Human tear lipocalin (TL; also known as Lcn1) is a secretory protein present in large amounts in fluids that cover epithelial surfaces such as tears and respiratory secretions. It is supposed to act as a physiological scavenger of hydrophobic, potentially harmful molecules, but there is evidence that it also inhibits bacterial growth. In the present study, we reconsidered the possibility that TL might interfere with microbial growth by scavenging of siderophores, as described for human neutrophil gelatinase-associated lipocalin (NGAL). Indeed, our experiments revealed that TL binds to microbial siderophores with high affinities. In contrast to NGAL, which was shown to have some specificity for bacterial catecholate-type siderophores, TL binds to a broad array of siderophores, including bacterial catecholate-type enterobactin and hydroxamate-type desferrioxamine B, and all major classes of fungal siderophores. By adding exogenous TL, bacterial and fungal growth could be inhibited under iron-limiting conditions. Thus, TL might be a novel member of the innate immune system especially involved in mucosal defense against fungal infections.

Several epithelial surfaces are continuously exposed to potential pathogens but rarely become infected because they are protected by specific secretions. These secretions contain a number of antimicrobial components and are constantly cleared by mechanical action. For instance, the human cornea has an elaborate system of defense provided by the tear film, which contains an assortment of proteins that derive antimicrobial activity by a variety of mechanisms. Human tear lysozyme hydrolyzes the glycosidic linkages in the polysaccharide components of the cell wall and kills Micrococcus leisodeikiticus and Bacillus subtilis. Secretory phospholipase A2 hydrolyzes phospholipids found mainly in microbial membranes and is the principal bactericide in tears for staphylococci and other gram-positive bacteria. Lactoferrin induces bacteriostasis by competing for free iron, but it also has bactericidal activity against several bacteria. Secretory immunoglobulin A inhibits the adhesion of specific bacteria and proteozoa to the corneal epithelium. Defensin peptides, which have a wide range of potential antimicrobial activities as a result of their formation of pores in the microbial cell membrane, have also been found in human tear fluid.

There is circumstantial and somewhat controversial evidence for the antibacterial activity of another protein found in high concentrations in tear fluid and other secretions, namely, tear lipocalin (TL), also called von Ebner's gland protein or Lcn1. TL is a member of the protein superfamily of lipocalins and is typically characterized by its ability to bind to a large variety of hydrophobic compounds, including fatty acids, phospholipids, glycolipids, cholesterol, retinol, arachidonic acid, and other lipid peroxidation products. By scavenging these molecules, TL may act as a general protection factor for epithelial surfaces.

Several facts prompted us to reconsider the antimicrobial activity of TL. First, there were some indications that its antibacterial activity depends on the ligands bound (10). TL purified by gel filtration showed antibacterial activity, which was not present after purification by reversed-phase high-pressure liquid chromatography (7, 36). Second, two other properties of TL might be relevant for antibacterial activity: cysteine proteinase inhibition and nonspecific magnesium-dependentendonuclease activity (38, 42). Third, very recently it was found that human neutrophil gelatinase-associated lipocalin (NGAL), another member of the lipocalin superfamily, avidly binds to the bacterial siderophore enterobactin and inhibits bacterial growth by interference with siderophore-mediated iron acquisition by members of the family Enterobacteriaceae (12).

Here we show that TL exhibits its antimicrobial activity by binding to microbial siderophores similarly to NGAL. However, in contrast to NGAL, TL was found to bind to a broad spectrum of siderophores, including all major classes of fungal siderophores. Growth inhibition tests showed that this siderophore binding results in a strong reduction of bacterial and fungal growth under iron-limiting conditions.

**MATERIALS AND METHODS**

**Chemicals, siderophores, strains, and media.** The fluorescent fatty acid derivative 11-[[5-dimethylaminol]-1-naphthalenyl][naphthyl]amino) undecanoic acid (DAUDA) was purchased from Molecular Probes (Leiden, The Netherlands). The recombinant TL used for the binding studies was produced in Escherichia coli as described previously (15) and was delipidated by chromatography on Lipidex 1000 (Sigma, Vienna, Austria) (11). The siderophores enterobactin, pyoverdine, aerobactin, and coprogen were purchased from Biophore Research Products (Tübingen, Germany). Ferricrocin and triacetylfusarinine C (TAFc) were purified from Aspergillus nidulans by high-pressure liquid chromatography by standard methods (38). Desferrioxamine B, thiomorulic acid, and ferrichrome were obtained from Sigma.

The following organisms were used for microbial growth inhibition tests: E.
coli (XL1-Blue), *Pseudomonas aeruginosa* (ATCC 9027), and *A. nidulans* ΔsidA (29). All bacteria were cultivated in M9 minimal medium (which contained the following per liter: 200 ml of 5 M9 minimal medium [64 g of Na₂HPO₄ · 7H₂O, 15 g of KH₂PO₄, 2.5 g of NaCl, 5 g of NH₄Cl], 2 ml of 1 M MgSO₄, 20 ml of 20% glucose, and 0.1 ml of 1 M CaCl₂) at 37°C. The fungi were cultivated as described by Pontecorvo et al. (30) at 30°C in minimal medium (MM) containing 1% glucose as the carbon source and 20 mM glutamine as the nitrogen source.

**Microbial growth inhibition tests.** To test the bacteriostatic activity of TL, 500 µl of M9 minimal medium in 48-well dishes (Nunc, Wiesbaden, Germany) was inoculated with a 1% overnight culture of *E. coli* or *P. aeruginosa*. TL was added at 5 µM, and the optical density at 600 nm (OD₆₀₀) was measured after 10, 25, and 36 h. Assays for antifungal activity were performed on MM agar in six-well plates (Techno Plastic Products, Trasadingen, Switzerland) containing 50 nM TAFC or 50 nM TAFC that had been preincubated with 500 nM TL in potassium phosphate (pH 7.2) at 37°C. The fungi were cultivated as described by Pontecorvo et al. (30) at 30°C in minimal medium (MM) containing 1% glucose as the carbon source and 20 mM glutamine as the nitrogen source.

**FIG. 1.** Schematic structures of bacterial and fungal siderophores. Italicized siderophores are the desferri forms.
cence of TL was excited at 295 nm, and emission was recorded at between 330 and 350 nm. For each measurement 250 μl of protein solution (5 μM) in 20 mM potassium phosphate (pH 7.2) was used, and the solution was mixed with 1 μl of a diluted ligand. The set of datum points was analyzed by standard mathematical methods, and a curve was fitted by nonlinear hyperbolic regression. **Endpoint saturation.** M9 minimal medium (500 μl) was inoculated with a 1% overnight *E. coli* culture containing 5 μM TL. Iron (FeCl₃) was added in increasing concentrations: 0.1, 1, 5, and 50 μM. The OD₆₀₀ was measured after incubation for 16 h at 37°C.

**Atomic absorption measurements.** All atomic absorption measurements were performed with a Unicam 939 QZ atomic absorption spectrophotometer. Before the iron content of the probe was measured, a solution containing 2.4 nM TAFC, 0.8 nM Fe (FeCl₃), and 2.4 nM TL was dialyzed in a Slide-A-Lyzer dialysis cassette (molecular weight cutoff, 3,500) obtained from Pierce (Rockford, Ill.) to remove the free iron in the buffer. Probes containing 2.4 nM TL in buffer and 2.4 nM Fe (FeCl₃), and 2.4 nM TAFC was dialyzed in a Slide-A-Lyzer dialysis cassette (molecular weight cutoff, 3,500) obtained from Pierce (Rockford, Ill.) to remove the free iron in the buffer. Probes containing 2.4 nM TL in buffer and 2.4 nM TAFC in buffer (20 mM potassium phosphate buffer [pH 7.2]) were used as a control.

**RESULTS**

**Binding of bacterial siderophores to TL.** In general, the binding of siderophores was measured by competitive DAUDA displacement assays. DAUDA, a fluorescent fatty acid analogue, exhibits little fluorescence in buffer but shows enhanced fluorescence and a blue shift when it is bound to TL. It is known that DAUDA binds to TL entirely within the hydrophobic pocket, at a single binding site, with a calculated dissociation constant (Kₐ) of 2.8 μM (10). When DAUDA is displaced from TL, there is a corresponding decrease in intensity and a redshift of the maximum peak.

Most bacterial siderophores are divided into two groups on the basis of their chemical compositions: hydroxamates and catecholates (27). Therefore, we tested enterobactin (26), the major catecholate-type siderophore of *E. coli*, and desferrioxamine B (13), a hydroxamate-type siderophore of *Streptomyces* (Fig. 1). In addition, aerobactin (9), another hydroxamate-type siderophore of enterobacteria, and pyoverdine (31), the hydroxamate-type siderophore of *P. aeruginosa*, were included in this study. Pyoverdins differ from other hydroxamates by the additional attachment of a conserved dihydroxyquinoline chromophore (31).

The displacement curves for various concentrations of ferric enterobactin, ferric aerobactin, and ferrioxamine B (iron-saturated desferrioxamine B) are shown in Fig. 2, top panel. All three siderophores were found to bind to TL. Ferric enterobactin and desferrioxamine B showed similar affinities, which were higher than that of ferric aerobactin. Pyoverdine could not be investigated by this assay due to the intrinsic fluorescence of the attached chromophore. Therefore, we investigated the binding of pyoverdine by obtaining atomic absorption measurements with Fe-saturated pyoverdine. Whereas the binding of Fe-saturated enterobactin could be easily detected by this method, no binding of pyoverdine was found (data not shown). Pyoverdine does not bind to TL due to the additional bulky chromophore. In addition, no significant binding of free iron to TL could be detected by atomic absorption.

**Binding of fungal siderophores to TL.** All of the most relevant fungal siderophores belong to the group of hydroxamates (Fig. 1). Therefore, rhodotorulic acid from *Rhodotorula pilimanae* (24), TAFC from *A. nidulans* (4, 29), coprogen from *Neurospora crassa* (37), and two ferrichromes, namely, ferrichrome from *Ustilago maydis* and ferriocrocin from *A. nidulans* (4, 21, 29), were used in our binding studies. From the DAUDA displacement curves in Fig. 2 (bottom panel), it is evident that all fungal siderophores tested bind to TL. Rhodotorulic acid showed the highest affinity to TL; TAFC, coprogen, and ferrichrome showed slightly lower affinities; and ferriocrocin showed the lowest affinity.

Since it is known that fatty acids are physiological ligands of TL, we compared the Kₐ of TAFC, which shows an affinity to TL in the medium range, to the Kₛ of fatty acids. By analysis with internal tryptophan fluorescence quenching, a Kₛ of 0.5 ± 0.1 μM was obtained for TAFC (Fig. 3). One of the most strongly TL-binding fatty acids is stearic acid; however, its affinity to TL was not measured directly but was calculated from relative displacement assays (10). Therefore, we performed a direct comparison of TAFC and stearic acid by displacement analysis in the same series of experiments. The results of our experiments demonstrate that TAFC has only a slightly lower affinity than the strongly binding ligand stearic acid (Fig. 4).

We found no significant difference in the binding of TL to iron-depleted or iron-saturated siderophores either for desfer-
rioxamine B or for TAFC. Together with the results from atomic absorption analysis that no free iron is bound by TL, it is evident that iron is bound to TL only in a complex with a siderophore and that siderophore binding to TL is independent of iron.

**Inhibition of bacterial growth by TL.** In order to test the interference of TL with siderophore-mediated iron uptake by microorganisms, we first explored its effect on the growth of *E. coli* in M9 minimal medium under iron-limiting conditions. The M9 minimal medium was not supplemented with iron, but trace amounts present in glassware and medium were sufficient for the bacteria to grow to saturation. However, after the addition of exogenous TL at a concentration of 5 µM, drastic inhibition of growth was observed (Fig. 5). This growth inhibition could be reversed by adding free iron, as indicated from endpoint saturation experiments (Fig. 5). In addition, no growth inhibition could be detected in non-iron-limited medium (data not shown). Therefore, our experiments clearly suggest that the bacteriostatic effect of TL is directly related to the inhibition of siderophore-mediated iron sequestration.

The same experiments were performed with *P. aeruginosa*, but no inhibition of growth could be detected (data not shown), thus supporting the results of our binding studies.

**Inhibition of fungal growth by TL.** Investigations concerning the inhibitory effects of TL on fungal growth were performed...
with a mutant strain of *A. nidulans* that contains a deletion of the l-ornithine \( N^2 \)-monooxygenase-encoding gene *sidA* (Δ*sidA*), which completely blocks siderophore biosynthesis. Thus, this mutant strain is unable to grow on minimal medium without supplementation with siderophores (5). *A. nidulans* Δ*sidA* was grown on a medium supplemented with TAFC or a medium containing TAFC preincubated with TL. As shown in Fig. 6, significant inhibition of growth was apparent after 48 and 72 h of growth on the medium containing the TL-preincubated TAFC compared to the growth on the TAFC-supplemented medium. TL does not have any antifungal activity beyond siderophore sequestration, since no growth inhibition was observed when a surplus of TAFC was used (data not shown). It is evident from the data for the 72- and 96-h time points that growth is not completely blocked but is retarded. The growth after prolonged incubation on the medium containing TL-preincubated TAFC was probably due to protease activity, since increased degradation of TL could be detected between 72 and 96 h by Western blot analysis (data not shown).

**DISCUSSION**

In the present study we demonstrated that the antimicrobial activity of TL depends on its ability to bind to microbial siderophores, thereby interfering with the iron acquisition systems of microorganisms. Since iron is an essential nutrient for microorganisms but is strongly bound to host proteins in mammals, microorganisms must compete with these proteins for this essential ion. Microbes have evolved powerful acquisition systems by producing highly specific iron chelators (siderophores) in response to iron deficiency. Thus, inhibition of this iron acquisition process by scavenging microbial siderophores is a powerful mechanism in the defense against microbial infections. Indeed, two human proteins have been identified as powerful mechanisms in the defense against microorganisms. Since iron is an essential nutrient for bacteria, these proteins must be at least as high as the affinity for the other ligands. 16-(9-Anthroyloxy)palmitic acid, a derivative of palmitic acid, has been shown to bind to TAFC, with a \( K_d \) of 0.8 \( \mu \text{M} \) (11). The \( K_d \) of stearic acid, which was described to be one of the ligands that binds to TAFC most strongly in vivo, was not measured directly but was calculated from relative displacement assays, which suggested a \( K_d \) of about 0.3 \( \mu \text{M} \) (10). Our results demonstrated that TL indeed has an affinity for siderophores comparable to that of strongly binding fatty acids. Next, the concentration and affinity of TL must be high enough to compete with microbial siderophore uptake systems. TL is constantly produced by several glands and is present in large amounts in tear fluid, nasal mucus, and tracheal secretions, e.g., tear fluid contains up to 3 mg of TL per ml (7, 22). In addition, its concentration increases during inflammation and/or infection (22, 34). The \( K_d \) values of fungal siderophore transporters were found to range from 0.9 to 2.3 \( \mu \text{M} \), which were for ferrichromes and TAFC, respectively (23, 41). Since we found comparable affinities of TL for these fungal siderophores, TL should be specific enough to efficiently outcompete all of the fungal siderophore receptors. Bacterial siderophore transporters are known to have higher affinities; e.g., enterobactin binds to its receptor with a \( K_d \) in the range of 0.4 to 100 \( \text{nM} \) (28). Therefore, TL might be a more efficient inhibitor of fungal growth than of bacterial growth in vivo.

Recent investigations demonstrated that TL binds to LMR-1, a novel endocytic receptor (39) which targets the TL-ligand complex and places them into specific intracellular vesicles, probably late endosomes or lysosomes (40). Targeting of the TL-siderophore-iron complex to these cellular compartments will be an important mechanism in the clearance process; furthermore, the acidic conditions of these vesicles might lead to ligand removal and degradation of the siderophores.

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