

A System for Production of Commercial Quantities of Human Lactoferrin: A Broad Spectrum Natural Antibiotic

Pauline P. Ward¹, Christopher S. Piddington², Grainne A. Cunningham¹, Xiaodong Zhou¹, Roger D. Wyatt³ and Orla M. Conneely^{1,*}

¹Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, ²PANLABS, Inc., 11804 North Creek Parkway South, Bothell, WA 98011-8805 and ³Agennix, Inc., 7505 Fannin St., Suite 510, Houston, TX 77054. *Corresponding author.

We previously reported the production of limited quantities of biologically active recombinant human lactoferrin in the filamentous fungus, *Aspergillus oryzae*. In the present study, we report a modification of this production system combined with a classical strain improvement program that has enabled production of levels of recombinant human lactoferrin in excess of 2 g/l. The protein was expressed in *Aspergillus awamori* as a glucoamylase fusion polypeptide which was secreted into the growth medium and processed to mature human lactoferrin by an endogenous KEX-2 peptidase. The recombinant protein retains full biological activity in terms of its ability to bind iron and human enterocyte receptors. Furthermore, the recombinant protein functions as a potent broad spectrum antimicrobial protein.

Received 6 December 1994; accepted 20 January 1995.

Human lactoferrin is a member of the transferrin family of iron-binding glycoproteins. Lactoferrin is the second most abundant protein in human milk (1 gram/l)^{1,2} and also is found at lower levels in other external secretions such as tears, nasal secretions, saliva, intestinal mucus³, and genital secretions^{4,5} where it is involved in the primary host defense system at the mucosal surface. Lactoferrin also is produced by the secondary granules of polymorphonuclear neutrophils⁶. The three dimensional structure of lactoferrin has been resolved by X-ray crystallographic analysis^{7,8}. The protein is folded into two repeated globular domains with each domain having the capacity to bind tightly, but reversibly, one ferric ion with the concomitant binding of an anion such as carbonate.

Lactoferrin is clearly a multi-functional protein to which several physiological functions have been described^{9,10}. The ability of lactoferrin to protect against microbial infection by a broad spectrum of bacteria is supported by both *in vitro*^{11,12} and *in vivo* evidence^{13,14}. Three different mechanisms, involving at least two separate domains of the protein, contribute to the antimicrobial function of lactoferrin. The first mechanism is a bacteriostatic effect related to a high iron-binding affinity that deprives bacteria of this essential growth nutrient¹⁵⁻¹⁷. The second antimicrobial function is a direct bactericidal activity¹⁸ and is provided by a highly cationic bactericidal domain located at the N-terminus of the protein, in a region distinct from the iron-binding sites¹⁹. This bactericidal domain increases bacterial membrane permeability and causes the release of lipopolysaccharide from the outer membrane of Gram negative bacteria^{20,21}. In addition to its bacteriostatic and bactericidal activities, lactoferrin released from activated neutrophils may also exert antimicrobial activity through regulation of systemic immune responses. High affinity lactoferrin receptors have been identified on cell types including lymphocytes²², monocytes²³, macrophages²⁴, and platelets²⁵. The exact molecular mechanism by which lactoferrin influences systemic immune responses remains to be elucidated but has been reported to involve regulation of cytokine release^{26,27}.

Lactoferrin may also have an important role in the regulation of iron uptake in the gastrointestinal tract. This is suggested by the discovery that lactoferrin, but not transferrin, could donate its bound iron to human duodenal cells²⁸. More recently, specific enterocyte receptors for lactoferrin have been identified and partially purified from a number of species [reviewed in ref. 10]. However, the molecular mechanism of

uptake of lactoferrin and its bound iron remains to be elucidated. Given the high affinity of lactoferrin for iron and its ability to retain bound iron over a broad pH range, the protein may also be involved in the sequestration and detoxification of free iron in the gastrointestinal tract.

The potential nutritional and therapeutic applications of human lactoferrin have not been exploited to date due to the lack of an efficient and cost effective method for production of sufficient quantities of functional lactoferrin for commercial use. We previously have described the production of human lactoferrin in *A. nidulans*²⁹ and *A. oryzae*³⁰. While these systems were used to produce moderate levels of intact, biologically active human lactoferrin, attempts to scale up production in *A. oryzae* have been largely unsuccessful. The successful large scale production of several recombinant proteins in alternative *Aspergillus* species, namely *A. niger* and *A. awamori*, has been reported^{31,32}. In the reported production of two mammalian proteins, significant improvements in yield were achieved when the heterologous protein was expressed as a chimera fused to genomic sequences encoding the complete or partial coding region of a highly expressed endogenous gene such as glucoamylase^{33,34}. The insertion of a natural protease cleavage site, such as KEX-2, between the glucoamylase and heterologous cDNAs, allows the efficient processing of the resultant chimeric protein³⁵.

We now report the production of recombinant human lactoferrin in *A. awamori* by expressing the protein as a chimera with the N-terminus of the *A. awamori* glucoamylase gene under the control of the *A. awamori* glucoamylase promoter. This approach, combined with a classical strain improvement and screening for high lactoferrin titer transformants, has yielded elevated levels of intact, biologically active recombinant lactoferrin.

Results

Expression and purification of recombinant lactoferrin from *A. awamori* transformants. *Aspergillus awamori* (ATCC 22342) transformants containing integrated lactoferrin expression plasmid, pPLF-19, were cultured as described in the Experimental Protocol and the culture medium was screened for lactoferrin using an ELISA assay³⁶. Transformants yielding >250 mg/l of lactoferrin were selected for mutagenesis and strain improvement. To date, initial rounds of mutagenesis have resulted in the generation of transformants secreting in excess of 2 g/l of intact recombinant lactoferrin into the

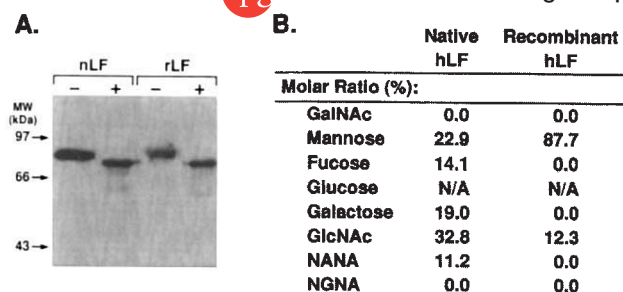


FIGURE 1. Glycosylation analysis of recombinant lactoferrin. Panel A. Western analysis of glycosylated and deglycosylated native and recombinant human lactoferrin. The first two columns contain native breast milk lactoferrin (nLF; 1 μ g) undigested (-) and digested (+) with N-glycosidase F. The last two columns contain purified recombinant human lactoferrin (rLF) (1 μ g) undigested (-) and digested (+) with N-glycosidase F. Panel B. Monosaccharide analysis of native and recombinant lactoferrin. Three separate hydrolysis reactions were performed on the native and recombinant human lactoferrin to determine amine, neutral and sialic acid monosaccharides (GLYKO, Novato CA).

A. awamori growth medium. Lactoferrin was purified to homogeneity from the growth medium of high titer transformants using CM-Sepharose as previously described³⁰. The efficiency of recovery, using this one step ion-exchange chromatography procedure, was >85%.

Characterization of recombinant human lactoferrin.

N-terminal sequence and glycosylation analysis. Lactoferrin was expressed in *A. awamori* as a fusion protein separated by a KEX-2 cleavage site from the catalytic and hinge region of the *A. awamori* glucoamylase gene. To determine if lactoferrin was correctly processed at the N-terminus, the first 11 N-terminal amino acids of the purified protein were sequenced using the automated Edman degradation procedure. The results of this analysis demonstrated that the sequence of the recombinant protein is identical to the corresponding amino acids in human breast milk lactoferrin and that the recombinant protein was correctly processed at the KEX-2 proteolytic cleavage site (results not shown). In addition, western immunoblot analysis, using a specific polyclonal IgG directed against human lactoferrin, detected a single 78 kDa protein in the culture medium of *A. awamori* transformants corresponding to the size of correctly processed lactoferrin. This further indicates that complete cleavage of lactoferrin from the lactoferrin/glucoamylase fusion protein has occurred (results not shown).

Human breast milk lactoferrin is a glycoprotein containing ~5% carbohydrate of the N-acetylactamine type, which are attached through glycosidic linkages at two sites on the protein³⁷. To determine if the nature of the carbohydrate-protein linkage and the extent of glycosylation are similar in the recombinant protein, native human breast milk lactoferrin and recombinant human lactoferrin were digested with N-glycosidase F, and analyzed by western immunoblotting using a specific polyclonal IgG directed against human lactoferrin. The results of this analysis are outlined in Figure 1 (A). Both native and recombinant human lactoferrin comigrate on the SDS-PAGE gel suggesting that the extent of glycosylation is similar for both proteins (lanes 1 and 3). Furthermore, digestion with N-glycosidase F altered the mobility of both proteins to the same degree (lanes 2 and 4) indicating that the attached carbohydrate is similarly N-linked to the protein. Analysis of the carbohydrate composition demonstrates that while the extent of glycosylation is similar for both proteins, there are differences in the monosaccharides released from recombinant lactoferrin and human lactoferrin isolated from human milk [Fig. 1 (B)]. The recombinant protein clearly contains high mannose type N-linked oligosaccharides and may display a simpler carbohydrate structure in contrast to the complex carbohydrate structure of human breast milk lactoferrin.

Iron-saturation and pH stability of iron-binding to native and recombinant lactoferrin. Lactoferrin is an iron-binding glycoprotein with the capacity to bind two moles of iron per mole of lactoferrin⁷. To determine if the binding of iron by recombinant lactoferrin was saturable and similar to that of human milk lactoferrin, an iron-saturation assay using ⁵⁹FeCl₃ was performed. The results of this analysis are outlined in Figure 2 (A). Both proteins bound ⁵⁹Fe with similar capacity and binding of iron was saturable at a 2:1 molar ratio of iron to lactoferrin as expected for the intact protein. Typically, saturation levels are reached at 92.5% of maximal binding. This is indicative of initial 7.5% iron remaining bound to the lactoferrin in the apolactoferrin preparation used for analysis. A key feature of the iron binding activity of lactoferrin that is different from other transferrins is its ability to retain bound iron over a broad pH range. To compare the pH stability of iron-binding by native and recombinant lactoferrin, both proteins were saturated with ⁵⁹Fe and dialyzed against buffers ranging in pH from 7-2. ⁵⁹Fe remaining bound to lactoferrin after dialysis was quantified using liquid scintillation counting. This analysis demonstrated identical pH dependent iron release profiles for both proteins [Fig. 2 (B)]. Both the native and recom-

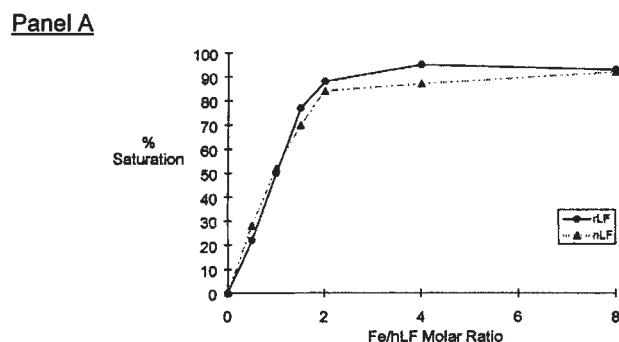
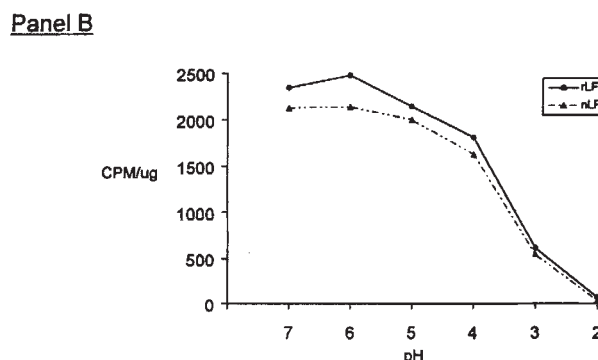


FIGURE 2. Iron-binding to recombinant lactoferrin. Panel A. Iron saturation of native and recombinant human lactoferrin. Native (nLF) and recombinant (rLF) human lactoferrin (500 μ g) were incubated with increasing concentrations of ⁵⁹Fe for 30 minutes at 22°C. Free ⁵⁹Fe was removed by passage of the samples over a NAP-10 column. ⁵⁹Fe bound lactoferrin was quantified by liquid scintillation counting. Panel B. pH dependent release of ⁵⁹Fe from native and recombinant human lactoferrin. ⁵⁹Fe saturated native (nLF) and recombinant (rLF) human lactoferrin (200 μ g) were dialyzed against buffers ranging from pH 7-2 for 48 hours at 4°C. ⁵⁹Fe remaining bound to lactoferrin samples after dialysis was quantified by liquid scintillation counting.



dent release of ⁵⁹Fe from native and recombinant human lactoferrin. ⁵⁹Fe saturated native (nLF) and recombinant (rLF) human lactoferrin (200 μ g) were dialyzed against buffers ranging from pH 7-2 for 48 hours at 4°C. ⁵⁹Fe remaining bound to lactoferrin samples after dialysis was quantified by liquid scintillation counting.

binant proteins retained most (>80%) of the iron over a pH range of 7–4 and released the iron between pH 4 and 2.

Binding of recombinant lactoferrin to human enterocyte receptors. Lactoferrin receptors have been identified on several cell types including human enterocytes¹⁰. The human colon carcinoma cell line, Caco-2, spontaneously differentiates into enterocyte-like cells upon formation of confluent monolayers³⁸. To determine if the recombinant protein was capable of binding lactoferrin receptors on Caco-2 cells, a receptor binding assay, using biotinylated iron-saturated recombinant human lactoferrin, was performed. The results of this analysis are summarized in Figure 3 (A). The binding of biotinylated recombinant lactoferrin to Caco-2 cell membranes was specific and saturable. Non-specific binding, determined using a 20-fold excess of unlabeled recombinant lactoferrin, was low, representing less than 5% of the specific binding component. Scatchard plot analysis³⁹ of the specific binding data revealed a dissociation constant (K_d) of $\sim 1.8 \times 10^{-7}$ M with 7.5×10^{14} binding sites/mg of membrane protein [Fig. 3 (A) inset]. The K_d is similar to that previously reported for human milk lactoferrin¹⁰.

The relative binding affinities of iron-saturated recombinant and natural lactoferrin were compared using competitive binding studies⁴⁰. Biotinylated iron-saturated recombinant lactoferrin (0.4 μ M) was incubated with 0–50-fold molar excess of unlabeled iron-saturated recombinant or natural lactoferrin or human transferrin. Both lactoferrin preparations displayed similar ability to displace binding of biotinylated recombinant lactoferrin to Caco-2 cell membranes at all concentrations tested [Fig. 3 (B)]. In contrast, iron-saturated human transferrin, a member of the same family of iron-binding glycoproteins sharing a 60% homology with lactoferrin⁴¹, was unable to displace binding of labeled lactoferrin.

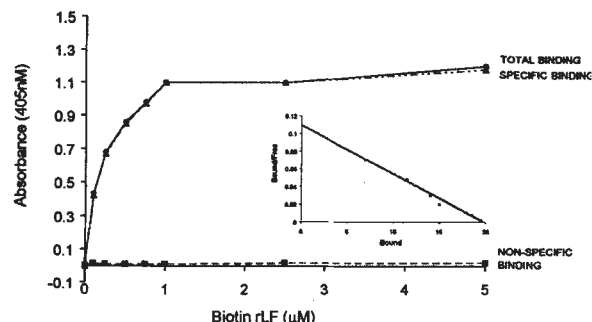
Lactoferrin functions as a potent natural antibiotic. Lactoferrin has previously been shown to inhibit the growth of Gram negative and Gram positive bacteria^{11–14,18–21}. Furthermore, a prophylactic effect by lactoferrin has been observed in mice which were injected with lethal doses of *E. coli*¹⁴. To determine if recombinant lactoferrin displayed similar antimicrobial action *in vivo*, mice were injected intraperitoneally (i.p.) with a lethal dose of *E. coli* 078:H11 with or without prior administration of recombinant iron-free lactoferrin (200 μ g–1 mg). The results outlined in Table 1 demonstrate that recombinant lactoferrin decreased the mortality rate of mice from 43% to 0% thus, exerting a similar prophylactic antimicrobial activity *in vivo* to native lactoferrin¹⁴.

To establish the spectrum of antimicrobial activity of recombinant lactoferrin, an *in vitro* microbroth dilution assay was performed. Clinical isolates (72), derived from 16 different strains of bacteria, were incubated in the presence or absence of iron-free recombinant lactoferrin (64–4,096 μ g/ml). The samples were incubated at 35°C for 20 hours. The results of this analysis are outlined in Table 2. Recombinant lactoferrin inhibited the growth of 66 of the 72 isolates. The effective minimal inhibitory concentration (MIC) of recombinant lactoferrin is in the μ M range and corresponds to 256–1,024 μ g/ml of the protein. Hence, recombinant lactoferrin functions as a potent broad spectrum antimicrobial protein in this *in vitro* assay system.

Discussion

The studies described herein demonstrate the feasibility of low-cost production of sufficient quantities of human lactoferrin for a variety of nutritional and therapeutic applications. The recombinant protein is biologically active as shown by three key criteria: iron and human enterocyte receptor binding and antimicrobial activities. Our studies demonstrate that recombinant lactoferrin is a potent antimicrobial factor *in vitro*

Panel A



Panel B

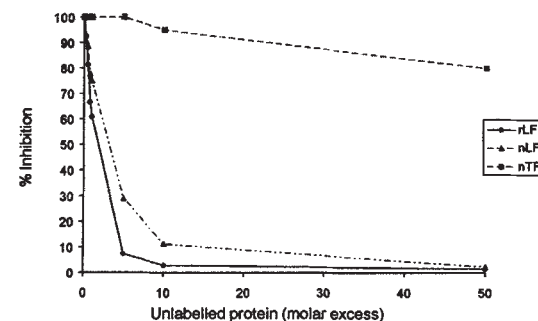


FIGURE 3. Binding of recombinant human lactoferrin (rLF) to human enterocyte cells. **Panel A.** Specific and saturable binding of biotin rLF to Caco-2 cell membranes. Iron saturated biotinylated rLF (0.05–5 μ M) was incubated with solubilized Caco-2 membranes (200 ng) in the presence or absence of a 20-fold molar excess of unlabeled ligand. The assay was carried out for 4 hours at 4°C. Binding of biotinylated rLF to the Caco-2 membranes was determined using a Biotin/Avidin microtiter assay. The inset shows a Scatchard analysis of the specific binding data. **Panel B.** Competition of iron saturated biotinylated recombinant human lactoferrin (rLF) binding to Caco-2 cell membranes. Iron saturated biotinylated rLF (0.4 μ M) was incubated with Caco-2 membranes (200ng) in the presence or absence of increasing molar excess concentrations of unlabeled iron-saturated rLF, native human lactoferrin (nLF) or human transferrin (nTF) for 4 hours at 4°C. Inhibition of biotinylated rLF binding to Caco-2 membranes was determined using a Biotin/Avidin microtiter assay.

TABLE 1. Antimicrobial action of recombinant human lactoferrin *in vivo*. ICR female mice (8–10 wks) were injected intraperitoneally with either recombinant apolactoferrin or BSA. After a period of 10 hours, they were injected with 2×10^8 cells of *E. coli* 078:H11. The mortality rate was monitored for 48 hours after the bacterial challenge.

Mortality	Treatment	No. Injected
BSA (1mg) & <i>E. coli</i> 078:H11	35	15
LF (1mg) & <i>E. coli</i> 078:H11	36	0
LF (200 μ g) & <i>E. coli</i> 078:H11	15	0

and *in vivo* and can inhibit the growth of a broad range of bacteria. The iron sequestration, bactericidal, and immune regulatory functions are likely to contribute to a synergistic antimicrobial response that is unique to lactoferrin and may account for the broad spectrum of antibiotic activity of this protein.

Lactoferrin is a glycosylated protein³⁷. While the core linkage and extent of glycosylation of the recombinant protein is similar to human breast milk lactoferrin, differences in specific carbohydrate residues between the two proteins were

observed. These differences do not affect the functional activity of the protein, an observation that is consistent with previous reports demonstrating a lack of contribution of carbohydrate to the functional activity of the protein^{8,42}. While altered carbohydrate does raise a potential for immunogenicity of recombinant glycoproteins in human biological fluids, in the case of lactoferrin, it has been demonstrated that heterogeneity exists in the carbohydrate attached to lactoferrin isolated from different species and from different tissues within a species^{37,43}. For example, bovine lactoferrin contains additional glycans of the oligomannosidic type not contained in human breast milk lactoferrin but similar to that observed in recombinant lactoferrin. Furthermore, there is evidence that human lactoferrin is glycosylated in a tissue specific manner^{37,43}. Since carbohydrate does not appear to affect the biological activity of lactoferrin, it is likely that differential glycosylation may contribute to the stability and half-life of the protein in certain biological fluids.

Significant improvements in yields of lactoferrin have been achieved by expressing the protein in *A. awamori* as a chimera with a fragment of the highly expressed *A. awamori* glucoamylase gene. These increases in production may be due, at least in part, to the specific strain utilized and to enhanced translation, stability and secretion of the chimeric protein. Comparable increases in the levels of two other mammalian proteins have been achieved using a generally similar approach. Bovine chymosin was secreted at high levels in *A. awamori* by fusing the precursor prochymosin cDNA with the complete gene encoding the *A. awamori* glucoamylase gene³³. Using a modification of this approach, improvements in secretion of human lysozyme (>1 gram/liter) in *A. niger* have been achieved by expressing the cDNA as a fusion with the N-terminal domain (498 amino acids) encoding the catalytic and hinge region of the *A. niger* glucoamylase gene³⁴. However, expression levels of recombinant proteins have been variable and greatly influenced by the specific characteristics and complexity of the heterologous protein as well as the specific strain in which the cDNA is expressed^{44,45}. Lactoferrin is a large (78 kDa) mammalian glycoprotein whose complexity is approximately two-fold greater than previously expressed mammalian proteins in these systems. In this case, the improved levels of expression of lactoferrin are likely due to a combination of a chimeric approach and expression in the *A. awamori* strain.

To date, the modified expression system, combined with limited mutagenesis, has resulted in increases in lactoferrin from ~20 mg/l to >2 g/l. Improvement of lactoferrin produc-

ing strains and optimization of production levels are expected to yield levels of lactoferrin in the 10–20 g/l range corresponding to production costs of ~5–10 cents per gram.

The broad spectrum antimicrobial properties of lactoferrin highlight the potential application of this protein as an enteral and parenteral antimicrobial factor. This recombinant lactoferrin production system provides, for the first time, an adequate source of human lactoferrin to examine the antimicrobial, therapeutic, and nutritional benefits of this protein.

Experimental Protocol

Construction of the *A. awamori* expression plasmid. The expression plasmid pPLF-19 was utilized for the production of recombinant lactoferrin in *A. awamori*. A 2.1 kb human lactoferrin fragment (nucleotide 1-2141), with NgoMI/EcoRI ends, was obtained by polymerase chain reaction (PCR) amplification of pGEMhLFC plasmid DNA³⁰. The oligonucleotide primers were as follows: 5' end oligonucleotide; 5' GGGGTA-CCGCGCCGGCCGTAGGAGAAGGAGTCT3'. 3' end oligonucleotide; 5' GAGGAATTCACAGGCTTCCAGGAG 3'. The amplified DNA was digested with NgoMI and EcoRI and was subcloned into Puc19 (New England Biolabs, Beverly, MA) generating pPLF-7. A 2.1 kb glucoamylase fragment encoding the promoter (1.1 kb), signal sequence and first 498 amino acids of the *A. awamori* glucoamylase gene was obtained by polymerase chain reaction (PCR) amplification of *A. awamori* genomic DNA (ATCC 22342). The oligonucleotide primers were as follows; 5' end oligonucleotide; 5' TATGCAGAGGAGCTCTCCCTGAC 3' (ATCC 10864; GenBank Accession # X56442) 3' end oligonucleotide; 5' GCGCTTGGAATCACATTCTTGCTGGTTCGAGGTCACGCT 3' (ref. 46). The 3' end oligonucleotide was engineered to incorporate an 18 bp oligonucleotide at the 3' end encoding a hexapeptide (Asn-Val-Ile-Ser-Lys-Arg) which contains a KEX-2 proteolytic cleavage site. The amplified DNA was repaired using the Klenow fragment of DNA polymerase I and subcloned into pPLF-7 which was digested with NgoMI, repaired with dCTP using the Klenow fragment of DNA polymerase I followed by Mung Bean nuclease digestion. The correct orientation containing the hexapeptide/lactoferrin fusion was verified by sequencing through the construction junction. Synthetic oligonucleotides were used to provide the last five carboxyl-terminal codons of lactoferrin (nt 2138-2153) and also to provide the first 160 bp of 3' untranslated sequences from the *A. niger* glucoamylase gene⁴⁷. The resulting plasmid, pPLF-18, was digested with Hind III and ligated with a 2.3 kb Hind III fragment encoding the phleomycin resistance marker (*Streptoalloteichus hindustanus* ble gene) under the control of the *A. niger* tubulin promoter⁴⁸. All PCR amplified products and construction junctions were sequenced using the commercially available sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH).

Aspergillus strain, transformation procedures and culture conditions. *Aspergillus awamori* was obtained from the American Type Tissue Collection (ATCC 22342). Complete Media (CM) for *A. awamori* transformations is composed of 50 ml of 20X Clutterbuck's salts (120 g Na₂NO₃, 10.4 g KCl, 10.4 g MgSO₄·7H₂O, 30.4 g KH₂PO₄), 2.0 ml Vogel's Trace Elements (per liter: 0.3 M citric acid, 0.2 M ZnSO₄, 25 mM Fe[NH₄]₂[SO₄]₆H₂O, 10 mM CuSO₄, 3 mM SO₄, 8 mM boric acid, 2 mM Na₂MoO₄·2H₂O), 5.0 g tryptone, 5.0 yeast extract, 10 g glucose. Agar (1.5%) was added for CM slants. PDA slants contained 39 g/L Potato Dextrose Agar (Difco, Detroit MI), 10.0 g/L glucose, 10.0 g/L agar, 0.1 g/L MgSO₄·7H₂O, 0.12 g/L KH₂PO₄, 0.25 g/L (NH₄)₂HPO₄. *Aspergillus awamori* strain ATCC 22342 was spheroplasted and transformed by a procedural modification of Tilburn et al⁴⁹. Conidia (2×10⁶/ml) were inoculated into CM media (50 ml) and grown for 22 hours at 30°C, 200 rpm. Mycelia were harvested by filtration through cheesecloth and added to 50 mls of KCM buffer⁵⁰ with 5 mg/ml of Novozyme 234 (Novo Biolabs, Bagsvaerd, Denmark) and incubated at 30°C, 90 rpm overnight for spheroplast generation. Undigested mycelia were removed by filtration using Miracloth and spheroplasts were harvested by centrifugation at 1800 rpm for 10 minutes in a bench-top centrifuge. The pellets were gently washed twice in KCM buffer and then resuspended in KCMC (KCM + 50 mM CaCl₂) buffer to a final density of 5×10⁷ cells/ml. Five µg of pPLF-19 plasmid DNA in 20 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added to 200 µl of spheroplasts and 50 µl of PCM⁵⁰ was gently pipetted into the DNA-spheroplast mixture and incubated on ice for 30 minutes. One ml of freshly made PCM was added to the transformation mix which was added to 50 mls of regeneration agar (CM + 1.3 M Mannitol, 3% agar). The mixture was cooled to 50°C and divided into five petri plates. Spheroplasts were allowed to regenerate for 3 to 5 hours at 30°C before overlaying with an equal amount of OL + 120 µg/ml phleomycin [OL: 1% peptone, 1% agar; phleomycin (CAYLA; Toulouse, FR)]. Putative transformants were transferred to PDA slants containing 125–150 µg/ml phleomycin. *A. awamori* transformants were screened for lactoferrin production in shake flasks. Conidia (1×10⁶/ml) were incubated into 30 mls of CSM medium in 250 ml flasks⁵². The cultures were grown at 30°C for 6 days after which the media was harvested by filtration using Miracloth (Calbiochem, San Diego, CA). Lactoferrin titers were determined using an ELISA assay³⁶.

Mutagenesis and screening. A combination of random and rational

Table 2. Spectrum of antimicrobial activity of lactoferrin in vitro. A standard inoculum of bacteria (1×10⁶ CFU/ml) was incubated in the presence or absence of increasing concentrations of recombinant apolactoferrin. After 20 hour incubations, MIC determinations were made using the microdilution procedure.

Species	No. of Isolates	No. Inhibited	MIC (µM)
<i>Escherichia coli</i>	14	14	3.28–6.56
<i>Salmonella enteritidis</i>	6	6	13.13
<i>Shigella dysenteriae</i>	3	3	1.64–6.56
<i>Shigella sonnei</i>	3	3	6.56–13.13
<i>Serratia marcescens</i>	6	3	13.13
<i>Enterobacter cloacae</i>	6	5	6.56–13.13
<i>Klebsiella pneumoniae</i>	6	4	13.13
<i>Pseudomonas aeruginosa</i>	6	6	6.56
<i>Meth R Staphylococcus aureus</i>	6	6	3.28–6.56
<i>Staphylococcus aureus</i> BL Neg	3	3	3.28
<i>Staphylococcus aureus</i> BL +	3	3	83.28
<i>Enterococcus faecalis</i>	6	6	6.56
QC <i>Escherichia coli</i>	1	1	6.56
QC <i>Staphylococcus aureus</i>	1	1	6.56
QC <i>Enterococcus faecalis</i>	1	1	6.56
QC <i>Pseudomonas aeruginosa</i>	1	1	6.56

selection strategies that targeted carbon flux towards lactoferrin production was utilized in the mutagenesis and screening program. Five rounds of mutagenesis were performed on *A. awamori* transformants producing recombinant lactoferrin at >250 mg/l. Mutagenesis consisted of two rounds of U.V. mutagenesis [1100 microwatts per square centimeter U.V. (short wave) intensity for 180 seconds] followed by four rounds of chemical mutagenesis using either 4-nitroquinoline-N-oxide (NQO) (1×10^6 spores/ml, 2 µg/ml NQO, pH 7.5 for 1 hour at 37°C) or Nitrous acid (NA) (1×10^6 spores/ml, 0.5M NA pH 4.5 for 30 minutes). After U.V. mutagenesis colonies were selected randomly or compact colonies were selected for glucose derepression using 2-deoxyglucose³². After NQO mutagenesis (2 rounds) colonies were selected which gave a small clear zone on YAGD (yeast extract, 5g/l; glucose 10g/l; trace elements, 1ml/l; vitamin mix, 2ml/l; 1M $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 ml/l; 2-6 Dichloro-4-nitroaniline, .0004%; Agar, 20g/l) containing CaCO_3 (0.5%) followed by selection of small colonies on YAGD medium containing 4–7% trisodium citrate. For nitrous acid mutagenesis, (1 round) colonies were selected randomly. 1,000 mutants were screened for lactoferrin production per round of mutagenesis based on random selection. The mutants were cultured in CSM medium as described above. After 6 days, the culture medium was harvested and lactoferrin titers were assayed by ELISA³⁶.

Monosaccharide analysis. The monosaccharides attached to recombinant and native human breast milk lactoferrin were analyzed using Fluorophore Assisted Carbohydrate Electrophoresis (FACE) technology (Glyko, Novato, CA). Three separate hydrolysis reactions (Amino sugar, neutral sugar and sialic acid) were performed on the lactoferrin samples (10 µg for each reaction). Amino sugar hydrolysis was carried out using 4N HCl for 3 hours at 100°C. The sample was dried and then re-N-acetylated using 100 mM ammonium carbonate, pH 9.6 (10 µl) and acetic anhydride (1 µl). The sample was incubated on ice for 20 minutes. Neutral sugar hydrolysis was carried out using 2N TFA for 5 hours at 100°C. Sialic acid hydrolysis was carried out using TFA (0.1N) for 1 hour at 80°C. The three hydrolysis reactions were dried using a speed vacuum (no heat). The labeling reaction was carried out using .3M aminoacridone in DMSO (2.5 µl) and 1 M BH3CN (5 µl).

Preparation of apo and iron-saturated lactoferrin. To generate apo-lactoferrin, purified recombinant lactoferrin and human breast milk lactoferrin were dialyzed against 0.1 M citric acid, pH 2.0, followed by extensive dialysis against H_2O . The pH of the solution was slowly raised to pH 7.6 using 5 mM sodium phosphate³¹. To generate iron saturated lactoferrin, a two fold molar excess of $\text{FeCl}_3 \cdot 3\text{H}_2\text{O}$ (400:1:8) was added to lactoferrin (500 µg) in binding buffer (0.025M Tris, pH 7.8; 0.01 M sodium bicarbonate; 0.1 M NaCl). Samples were incubated at 22°C for 30 minutes. Free iron and NTA were removed by passage over a NAP-10 column (Pharmacia) which had been equilibrated with 15 ml of binding buffer. The amount of iron bound to lactoferrin was quantified using liquid scintillation counting.

pH stability of iron-binding. To determine the pH stability of iron-binding to native and recombinant lactoferrin, the ⁵⁹Fe-saturated proteins (500 µg) were dialyzed against buffers ranging from pH 7.0 to pH 2.0 for 48 hours at 4°C³². Iron-bound to the lactoferrin samples after dialysis was quantified using liquid scintillation counting.

Tissue culture. Human colon carcinoma Caco-2 cells (ATCC HTB37) were used between passages 50 and 85. Cells were routinely cultured in Dulbecco's modified Eagle media (DMEM) (Gibco, Grand Island N.Y.) supplemented with 10% fetal bovine serum and containing penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C and 10% CO_2 . Differentiated Caco-2 cells were obtained by culturing the cells for 10–12 days post confluency on 100 mM plastic tissue culture dishes (2×10^6 cells/plate).

Receptor binding assay. Receptor binding assays were performed essentially as described by Rejman et al³³. All procedures were performed at 4°C unless otherwise stated. Differentiated Caco-2 cells (>14 days old) were harvested from tissue culture plates using 1 X PBS, pH 7.4. Cells were harvested by centrifugation at 1000 X g for 10 minutes. The cells were resuspended in membrane buffer (MB) (25 mM Tris-Cl, 1 mM CaCl_2 , 1 mM MgCl_2 , 0.02% Na azide, pH 7.2) supplemented with 0.25 M sucrose. Crude membrane preparations were obtained by sonication of the solubilized Caco-2 cells (2 X 1 minute bursts) followed by centrifugation at 30,000 X g for 30 minutes. The membranes were washed twice in MB and resuspended in 1 X PBS. Microtitre plates (Falcon Pro-BIND #3915) were coated overnight with 200 ng of membrane protein in coating buffer (0.1 M sodium carbonate/bicarbonate, pH 9.6). The plates were washed three times with wash buffer (100 mM Tris, 150 mM NaCl, .1% Tween 20, pH 7.4) prior to blocking with 250 µl of blocking buffer (100 mM Tris-Cl, 150 mM NaCl, .1% BSA, pH 7.4). Recombinant lactoferrin was biotinylated (Molecular probes, Eugene OR) and the iron saturated labeled protein (0–5 µM) was incubated in the presence or absence of a 20 fold molar excess of unlabeled recombinant lactoferrin in 100 µl of binding buffer (100 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4). Unbound biotinylated lactoferrin was removed by washing four times in wash buffer. Lactoferrin-receptor binding was detected using the ABC reagent according to manufacturer's instructions (Vectastain, Vector Labs, Burlingame, CA) followed by color detection using the OPD substrate (Sigma, St Louis, MO). Absorbency at 405 nm was read on an automated Dynatech machine (MR7000) and compared to biotinylated lactoferrin standards for concentration determinations. Non-specific binding of biotinylated lactoferrin (0.05–5 µM) to the microtitre plates was determined by carrying out receptor-binding assays with heat inactivated membrane (200 mg; 100°C for 5

minutes). These values were subtracted from the corresponding values obtained using non-heat-treated membrane.

In vivo antibacterial activity of lactoferrin. The enterotoxigenic strain; *E. coli* 078:H11 (ATCC # 35401) was used for the *in vivo* antibacterial studies. The cells were grown in nutrient broth (DIFCO) and cells in the exponential growth were harvested and resuspended in phosphate buffered saline (pH 7.4) at a concentration of 1×10^8 cells/ml. ICR female mice (8–10 wks—Harlan Sprague-Dawley) were injected intraperitoneally with either recombinant apolactoferrin (1 mg or 200 µg) or bovine serum albumin (1 mg) in 0.2 ml pyrogen-free, phosphate buffered saline (pH 7.4). After a period of 10 hours, the mice were injected, intraperitoneally, with *E. coli* 078:H11 (2×10^8 cells).

In vitro antimicrobial action of recombinant human lactoferrin. The antimicrobial activity of recombinant lactoferrin against 72 clinical isolates of bacteria was determined using an *in vitro* microtitre broth dilution assay. A standard inoculum of bacteria (1×10^5 /ml) was incubated in the presence of recombinant apo-lactoferrin in 1% bacto-peptone broth (pH 7.5). The samples were incubated at 35°C for 20 hours after which MICs were determined.

Acknowledgments

We gratefully acknowledge Aileen Ward, Chris Houston, and Peter Campbell for excellent technical assistance and Irene A. Harrison and Rebecca Rivera for their help in the preparation of the manuscript. Classical strain improvement was carried out by PANIABS Ferm Tech Division, Taiwan. Screening of clinical isolates of bacteria *in vitro* was carried out by Clinical Microbiology Institute, Inc., Tualatin, OR.

References

- Masson, P. L. and Heremans, J. F. 1971. Lactoferrin in milk from different species. *Comp. Biochem. Physiol.* **39**:119–129.
- Hennart, P. F., Brasseur, D. J., Delogne-Desnoeck, J. B., Dramaix, M. M. and Robyn, C. E. 1991. Lysozyme, lactoferrin and secretory immunoglobulin A content in breast milk: Influence of duration of lactation, nutrition status, prolactin status, and parity of mother. *Am. J. Clin. Nutr.* **53**:32–39.
- Masson, P. L., Heremans, J. F. and Dive, C. 1966. An iron-binding protein common to many external secretions. *Clin. Chim. Acta.* **14**:735–739.
- Pentecost, B. T. and Teng, C. T. 1987. Lactoferrin is the major estrogen inducible protein of mouse uterine secretions. *J. Biol. Chem.* **262**:10134–10139.
- Yu, L.-C. and Chen, Y.-H. 1993. The developmental profile of lactoferrin. *Biochem. J.* **296**:107–111.
- Masson, P. L., Heremans, J. F. and Schonne, E. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* **130**:643–658.
- Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W. and Baker, E. N. 1989. Structure of human lactoferrin: Crystallographic structure analysis and refinement at 2.8 Å resolution. *J. Mol. Biol.* **209**:711–734.
- Anderson, B. F., Baker, H. M., Norris, G. E., Rumball, S. V. and Baker, E. N. 1990. Apolactoferrin structure demonstrates ligand-induced conformational change in transferrins. *Nature* **344**:784–787.
- Sanchez, L., Calvo, M. and Brock, J. H. 1992. Biological role of lactoferrin. *Arch. Dis. Child.* **67**:657–661.
- Iyer, S. and Lonnerdal, B. 1993. Lactoferrin, lactoferrin receptors and iron metabolism. *Eur. J. Clin. Nutr.* **47**:232–241.
- Oram, J. D. and Reiter, B. 1968. Inhibition of bacteria by lactoferrin and other iron-chelating agents. *Biochem. Biophys. Acta.* **170**:351–365.
- Arnold, R. R., Brewer, M. and Gauthier, J. J. 1980. Bactericidal activity of human lactoferrin: Sensitivity of a variety of microorganisms. *Infect. Immun.* **28**:893–898.
- Trumpler, U., Straub, P. W. and Rosenmund, A. 1989. Antibacterial prophylaxis with lactoferrin in neutropenic patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **8**:310–313.
- Zagulski, T., Lipinski, P., Zagulska, A., Broniek, S. and Jarzabek, Z. 1989. Lactoferrin can protect mice against a lethal dose of *Escherichia coli* in experimental infection *in vivo*. *Br. J. Exp. Pathol.* **70**:697–704.
- Bishop, J. A., Schanbacher, F. L., Ferguson, L. C. and Smith, K. L. 1976. *In vitro* growth inhibition of mastitis causing coliform bacteria by bovine apolactoferrin and reversal of inhibition by citrate and high concentrations of apolactoferrin. *Infect. Immun.* **14**:911–918.
- Bullen, J. J., Rogers, H. J. and Griffiths, E. 1978. Role of iron in bacterial infection. *Curr. Top. Microbiol. Immunol.* **80**:1–35.
- Reiter, B. 1978. Review of non-specific and microbial factors in milk. *Ann. Rech. Vet.* **9**:205–224.
- Arnold, R. R., Cole, M. F. and McGhee, J. R. 1977. A bactericidal effect for human lactoferrin. *Science* **197**:263–265.
- Bellamy, W., Takase, M., Yamauchi, K., Wakabayashi, H., Kawase, K. and Tomita, M. 1992. Identification of the bactericidal domain of lactoferrin. *Biochem. Biophys. Acta.* **1121**:130–136.
- Ellison, R. T., Giehl, T. J. and LaForce, F. M. 1988. Damage of the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. *Infect. Immun.* **56**:2774–2781.
- Yamauchi, K., Tomita, M., Giehl, T. J. and Ellison, R. T. 1993. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. *Infect. Immun.* **61**:719–728.
- Mazurier, J., Legrand, D., Hu, W.-L., Montreuil, J. and Spik, G. 1989. Expression of human lactotransferrin receptors in phytohemagglutinin stimulated human peripheral blood lymphocytes: Isolation of the receptors by antigen-affinity chromatography. *Eur. J. Biochem.* **179**:481–487.
- Birgens, H. S., Hansen, N. E., Karle, H. and Kristensen, L. O. 1983. Receptor

- binding of lactoferrin by human monocytes. *Br. J. Haematol.* **54**:383-391.
24. van Snick, J. L. and Masson, P. L. 1976. The binding of human lactoferrin to mouse peritoneal cells. *J. Exp. Med.* **144**:1568-1580.
 25. Leveugle, B., Mazurier, J., Legrand, D., Mazurier, C., Montreuil, J. and Spik, G. 1993. Lactotransferrin binding to its platelet receptor inhibits platelet aggregation. *Eur. J. Biochem.* **213**:1205-1211.
 26. Machnicki, M., Zimecki, M. and Zagulski, T. 1993. Lactoferrin regulates the release of tumour necrosis factor alpha and interleukin 6 *in vivo*. *Int. J. Exp. Path.* **74**:433-439.
 27. Crouch, S. P. M., Slater, K. J. and Fletcher, J. 1992. Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. *Blood* **80**:235-240.
 28. Cox, T. M., Mazurier, J., Spik, G., Montreuil, J. and Peters, T. J. 1979. Iron binding proteins and influx of iron across the duodenal brush border. Evidence for specific lactotransferrin receptors in the human intestine. *Biochem. Biophys. Acta.* **558**:129-141.
 29. Ward, P. P., May, G. S., Headon, D. R. and Conneely, O. M. 1992. An inducible expression system for the production of human lactoferrin in *Aspergillus nidulans*. *Gene* **122**:219-223.
 30. Ward, P. P., Lo, J.-Y., Duke, M., May, G. S., Headon, D. R. and Conneely, O. M. 1992. Production of biologically active recombinant human lactoferrin in *Aspergillus oryzae*. *Bio/Technology* **10**:784-789.
 31. Jeenes, D. J., Mackenzie, D. A., Roberts, I. N. and Archer, D. B. 1991. Heterologous protein production by filamentous fungi. *Biotech. Gen. Eng. Rev.* **9**:327-367.
 32. Dunn-Coleman, N. S., Bloebaum, P., Berka, R. M., Bodie, E., Robinson, N., Armstrong, G., Ward, M., Przetak, M., Carter, G. L., LaCost, R., Wilson, L. J., Kodama, K. H., Bower, B., Lamsa, M. and Heinsohn, H. 1991. Commercial levels of chymosin production by *Aspergillus*. *Bio/Technology* **9**:976-981.
 33. Ward, M., Wilson, L. J., Kodama, K. H., Rey, M. W. and Berka, R. M. 1990. Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. *Bio/Technology* **8**:435-440.
 34. Jeenes, D. J., Mackenzie, D. A. and Archer, D. B. 1993. A truncated glucoamylase gene fusion for heterologous protein secretion from *Aspergillus niger*. *FEMS Microbiol. Lett.* **107**:267-272.
 35. Devchand, M., Gwynne, D., Buxton, F. and Davies, R. 1989. An efficient cell-free translation system from *Aspergillus nidulans* and *in vitro* translocation of prepro-a mating factor across *Aspergillus* microsomes. *Curr. Genet.* **14**:561-566.
 36. Vilja, P., Krohn, K. and Tuohimaa, P. 1985. A rapid and sensitive noncompetitive avidin-biotin assay for lactoferrin. *J. Immunol. Meth.* **76**:73-83.
 37. Spik, G., Codderville, B. and Montreuil, J. 1988. Comparative study of the primary structures of sero-, lacto- and ovotransferrin glycans from different species. *Biochem. J.* **70**:1459-1469.
 38. Pinto, M., Robine-Leon, S., Appay, M.-D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffer, K., Fogh, J. and Zweibaum, A. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell.* **47**:323-330.
 39. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* **51**:660-672.
 40. Clark, J. H., Mitchell, W. C. and Guthrie, S. C. 1986. Triphenylethylene antiestrogen binding sites (TABS) specificity. *J. Steroid Biochem.* **26**:433-437.
 41. Metz-Boutigue, M.-H., Jolles, J., Mazurier, J., Schoetger, F., Legrand, D., Spik, G., Montreuil, J. and Jolles, P. 1984. Human lactotransferrin: amino acid sequence and structural comparisons with other transferrins. *Eur. J. Biochem.* **145**:659-676.
 42. Kawakami, H. and Lonnedal, B. 1991. Isolation and function of a receptor for human lactoferrin in human fetal intestinal brush-border membrane. *Am. J. Physiol.* **261**:G841-G846.
 43. Derisbourg, P., Wieruszkeski, J.-M., Montreuil, J. and Spik, G. 1990. Primary structure of glycans isolated from human leucocyte lactotransferrin. Absence of fucose residues questions the proposed mechanism of hyposideraemia. *Biochem. J.* **269**:821-825.
 44. Saunders, G., Picknett, T. M., Tuite, M. F. and Ward, M. 1989. Heterologous gene expression in filamentous fungi. *Trends in Biotechnol.* **7**:283-287.
 45. Contreras, R., Carrez, D., Kinghorn, J. R., van der Hondel, C. A. M. J. J. and Fier, W. 1991. Efficient KEX2-like processing of a glucoamylase-interleukin-6 fusion protein by *Aspergillus nidulans* and secretion of mature interleukin 6. *Bio/Technology* **9**:378-381.
 46. Nunberg, J. H., Meade, J. H., Cole, G., Lawyer, F. C., McCabe, P., Schweickart, V., Tal, R., Wittman, V. P., Flatgaard, J. E. and Innis, M. A. 1984. Molecular cloning and characterization of the glucoamylase gene of *Aspergillus awamori*. *Mol. Cell. Biol.* **4**:2306-2315.
 47. Boel, E., Hansen, M. T., Hjort, I., Hoegh, I. and Fiil, N. P. 1984. Two different types of intervening sequences in the glucoamylase gene from *Aspergillus niger*. *EMBO J.* **3**:1581-1585.
 48. Gatignol, A. 1987. Phleomycin resistance encoded by the *ble* gene. *Mol. Gen. Genet.* **207**:342-348.
 49. Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabicky-Zissima, J. H., Lockington, R. A. and Davis, R. W. 1983. Transformation by integration in *Aspergillus nidulans*. *Gene* **26**:205-221.
 50. Carboral, J. M., Diez, B., Barredo, J. L., Alvarez, E. and Martin, J. F. 1987. High frequency transformation of penicillium chrysogenum. *Bio/Technology* **5**:494-497.
 51. Masson, P. L. and Heremans, J. F. 1968. Metal-combining properties of human lactoferrin (Red Milk Protein). I. The involvement of bicarbonate in the reaction. *Eur. J. Biochem.* **6**:579-584.
 52. Stowell, K. M., Rado, T. A., Funk, W. D. and Tweedie, J. W. 1991. Expression of cloned human lactoferrin in baby-hamster kidney cells. *Biochem. J.* **276**:349-355.
 53. Rejman, J. J., Turner, J. D. and Oliver, S. P. 1994. Characterization of lactoferrin binding to the Mac-T bovine mammary epithelial cell line using a biotin-avidin technique. *Int. J. Biochem.* **26**:201-206.



Serving Life Science in Europe

The Solution to QPCR

AMPLISENSOR™

A SIMPLE FOUR STEP PROCEDURE

Eliminates

Gels or beads
Radioactivity
2nd step immunological detection
Cross contamination
Post amplification sampling

Offers

Reproducibility
True reaction kinetics
Cycle by cycle control
Sensitivity to 10 copies

KITS available now for:

HIV, HBV, HCV, CMV
HLA Typing
Genetic Diseases

Most other research applications can adopt
Amplisensor™ either using our universal
system or our comprehensive custom service.

Amplisensor is covered by US patent 5348853.

PCR is covered by patents owned by Hoffmann La Roche.

For further information please call your local
ams office:

ams Biotechnology (Europe) Ltd

Telephone:

Fax:

(UK) +44(0) 1993 706500

+44(0) 1993 706006

(S) +46(0) 8 6300232

+46(0) 8 7569490

(CH) +41(0) 91 505522

+41(0) 91 591785

(D) +49(0) 69 779099

Write in No. 251 on Reader Service Card