr. Transgenic farm animals—a critical analysis. *Theriogenology* **38**, 337–357 (1992).
26. Marais, R. et al. A cell surface tethered enzyme improves efficiency in gene-directed enzyme producing therapy. *Nat. Biotechnol.* **15**, 1373–1377 (1997).
27. Reiter, B. Review of the progress of dairy science: antimicrobial systems in milk. *J. Dairy Res.* **45**, 131–147 (1978).
28. Takahashi, N. *et al.* Nonspecific antibacterial factors in milk from cows immunized with human oral bacterial pathogens. *J. Dairy Sci.* **75**, 1810–1820 (1992).
29. Platenburg, G.J. *et al.* Expression of human lactoferrin in milk of transgenic mice. *Transgenic Res.* **3**, 99–108 (1994).
30. Yarus, S., Rosen, J.M., Cole, A.M. & Diamond, G. Production of active bovine tracheal antimicrobial peptide in milk of transgenic mice. *Proc. Natl. Acad. Sci. USA* **93**, 14118–14121 (1996).
31. Maga, E.A., Anderson, G.B., Cullor, J.S., Smith, W. & Murray, J.D. Antimicrobial properties of human lysozyme transgenic mouse milk. *J. Food Prot.* **61**, 52–56 (1998).
32. Harmon, R.J., Schanbacher, F.L., Ferguson, L.C. & Smith, K.L. Concentration of lactoferrin in milk of normal lactating cows and changes occurring during mastitis. *Am. J. Vet. Res.* **36**, 1001–1007 (1975).
33. Drews, R. *et al.* Proteolytic maturation of protein C upon engineering the mouse mammary gland to express furin. *Proc. Natl. Acad. Sci. USA* **92**, 10462–10466 (1995).
34. Chandler, R.L. Experimental bacterial mastitis in the mouse. *J. Med. Microbiol.* **3**, 273–282 (1970).

© 2001 Nature Publishing Group http://biotech.nature.com