

Effect of chelating agents on the growth, surface polypeptide synthesis and interaction of *Streptococcus agalactiae* with human epithelial cells

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Abstract. In the present study, the influence of the chelating agents of ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (β -aminoethyl ether) (EGTA) and 1,10-phenanthroline (PHEN) on growth rate, cytoadherence ability and surface protein expression was examined in a clinical strain of *Streptococcus agalactiae* isolated from a blood sample. EDTA and EGTA at 10 mM did not inhibit the growth of *S. agalactiae*, while PHEN significantly arrested bacterial proliferation in a concentration range of 10-0.01 mM. The *in vitro* interaction between *S. agalactiae* and A549 cells was a time-dependent process; adherence and bacterial intracellular viability were more pronounced after 3 h of contact. The pre-treatment of bacterial cells with EDTA adversely influenced the adhesive properties of *S. agalactiae* to A549 cells after 2 and 3 h, whereas EGTA only blocked this process after 3 h. Viable intracellular bacteria were just detected after 3 h of interaction, and EDTA and EGTA inhibited intracellular viability in a similar fashion. Conversely, PHEN inhibited neither the adherence nor the intracellular viability of the microorganism. Furthermore, EDTA robustly suppressed surface polypeptide synthesis, suggesting a decline in the possible bacterial ligands responsible for *S. agalactiae* adhesive properties.

Introduction

Streptococcus agalactiae, known as group B *Streptococcus* (GBS), is the major cause of bacterial sepsis, pneumonia and

meningitis in neonates and has emerged as an increasingly common cause of invasive disease in immunocompromized individuals (1). About 50% of infants born to infected women became colonized with *S. agalactiae* during delivery and 1% of the colonized infants develop severe GBS infection. The main route of neonatal infection is the ascending spread of *S. agalactiae* into the amniotic fluid followed by the aspiration of contaminated amniotic fluid by the fetus. The bacteria can then colonize and infect the lungs, resulting in pneumonia. Subsequent transmigration of *S. agalactiae* across the epithelial border allows the bacteria to invade the bloodstream and eventually reach the meninges (1). The adherence of *S. agalactiae* to the epithelial cells of the mother (intestinal and vaginal) and infant (lung) may be essential for virulence (2,3).

Metal-chelating agents are able to selectively disturb the essential metal metabolism of cells, interfering with metal acquisition and bioavailability for crucial reactions. Chelating agents are also inhibitors of several bacterial adhesins (4,5); for instance, ethylenediaminetetraacetic acid (EDTA) is capable of inhibiting adhesion molecules of divergent specificities from members of the genera *Actinomyces*, *Capnocytophaga*, *Fusobacterium*, *Porphyromonas* and *Prevotella*. Some of the adhesins are lectins capable of binding carbohydrates, whereas others are specific for proteins and/or hydrophobins (6). The metal chelator EDTA is known to have activity against biofilms of gram-positive bacteria such as *Staphylococcus aureus*. EDTA can also kill planktonic cells of *Pseudomonas aeruginosa* and causes a rapid dispersion of microorganisms, suggesting that magnesium, calcium and iron are involved in *P. aeruginosa* biofilm maintenance (7). In addition, these chelating compounds may inhibit the hydrolytic activity of different types of metal-dependent enzymes, such as metallo-type proteases. We recently demonstrated that clinical strains of *S. agalactiae* secrete metalloproteases into the extracellular environment. These *S. agalactiae* metalloproteases were able to cleave several key host proteins, such as fibronectin, laminin, type IV collagen, fibrinogen and albumin, suggesting a possible participation during bacterial tissue dissemination. Chelating agents robustly inhibited the metalloprotease activities released

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by *S. agalactiae*, resulting in the blockage of the hydrolysis of these proteinaceous substrates (8).

In the present study, we aimed to investigate the effect of chelating agents such as EDTA, ethylene glycol-bis (β -aminoethyl ether) (EGTA) and 1,10-phenanthroline (PHEN) on the growth rate of a clinical sample (designated 90186 strain) belonging to the serotype V of *S. agalactiae*, which was isolated from blood of an adult patient. Interaction assays were performed to examine the influence of the metal-chelating compounds on interaction with lung epithelial cells, and the effect of the chelating agents on surface protein expression was also analyzed.

Materials and methods

Chemicals. Brain heart infusion medium (BHI) was obtained from Difco Laboratories (Detroit, MI, USA). Blood agar base (BAB) was purchased from Oxoid (Basingstoke, Hampshire, England). The chelating agents PHEN, EDTA and EGTA, M199 medium, collagenase IV, all antibiotics and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Gaithersburg, MD, USA). The other reagents were of analytical grade.

Bacterial strain and growth conditions. The *S. agalactiae* 90186 strain (serotype V), partially investigated for its adhesive properties (9), was used throughout this study. The strain was obtained from the Culture Collection of the Laboratório de Cocos Patogênicos (Departamento de Microbiologia Médica, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Brazil), identified as *S. agalactiae* and serotyped as previously described (10). Microorganisms were stored after lyophilization and recovered in BHI broth. The *S. agalactiae* strain was grown in Erlenmeyer flasks containing 200 ml of M199 medium for 16-18 h at 37°C.

Effect of chelating agents on *S. agalactiae* growth. Experiments were performed in 10x100-mm glass tubes containing 1 ml of M199 medium. The inoculum consisted of 10% of a 6 h culture of *S. agalactiae* (isolated 90186-blood) containing $\sim 2.0 \times 10^8$ cells. EDTA and EGTA were dissolved in water at 100 mM, while PHEN was dissolved in DMSO at 100 mM. All drugs were filter-sterilized. The bacteria were incubated at 37°C for 1 h in the absence or presence of the chelating agents at different concentrations (10, 1.0, 0.1, 0.01 and 0.001 mM). Cell growth was estimated by plating and counting the resulting colonies, which manifested in BAB plates containing 5% sheep-desfibrinated blood. Untreated and DMSO-treated *S. agalactiae* cultures were followed concomitantly as controls (8).

Epithelial cell culture. A549 is a human type II alveolar epithelial-like lineage derived from the lung carcinomatous tissue of human patients, and is widely used as a model for respiratory pathogen infection. The cultures were maintained and grown to confluence at 37°C in 25-cm² culture flasks containing DMEM supplemented with 10% FCS in a 5% CO₂ atmosphere.

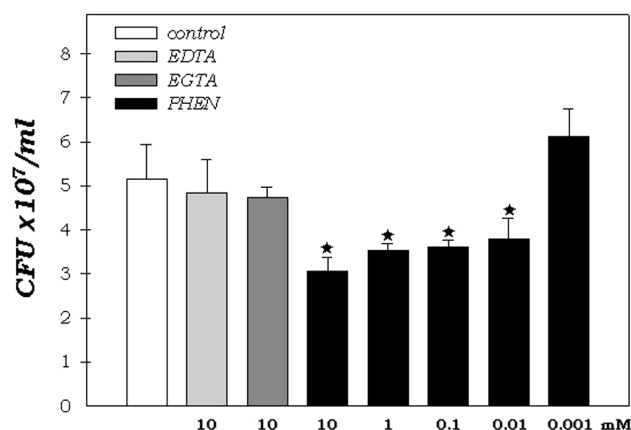


Figure 1. Effect of chelating agents on the growth of *S. agalactiae* (90186-blood strain). After growth in M199 medium, bacterial cells were pre-treated for 1 h in the absence (control) or presence of EDTA (10 mM), EGTA (10 mM) or PHEN (10, 1.0, 0.1, 0.01 and 0.001 mM). Cells were harvested, washed with PBS and inoculated in fresh solid medium to measure the colony-forming units. Values represent the means \pm standard error of three independent experiments, performed in triplicate. GBS cells treated with chelating agents that had a growth rate significantly different from the controls are shown (* $P < 0.05$, Student's t-test).

Bacterial binding and intracellular viability assays. Confluent cultures of A549 cells were allowed to interact with *S. agalactiae* [5×10^7 colony-forming units (CFU)] treated or untreated for 1 h with 10 mM EDTA, 10 mM EGTA or 0.001 mM PHEN. For the bacterial binding assays, infected monolayers were rinsed three times with M199 then lysed in a 0.5-ml solution of 25 mM Tris, 5 mM EDTA, 150 mM NaCl and 1% Igepal. The viability of total bacteria (intracellular plus surface adherent) was estimated by plating epithelial lysates and counting the resulting colonies emerging in BAB plates containing 5% sheep-desfibrinated blood. To measure bacterial internalization, the infected monolayers were incubated for 1 h, rinsed three times with M199 medium and incubated for an additional 2-h period in M199 containing bactericidal amounts of gentamicin (100 μ g/ml) and penicillin G (5 μ g/ml). The number of internalized bacteria was determined as outlined above. Adherence rates were determined as: [CFU of total cell-associated (intracellular viable plus surface adherent) GBS-CFU intracellular GBS] (9).

Biotinylated *S. agalactiae* extract preparation. Biotinylated bacterial extracts were prepared using a commercial kit (Amersham-Pharmacia, UK). Briefly, *S. agalactiae* treated or untreated for 1 h with 10 mM EDTA, 10 mM EGTA or 0.001 mM PHEN was washed twice with cold PBS and incubated with 40 mM sodium bicarbonate buffer pH 8.6 (250 μ l/well) containing 150 mM NaCl and 2 μ l of biotin reagent, according to the supplier's instructions. Cells were incubated for 30 min at 4°C under gentle agitation. After a washing step with PBS, cellular integrity was monitored by optical microscopy observation. Cell extracts were obtained by adding 200 μ l/well of lysis buffer (25 mM Tris-HCl pH 7.5 containing 250 mM NaCl, 5 mM EDTA, 1% Igepal CA-630, 1 mM phenylmethylsulfonyl fluoride and 40 μ M leupeptin) for 30 min at 4°C. Following the centrifugation of cell extracts at 6000 g for 15 min at 4°C, the supernatants were subjected to electrophoresis in 10% polyacrylamide separating gel (SDS-

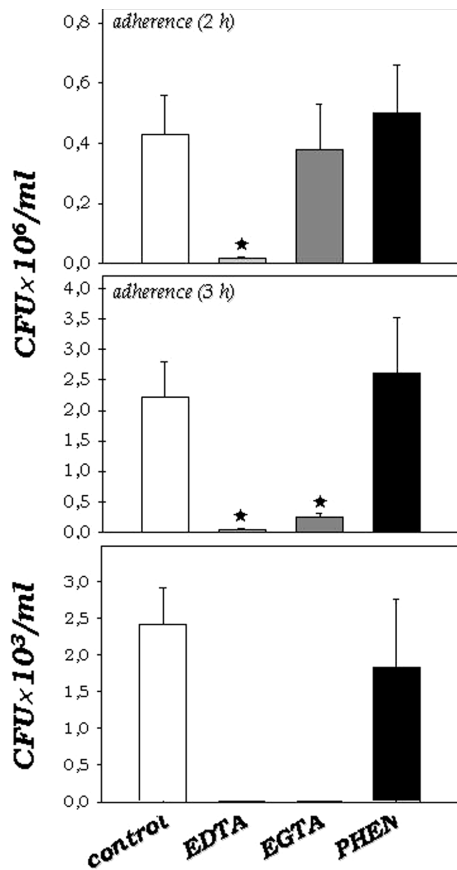


Figure 2. Interaction of *S. agalactiae* (90186-blood strain) with A549 epithelial cell lineage. Confluent cultures were allowed to interact with *S. agalactiae* cells pre- or untreated for 1 h with 10 mM EDTA, 10 mM EGTA or 0.001 mM PHEN. Adhered bacteria and intracellular viability were calculated as described in Materials and methods. Each value is the mean \pm SEM of four samples. Systems treated with chelating agents that had an interaction rate significantly different from the controls are shown (* $P < 0.05$, Student's t-test).

PAGE) under non-reducing conditions (11). Fractionated samples were transferred to Immobilon-P membranes (Amicon) using the Bio-Rad mini-gel electrophoresis system. Non-specific sites of the membranes were blocked with 3% BSA in PBS overnight at 4°C. The membranes were incubated with horse-radish peroxidase-conjugated streptavidin (1:1500). Bound complexes were detected using the enhanced chemiluminescence system (ECL, Amersham-Pharmacia, UK).

Statistical analysis. The experiments were repeated at least three times, and conditions were performed in triplicate. Representative images of these experiments are shown. Statistical analysis of the data was performed using the Student's t-test, version EPI-INFO 6.04 (database and statistics program for public health) computer software. $P \leq 0.05$ was considered statistically significant.

Results and Discussion

Chelating agents have a broad variety of reactions in biological systems. EDTA has traditionally been considered a potentiator of antimicrobial activity against gram-negative bacteria (7). However, very little interactive effect was observed between chelating agents against gram-positive bacteria. Our results show that EDTA and EGTA at 10 mM did not inhibit

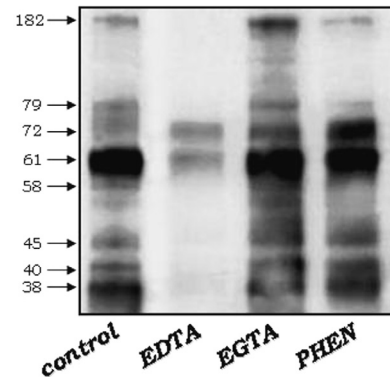


Figure 3. Modulation of *S. agalactiae* (90186-blood strain) surface-biotinylated proteins by chelating agents. *S. agalactiae* cells pre- or untreated for 1 h with 10 mM EDTA, 10 mM EGTA and 0.001 mM PHEN were biotinylated using a commercial kit (Amersham-Pharmacia, UK). Bacterial surface-bound proteins were extracted using lysis buffer and resolved in non-reducing conditions on a 10% polyacrylamide separating gel. After being transferred to PVDF membranes, proteins were probed with a streptavidin-peroxidase conjugate and revealed using a chemiluminescence reagent. Notably, bacterial cells treated with EDTA showed a drastic inhibition in surface protein expression as compared to the control cells. The numbers represent the molecular masses of the standard proteins, expressed in kDa.

S. agalactiae proliferation *in vitro*. However, PHEN at this concentration promoted a significant decrease of ~42% in bacterial growth (Fig. 1), and effectively inhibited *S. agalactiae* growth at up to 0.01 mM (Fig. 1). The mechanism by which this specific chelating agent acts is not fully understood, but is generally attributed to its ability as a chelator. Thus, essential divalent cations, such as magnesium, calcium, iron and particularly zinc ions, remain unavailable in the presence of PHEN, inhibiting *S. agalactiae* development and maintenance. Recently, Reidmiller *et al* (12) described the antimicrobial properties of the chelating agent EDTA on streptococcal bovine mastitis isolates. However, this inhibitory phenomenon was observed only when EDTA was used at higher concentrations of 30-100 mM.

The adherence of *S. agalactiae* to epithelial cells is required for the colonization of the rectal and vaginal tracts of the mother and for persistence in the lungs of the neonate. We demonstrated that *S. agalactiae* cells adhered to and were ingested by A549 cells. The interaction process between *S. agalactiae* and A549 cells was a time-dependent phenomenon in which adherence and bacterial intracellular viability were more pronounced after 3 h of bacteria-mammalian cell contact (Fig. 2). The pre-treatment of bacterial cells for 1 h with EDTA at 10 mM negatively influenced the adhesive properties of *S. agalactiae* to A549 cells after 2 and 3 h, whereas EGTA (10 mM) only blocked this process after 3 h. Viable intracellular bacteria were just detected after 3 h of interaction, and EDTA and EGTA robustly inhibited intracellular viability in a similar fashion. In contrast, PHEN at 0.001 mM inhibited neither the adherence nor the intracellular viability of *S. agalactiae* with A549 cells (Fig. 2). The finding that metal-binding agents such as EDTA and EGTA inhibit the adherence and invasion of *S. agalactiae* cells suggests that these chelators bind to essential ion(s) for their adhesive properties. In this context, EDTA and EGTA are able to form a powerful complex with Ca^{2+} ; this multi-functional divalent cation has been implicated in a large number of biological

processes in eukaryotic and prokaryotic cells (13,14). In bacteria, Ca²⁺ is implicated in processes as important and diverse as cell cycle and division, competence, pathogenesis, motility, and chemotaxis and quorum sensing (4).

S. agalactiae express specific surface proteins which mediate interactions with the components of the host extracellular matrix and host surface components, triggering the adherence, colonization and/or invasion of host cells and tissues as well as host defence evasion. The availability of the whole genome sequence of the human pathogen *S. agalactiae* has provided insights into the repertoire of the cell surface proteins present in this bacterial species. Thirty-five cell wall-associated proteins that may play a role in the adhesion, invasion, inhibition of phagocytosis and evasion of host immune defence mechanisms have been annotated (15,16). As is well known, chelating agents repress the synthesis of surface receptor in several biological systems (13,17), disturbing the physical contact between microorganisms and host cells. Our results show that EDTA vigorously suppressed the synthesis of several surface polypeptides in comparison with the control cells (Fig. 3). Of note, pre-treatment with EGTA and PHEN stimulated the expression of certain surface polypeptides, for instance the 182, 79 and 72 kDa molecules observed in EGTA-treated cells and the 72 kDa polypeptide in PHEN-treated ones (Fig. 3). Chelating agents are clearly inhibitors of several bacterial surface polypeptides that act as adhesins. In this sense, the Fim H adhesin of *Escherichia coli* was sensitive to EDTA (5). Lü-Lü *et al.* (17) noted that the glucan-binding lectin of *Streptococcus sobrinus* is also inhibited by chelating agents. EDTA powerfully repressed the expression of several surface polypeptides in *S. agalactiae*. These results may partially explain the drastic inability of *S. agalactiae* cells to bind to A549 cells *in vitro*. Conversely, EGTA- and PHEN-treated *S. agalactiae* cells displayed analogous surface polypeptide profiles as compared to non-treated cells. Nevertheless, EGTA was capable of inhibiting the adhesion and intracellular viability of *S. agalactiae* after 3 h of interaction with A549 cells. Thus, EGTA probably operates by inhibiting the interaction process using a dissimilar mechanism from that of EDTA. In a previous report by our research group, we observed that EGTA fully inhibited hydrolytic surface activities and released metallo-type proteases produced by the *S. agalactiae* 90186 strain (8). Proteases play several roles during different phases of bacteria-host cell interplay, including the cleavage of host surface receptors, and/or act as an adhesive molecule (18). Based on these findings, we cannot discount the hypothesis that EGTA inhibits *S. agalactiae*-A549 interaction by directly acting on the metalloproteases produced by this bacterial pathogen.

In summary, our results show that *S. agalactiae* efficiently adhere to human epithelial lung cells *in vitro*. We demonstrated that *S. agalactiae* is able to invade A549 cells, possibly remaining viable for several hours inside the endocytic vacuoles without causing any significant damage to the host cells (data not shown). Knowledge of the specific receptors responsible for the interaction between epithelial cells and *S. agalactiae* and the modulation of host tissues by metal-chelating agents may be essential to understanding the hematogenous routes of infection employed by this opportunistic pathogen.

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