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EFFECT OF NYSTATIN ON INVASION OF *SERRATIA GRIMESII* AND *SERRATIA PROTEAMACULANS* BACTERIA INTO EPITHELIAL CELLS

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BACKGROUND: Bacteria use various endocytic pathways during entering non-phagocytic cells. The involvement of caveolae/lipid rafts in bacterial invasion has been demonstrated for many bacterial pathogens. However, for bacteria of the genus *Serratia*, the involvement of membrane microdomains in the process of bacterial internalization has been poorly studied.

AIM: To evaluate the involvement of caveolae/lipid rafts in the invasion of *S. grimesii* and *S. proteamaculans* bacteria into non-phagocytic epithelial Caco-2 and M-HeLa cells using nystatin.

MATERIALS AND METHODS: M-HeLa and Caco-2 epithelial cells were incubated with 50 μM nystatin for 1 hour at 37 ºC, after which they were infected with the bacteria *S. grimesii* strain 30063 and *S. proteamaculans* strain 94, the multiplicity of infection was 100 bacteria per cell. The number of intracellular bacteria was assessed using gentamicin protection assay. The level of caveolin-1 in cells was visualized using confocal microscopy and Western blotting. The expression of Toll-like receptors genes were measured by real-time RT-PCR.

RESULTS: Treatment of epithelial cells with nystatin reduces the internalization of *S. grimesii* and *S. proteamaculans* into M-HeLa cells by 30% and does not affect penetration into Caco-2 cells. At the same time, nystatin does not affect the redistribution / the integrity impairment of lipid rafts and does not lead to the cytoskeleton reorganization of eukaryotic cells. The addition of nystatin increases the level of caveolin-1 in M-HeLa cells (caveolin-1 is not expressed in Caco-2), which leads to a change plasma membrane fluidity. Nystatin promotes the secretion of proinflammatory cytokines interleukin-6 and interleukin-8 in both cell lines. Infection of M-HeLa cells pretreated with nystatin with the studied bacteria leads to an increase in the expression of *tlr2* and *tlr4* genes, but does not exceed the level of their expression in control samples. Therefore, it is impossible to speak unambiguously about the participation of Toll-like receptors in the invasion of *Serratia* bacteria.

CONCLUSIONS: The results obtained suggest that the interaction of bacteria with eukaryotic cells induces the expression of caveolin-1, which leads to a change plasma membrane components mobility. This may be due to the fact that β1-integrin is involved in the invasion of the studied bacteria, which should be stabilized at the plasma membrane upon binding of the ligand due to the formation of a cholesterol- and sphingolipid-rich membrane microenvironment.

Keywords: bacterial invasion; *Serratia*; nystatin; plasma membrane; TLR.

ВЛИЯНИЕ НИСТАТИНА НА ИНВАЗИЮ БАКТЕРИЙ *SERRATIA GRIMESII* И *SERRATIA PROTEAMACULANS* В ЭПИТЕЛИАЛЬНЫЕ КЛЕТКИ

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Обоснование. При проникновении в нефагоцитирующие клетки бактерии задействуют различные эндоцитарные пути. Для многих бактериальных патогенов было показано участие кавеол/липидных рафтов в процессе бактериальной инвазии. Однако для бактерий рода *Serratia* мало изучено вовлечение мембранных микродоменов в процесс интернализации бактерий.

List of abbreviations

IL — interleukin; CFU — colony-forming unit; LPS — lipopolysaccharide; CTSB — cholera toxin subunit B; RT-PCR — reverse transcription-polymerase chain reaction; cAMP — cyclic adenosine monophosphate; DAPI — 4,6-diamino-2-phenylindole; DMEM — Dulbecco's Modified Eagle's Medium; DPBS — Dulbecco's phosphate-saline solution; LB — Luria–Bertani medium; MOI — multiplicity of infection; PBS — phosphate-buffered saline; TLR — toll-like receptor

Цель — установить участие кавеол/липидных рафтов в инвазии бактерий *S. grimesii* и *S. proteamaculans* в нефагоцитирующие эпителиоподобные клетки Caco-2 и M-HeLa с помощью нистатина.

Материалы и методы. Эпителиальные клетки M-HeLa и Caco-2 инкубировали с 50 мкмоль/л нистатина в течение 1 ч при 37 °C, после чего заражали бактериями *S. grimesii* штамм 30063 и *S. proteamaculans* штамм 94, множественность заражения составляла 100 бактерий на клетку. Количество внутриклеточных бактерий оценивали с использованием гентамицина. Уровень кавеолина-1 в клетках визуализировали с помощью конфокальной микроскопии и вестерн-блоттинга. Изменение экспрессии генов, кодирующих Toll-подобные рецепторы, измеряли методом обратной транскрипции и полимеразной цепной реакции в режиме реального времени.

Результаты. Обработка эпителиальных клеток нистатином приводит к уменьшению интернализации бактерий *S. grimesii* и *S. proteamaculans* в клетки M-HeLa на 30 % и не влияет на проникновение в клетки Caco-2. При этом нистатин не оказывает влияния на перераспределение/нарушение целостности липидных рафтов, не приводит к реорганизации цитоскелета эукариотических клеток. Добавление нистатина увеличивает уровень кавеолина-1 в клетках M-HeLa (в Caco-2 кавеолин-1 не экспрессируется), что приводит к изменению текучести плазматической мембраны. Нистатин способствует секреции провоспалительных цитокинов интерлейкина-6 и интерлейкина-8 в обеих клеточных линиях. Заражение предварительно обработанных нистатином клеток M-HeLa исследуемыми бактериями приводит к увеличению экспрессии генов *tlr2* и *tlr4*, но не превосходит уровень их экспрессии в контрольных образцах, поэтому нельзя однозначно говорить об участии Toll-подобных рецепторов в инвазии бактерий *Serratia*.

Заключение. Полученные данные позволяют предположить, что взаимодействие бактерий с эукариотическими клетками индуцирует экспрессию кавеолина-1, что приводит к изменению подвижности компонентов плазматической мембраны. Это может быть связано с тем, что в инвазии исследуемых бактерий участвует β1-интегрин, который должен стабилизироваться на плазматической мембране при связывании с лигандом за счет образования мембранного микроокружения, богатого холестерином и сфинголипидами.

Ключевые слова: бактериальная инвазия; *Serratia*; нистатин; плазматическая мембрана; TLR.

Background

Although a wide variety of antimicrobial drugs are now available, the risk of bacterial infection remains high. According to the World Health Organization (report of 01.2022), the figures obtained over the past decade indicate a high prevalence of hospital-acquired infections, many of which are caused by multidrug-resistant microorganisms. Moreover, the pathological process of infection development includes both tissue (intercellular) invasion of microorganisms and penetration into cells that are not professional phagocytes. Microbial penetration into host cells provides them with protection not only from antibacterial drugs but also from the host immune system. After internalization, the microorganisms can remain inactive and persistent, causing recurrent infections and chronic diseases [1].

The ability to penetrate the host epithelial cells is an important virulence factor for several pathogenic bacteria. However, not only pathogenic bacteria but also opportunistic bacteria, such as *Serratia marcescens* (in hospital infections, it mainly affects the urinary tract), can invade [2]. Other members of the genus *Serratia*, namely *S. grimesii* and *S. proteamaculans*, how also shown to be capable of invading epithelial cells [3, 4]. The study of invasion mechanisms is important not only for understanding the fundamental processes of interaction between bacterial and eukaryotic cells but also for assessing the effectiveness of existing antimicrobial drugs and developing new ones.

The plasma membrane of the cell is the first barrier to bacterial penetration into the epithelial cell; microorganisms use it actively for processes of adhesion and invasion into the host cell. Microdomains enriched in sphingolipids and cholesterol (and therefore less fluid than the rest of the membrane), namely caveolae and lipid rafts, are present on the plasma membrane of eukaryotic cells. Caveolae possess an invaginated structure (owing to the presence of the caveolin-1 protein) and lipid rafts, which are flat membrane regions. As a rule, various proteins involved in the transmission of cellular signals can be anchored to these microdomains [5]. Interestingly, bacterial infectious agents can induce the formation of lipid rafts [6, 7]. The involvement of caveolae/lipid rafts in penetration into epithelial cells has been documented in certain bacteria [6, 8, 9]. For instance, in the process of internalization of *Pseudomonas aeruginosa*, the participation of lipid rafts in the activation of signaling pathways leading to invagination of the membrane of epithelial-like nonphagocytic cells has been proven. In addition, the direct role of glycosphingolipids, which are the main components of lipid rafts, in lectin A-induced invasion of *P. aeruginosa* has been reported [10].

Nystatin (a polyene antibiotic that has a high affinity for cell membrane sterols owing to the presence of double bonds) has been shown to inhibit caveolin-dependent endocytosis [11, 12]; however, it does not affect clathrin-dependent endocytosis [13]. Nystatin can bind to cholesterol and extract it from the plasma membranes of eukaryotic cells, which leads to the flattening of caveolae and the destruction of lipid rafts [14].

This study aimed to use nystatin to determine whether caveolae/lipid rafts are involved in the invasion of *S. grimesii* and *S. proteamaculans* into nonphagocytic epithelial-like Caco-2 and M-HeLa cells.

Materials and methods

1. Bacterial strains and culture conditions

S. grimesii strain 30063 was obtained from the German Collection of Microorganisms and Cell Cultures (DSM 30063). *S. proteamaculans* strain 94 was kindly provided by I.V. Demidyuk (Institute of Molecular Genetics, Russian Academy of Sciences). The bacteria were cultured in Luria–Bertani medium (LB, Dia-M, Russia) for 24 ± 2 h with vigorous stirring at 37 °C (*S. grimesii*) or 30 °C (*S. proteamaculans*). To determine the number of colony-forming units (CFU/mL), the optical density of the bacterial suspension was measured spectrophotometrically at 600 nm, followed by dilution and plating on Petri dishes with LB agar according to Miller (Dia-M, Russia). The volume of bacterial suspension corresponding to the multiplicity of infection (MOI) of 100 CFU per cell was centrifuged for 10 min at 13,000 rpm, the supernatant was removed, and the bacterial pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM, Biolot, Russia).

2. Cell culture and cultivation conditions

The cell lines M-HeLa (human cervical epithelioid carcinoma) and Caco-2 (human colon adenocarcinoma) were obtained from the Russian Collection of Cell Cultures (Institute of Cytology, Russian Academy of Sciences). The cells were cultured in DMEM in the presence of 10% fetal bovine serum (Gibco, Australia) without the addition of antibiotics at 37 °C and 5% CO_2. For the experiments, the cells were trypsinized (Trypsin–Versene solution, Biolot, Russia), plated in 24-well plates at 100–200 thousand cells per well, and incubated for 24–48 h to 70%–95% confluency. The cells were countered in a Goryaev chamber using trypan blue (Biolot, Russia).

Before incubation with nystatin (suspension in Dulbecco's phosphate-buffered saline [DPBS], Sigma-Aldrich, USA), epithelial cells washed twice with phosphate-buffered saline (PBS) pH 7.4 (Biolot, Russia) and then with 50 μM (205 U) nystatin in DMEM medium (without serum or antibiotics) or a similar amount of Dulbecco's solution (DPBS, Biolot, Russia) were added and incubated for 1 h at 37 °C and 5% CO_2. Subsequently, the cells washed twice with PBS and 500 μL of DMEM (without serum and antibiotics) or 0.05% Tween20 (Sigma-Aldrich, USA) in DMEM medium (without serum and antibiotics) were added. Bacteria were then added, as described in the quantitative invasion method.

3. Method for quantitative assessment of invasion

To quantitatively assess invasion, 100 μL of bacterial suspension in DMEM medium was added to each well with eukaryotic cells in accordance with an MOI of 100 CFU per cell. The plate was centrifuged for 5 min at 2000 rpm and then incubated for 2 h at 37 °C and 5% CO_2. The cells were subsequently washed thrice with PBS, trypsinized, and an equal volume of DMEM medium containing 100 μg/mL gentamicin (Biolot, Russia) was added and incubated for 1.0–1.5 h to eliminate noninvasive extracellular bacteria. The cells were then lysed by adding 4.5% sodium deoxycholate solution (Applichem, Germany) to the cell suspension in a 1:2 ratios. Then, 10-fold dilutions of lysate aliquots were prepared in LB medium and plated on LB agar. The plates with the inoculations were incubated for 48 h at room temperature, after which the grown colonies were counted. The obtained CFU value (considering the dilutions) corresponded to the number of invading bacteria.

All experiments were performed at least four times, with three replicates per experiment. The values obtained in the control samples were taken as 100%. Statistical processing was performed using SigmaPlot 12.0 (USA) and Microsoft Excel 2010 (USA) programs. The data were presented as $M \pm SE$ (mean values and standard deviations). To assess differences, Student's t-test was used; differences were considered significant at $p < 0.05$.

4. Confocal microscope

Caco-2 and M-HeLa cells grown on glass slides were incubated with nystatin for 1 h at 37 °C and 5% CO_2. The cells on the slides were then washed with PBS and fixed in 4% formaldehyde (Sigma-Aldrich, USA) for 15 min at room temperature. Lipid rafts were stained using the cholera toxin subunit B conjugated with the Alexa488 fluorescent label (Sigma-Aldrich, Germany) without prior permeabilization for 15 min at 4 °C. For cell staining with antibodies, the cell membrane was permeabilized with 0.1% Triton X-100 (Helicon, Russia) for 10 min at room temperature. Nonspecific binding of antibodies was blocked with 1% bovine serum albumin (Helicon, Russia) for 1 h at room temperature. Antibodies were then added to α-tubulin at a dilution of 1:1000 (#T5168, Sigma-Aldrich, USA) or caveolin-1 at a dilution of 1:1000 (#GB11409, Servicebio, China). Subsequently, the cell preparations were incubated at 4 °C for 16–18 h, after which antispecies antibodies conjugated with AlexaFluor488 1:500 (#sc-2010, Santa Cruz Biotechnology, USA) or AlexaFluor555 1:1000 (#ab150078, Abcam, USA) were added and incubated in the dark for 1 h at room temperature. Cells stained only with antispecies antibodies were used as a negative control. Cytoskeletal F-actin was stained with rhodamine–phalloidin solution (Invitrogen, USA) for 15 min

at 37 °C; to visualize cell nuclei, preparations were stained with 4,6-diamino-2-phenylindole dihydrochloride (Sigma-Aldrich, Germany). Preparations were examined using an Olympus FV3000 microscope (Olympus, Japan) at 405 nm, 488 nm, and 561 nm.

5. Western blotting

Eukaryotic cells were preincubated with 50 μmol/L nystatin or DPBS for 1 h at 37 °C and 5% CO_2. The medium was then removed, sample preparation stain containing 2-mercaptoethanol was added, and the cells were heated for 5 min at 56 °C. Subsequently, the samples were heated for 5 min at 98 °C.

Proteins were separated using polyacrylamide gel electrophoresis under denaturing conditions; prestained proteins (#26619, Bio-Rad, USA) were used as molecular weight markers. The proteins were then transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad, Germany) using the semi-dry method. Nonspecific binding was blocked with a 3% solution of dry skim milk (Serva, Germany) in 0.05% Tween20 in PBS. After transferring, the membrane was cut and the corresponding parts were incubated with either rabbit anti-caveolin-1 antibody (#GB11409, Servicebio, China) at a dilution of 1:800 or murine anti-β-actin antibody (#GB15001, Servicebio, China) at a dilution of 1:1000. The dilutions were maintained for 2 h at room temperature. The membranes were washed with 0.05% Tween20 solution in PBS and incubated with horseradish peroxidase-conjugated secondary antibodies against murine (#ab97023, Abcam, USA) or rabbit (#31460, Thermo Fisher Scientific, USA) immunoglobulin at a dilution of 1:20,000 for 1 h at room temperature. The signals were detected using SuperSignal West Femto Maximum Sensitivity Substrate chemiluminescence reagents (Thermo Scientific, USA) on a ChemiDoc Imaging Systems instrument (Bio-Rad, USA).

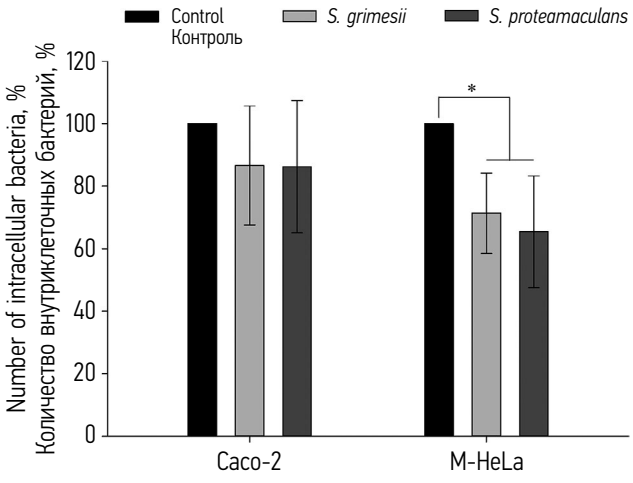

Fig. 1. The number of intracellular bacteria in Caco-2 and M-HeLa cells preincubated with 50 μM nystatin for 1 h relative to control cells preincubated with DPBS (control). Means and standard deviations are shown. *$p < 0.05$

Рис. 1. Количество внутриклеточных бактерий в клетках Caco-2 и M-HeLa, предварительно инкубированных с 50 мкмоль/л нистатина в течение 1 ч, относительно контрольных клеток, прединкубированных с фосфатно-солевым раствором Дульбекко (контроль). Показаны средние значения и стандартные отклонения. *$p < 0,05$

6. Real-time reverse transcription-polymerase chain reaction (RT-PCR)

After preincubation with 50 μmol/L nystatin or DPBS and bacteria, epithelial cells were washed thrice with PBS and trypsinized with 0.25%. The cells were then pelleted via centrifugation at 1500 rpm for 10 min, and the supernatant was removed. Total RNA was isolated from the resulting pellet using the diaGene cell culture RNA isolation kit (Dia-M, Russia). Reverse transcription and amplification were performed using BioMaster RT-PCR SYBR Blue (Biolabmix, Russia) on a CFX96 Touch device (Bio-Rad, USA). The stages included initial cDNA synthesis at 45 °C for 30 min, denaturation at 95 °C for 5 min, and 40 amplification cycles (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s). Each sample was analyzed in quadruplicate at least twice. Primers (forward and reverse, respectively) to *tlr2* were CTGGACAATGCCACATAC and CTAATGTAGGTGATCCTG, those to *tlr4* were CAGAACTGCAGGTGCTGG and GTTCTCTAGAGATGCTAG, and those to beta-actin were GCCGGGACCTGACTGACTAC and TTTCCTTAATGTCACGCACGAT. The PCR analysis results were interpreted using the ΔΔCT method based on the assessment of the expression level of the target gene in relation to the reference gene (β-actin).

Results

1. Cholesterol sequestration by nystatin reduces bacterial invasion by enhancing caveolin-1 expression

Caveolae/lipid rafts have been shown to be involved in the invasion and intercellular spread of many pathogenic and opportunistic bacteria [15, 16]. Hence, whether caveolae/lipid rafts are involved in the internalization of *S. grimesii* and *S. proteamaculans* into epithelial cells was examined. Preliminary incubation of Caco-2 cells with 50 μM nystatin for 1 h did not affect the invasion of cells by either *Serratia* species (Fig. 1). A four-fold increase in nystatin concentration also did not affect the invasion of the studied bacteria into colon adenocarcinoma cells (data not provided). Nystatin treatment of M-HeLa cells inhibited the invasion of the studied bacteria by approximately 30%. These results suggest that *S. grimesii* and *S. proteamaculans* use caveolae and/or lipid rafts to enter M-HeLa cells.

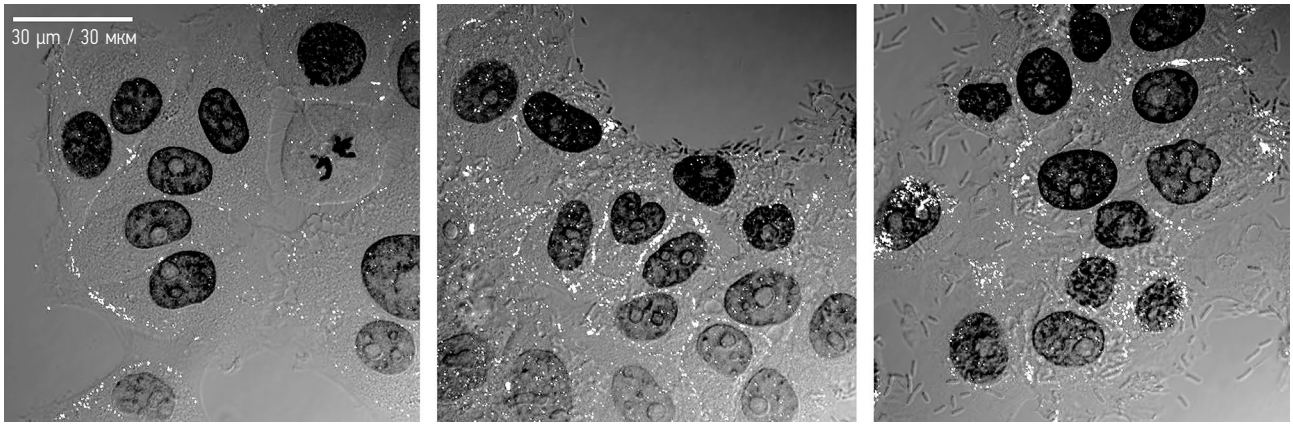

Fig. 2. Confocal fluorescence microscopy of M-HeLa cells without bacteria and after 2-h incubation with *S. grimesii* or *S. proteamaculans*. Nuclei are stained with DAPI (black), caveolin — indirect immunofluorescence (white)

Рис. 2. Конфокальная флуоресцентная микроскопия клеток M-HeLa без бактерий и после двухчасовой инкубации с *S. grimesii* и *S. proteamaculans*. Ядра окрашены 4,6-диамино-2-фенилиндолом (черный), кавеолин — непрямая иммунофлуоресценция (белый)

Expression of caveolin-1 can modulate the mobility of caveolae-associated proteins, resulting in alterations in plasma membrane fluidity [17]. As confocal micrographs did not reveal unambiguous colocalization of bacteria and caveolin-1 (Fig. 2), whether the invasion-inhibitory effect of nystatin was associated with changes in caveolin-1 expression and plasma membrane fluidity in M-HeLa cells was examined. Western blotting (Fig. 3*a*) indicated that caveolin-1 was not expressed in Caco-2 cells, which is consistent with literature data [18]. In contrast, the cytoplasmic membrane of M-HeLa cells contained caveolae, and the amount of caveolin-1 increased by 1.5 times after incubation with nystatin. Thus, the treatment of M-HeLa cells with nystatin can be assumed to induce a change (probably a decrease) in the cell membrane fluidity owing to the increase in the expression of caveolin-1. To enhance membrane fluidity, the cells were first preincubated with 50 μmol/L nystatin for 1 h, and then, the medium containing 0.05% Tween20 was added [19]. Subsequently, the cells were infected with the studied bacteria, and the invasion was measured. The addition of the detergent did not affect either the viability of eukaryotic cells or the viability and growth rate of bacteria. According to Fig. 3*b*, in the presence of Tween20, invasion into control

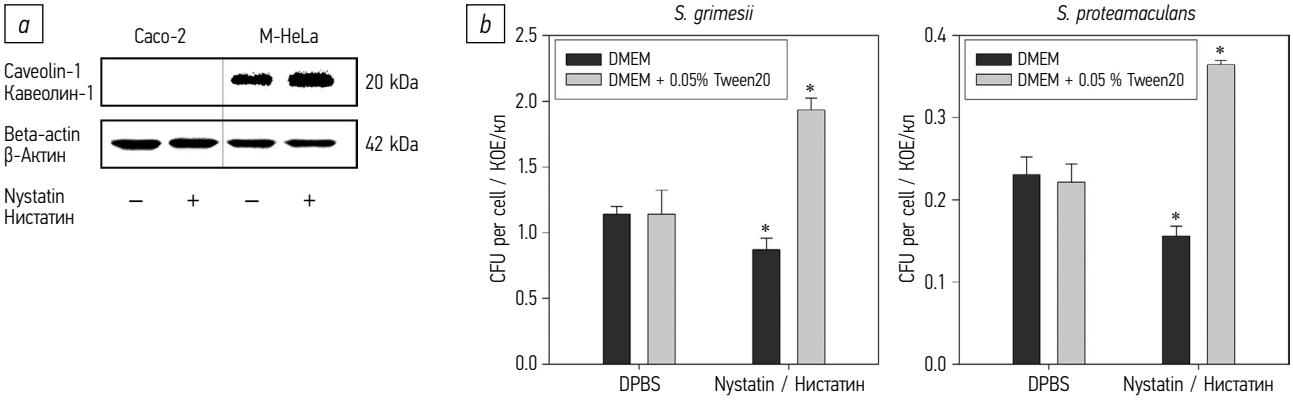

Fig. 3. The protein level of caveolin-1 in Caco-2 and M-HeLa cells treated with 50 μM nystatin (+) or DPBS (–) was detected by western blot analysis assays (*a*). The number of intracellular bacteria per M-HeLa cell preincubated with 50 μM nystatin or DPBS (control), after that bacteria were added in DMEM or DMEM containing 0,05% Tween20. Means and standard deviations are shown. *$p < 0.05$ (relative to corresponding control samples) (*b*)

Рис. 3. Экспрессия кавеолина-1 в клетках Caco-2 и M-HeLa, предварительно инкубированных с 50 мкмоль/л нистатина (+) или фосфатно-солевым раствором Дульбекко (–), детектирована с помощью вестерн-блоттинга (*a*). Количество внутриклеточных бактерий, приходящихся на 1 клетку M-HeLa, которые предварительно инкубировали с 50 мкмоль/л нистатина или фосфатно-солевым раствором Дульбекко (контроль), после чего к ним добавляли бактерии в среде DMEM или DMEM, содержащей 0,05 % Tween20. Показаны средние значения и стандартные отклонения. *$p < 0,05$ (относительно соответствующих контрольных образцов) (*b*)

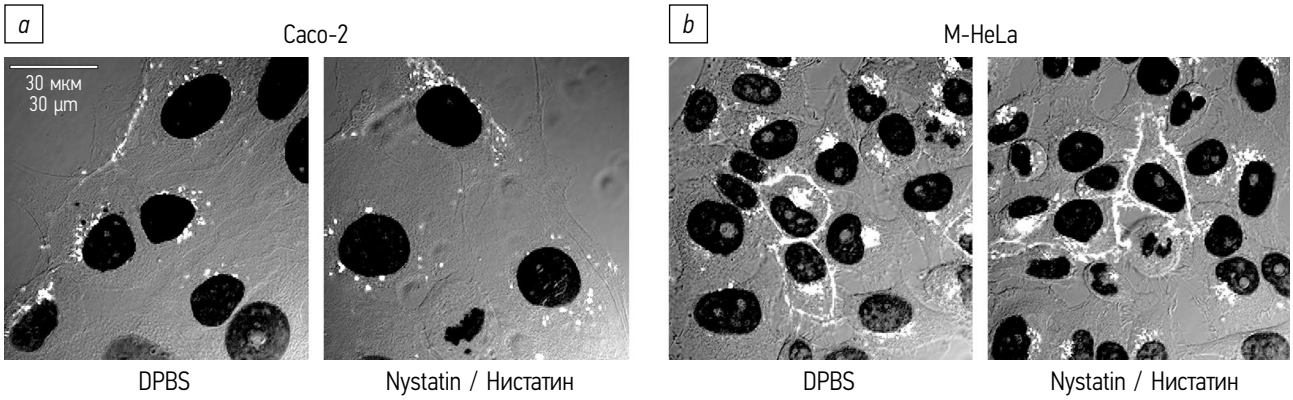

Fig. 4. Confocal fluorescence microscopy: monitoring of lipid rafts distribution. Caco-2 (*a*) and M-HeLa (*b*) cells incubated with 50 μM nystatin at 37 °C for 1 hour. GM1 was stained with CTxB-FITC (white), cell nuclei were stained with DAPI (black)

Рис. 4. Конфокальная флуоресцентная микроскопия липидных рафтов. Изображение клеток Caco-2 (*a*) и M-HeLa (*b*), инкубированных с 50 мкмоль/л нистатина при 37 °C в течение 1 ч. GM1 окрашивали CTxB-FITC (белый), ядра клеток окрашены 4,6-диамино-2-фенилиндолом (черный)

cells preincubated with DPBS did not change, and the number of intracellular bacteria in cells treated with nystatin increased by 1.6 times (compared with the control). These results imply that differences in the invasion efficiency of *S. grimesii* and *S. proteamaculans* into Caco 2 and M-HeLa cells are related to variations in the plasma membrane properties of carcinoma cells.

2. Nystatin does not affect lipid raft assembly and does not cause cytoskeletal rearrangements

Nystatin is known to have a higher affinity for ergosterol than for cholesterol, but it has been shown to sequester or remove cholesterol from lipid rafts [20–22]. To assess the effect of nystatin on lipid raft status, cells were stained with cholera toxin subunit B conjugated to the fluorescent label Alexa488 (CTxB-FITC) as CTxB binding to ganglioside GM1 is a marker for identifying lipid rafts. According to Figure 4, treatment of cells with 50 μmol/L nystatin did not inhibit cellular uptake of the marker (control samples were supplemented with the appropriate amount of DPBS).

Pretreatment of M-HeLa and Caco-2 cells for 2 h with methyl-β-cyclodextrin, which specifically removes cholesterol from the plasma membrane and disrupts the formation of lipid rafts, has previously been shown to increase the amount of stress fibrils in the cells [23]. Therefore, the effect of nystatin on the reorganization of the cytoskeleton of carcinoma cells was examined (Fig. 5).

Incubation of cells with nystatin did not result in the reorganization of actin microfilaments and microtubules of cytoskeleton in either Caco 2 or M-HeLa cells.

3. Cell-specific agonistic and antagonistic activity of nystatin toward toll-like receptors (TLRs)

The plasma membrane of epithelial cells, including HeLa and Caco-2 [24, 25], contains pattern recognition receptors, such as TLRs, which recognize various microbial structural components. Thus, TLR2 responds to lipoproteins and peptide glycan, and TLR4 is activated by lipopolysaccharides (LPS) on the outer membrane of gram-negative bacteria. LPS molecules are recognized by CD14 and transferred to the MD2–TLR4 complex before TLR4 activation, and a portion of the LPS–MD2–TLR4 complex enters the cell via endocytosis [26]. Both TLR2 and TLR4 and accessory proteins, as well as the stimulated LPS–MD2–TLR4 complex, have been shown to be associated with lipid rafts/caveolae [27, 28]. This association is probably a prerequisite for TLR-mediated signaling and target gene expression [29, 30].

Nystatin has been reported to inhibit LPS-induced NF-kB activation and expression of genes encoding immunomodulatory cytokines [29]. At the same time, studies have shown that nystatin exhibits TLR2- and TLR4-agonistic activity [31] and stimulates interleukin-8 (IL-8) secretion by TLR2-expressing cells [32]. In this regard, the effect of nystatin on the production of proinflammatory cytokines IL-6 and IL-8 by M-HeLa and Caco-2 cells was investigated. As inferred from Fig. 6, the addition of 50 μmol/L nystatin resulted in a 1.5-fold increase in cytokine production (IL-6 was not detected in Caco-2 cells).

Since nystatin can exhibit both agonistic and antagonistic activity toward TLR2 and TLR4, its effect on the expressions of genes encoding these receptors (Fig. 7*a*) was compared. Depending on the cell line, nystatin either increased (Caco 2) or decreased (M-HeLa) the expressions of *tlr2* and *tlr4*.

TLR4 is activated by LPS of gram-negative bacteria to induce the production of proinflammatory mediators aimed at the destruction of bacteria.

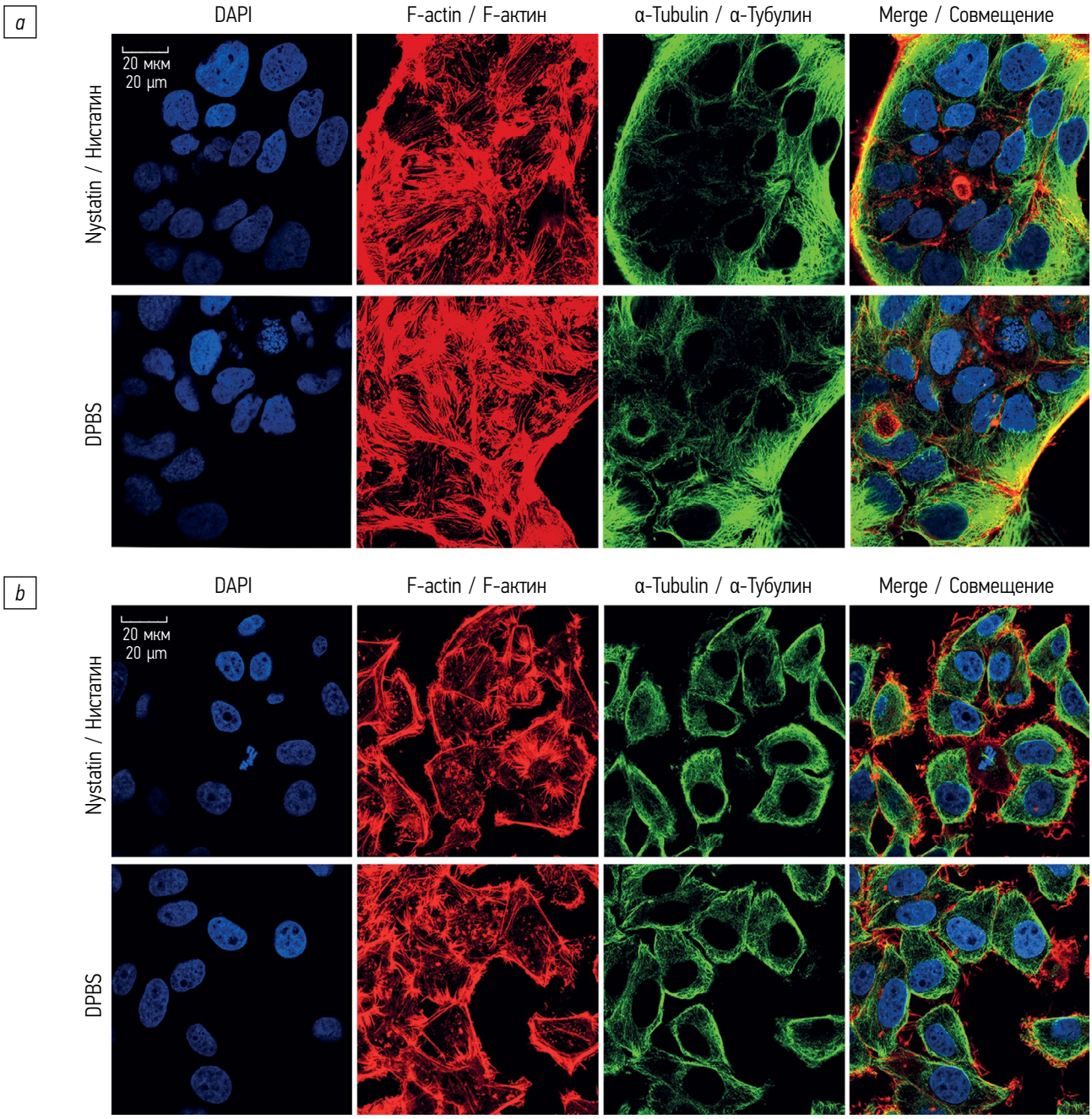

Fig. 5. Visualization the cytoskeleton of Caco-2 (*a*) and M-HeLa (*b*) cells treated with 50 μM nystatin or DPBS for 1 h by confocal fluorescence microscopy. Nuclei are stained with DAPI (blue), F-actin is stained with RF (red), α-tubulin is indirect immunofluorescence (green)

Рис. 5. Конфокальная флуоресцентная микроскопия клеток Caco-2 (*a*) и M-HeLa (*b*), визуализирующая цитоскелет эпителиальных клеток, обработанных 50 мкмоль/л нистатина или фосфатно-солевого раствора Дульбекко в течение 1 ч. Ядра окрашены 4,6-диамино-2-фенилиндолом (синий), F-актин окрашен RF (красный), α-тубулин — непрямая иммунофлуоресценция (зеленый)

For uropathogenic *Escherichia coli*, a decrease in their internalization has been shown to be linked to an increase in TLR4 expression, which leads to a decrease in the amount of the phosphorylated form of the small guanosinetriphosphatase Rac-1, which is involved in bacterial invasion [33]. In our study, the treatment of M-HeLa cells with nystatin reduced the invasion of *S. grimesii* and *S. proteamaculans*, which might be accompanied by an increase in *tlr4* expression. However, the expressions of both *tlr4* and *tlr2* decreased after the addition of the antibiotic. Therefore, the effects of *S. grimesii* and *S. proteamaculans* on the expressions of *tlr4* and *tlr2* in control and nystatin-treated M-HeLa cells were determined (Fig. 7*b*). Bacterial invasion increased the gene expression

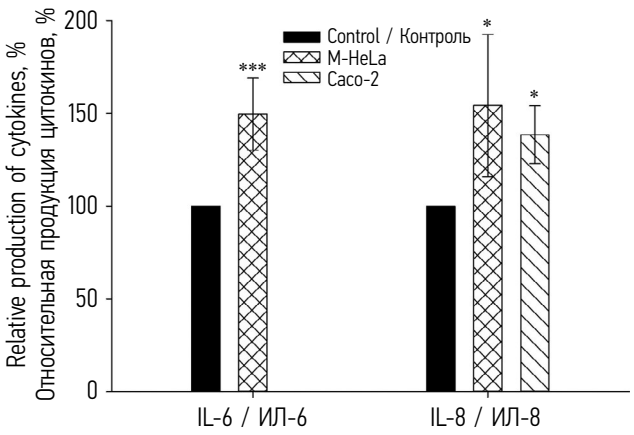

Fig. 6. Relative production of cytokines IL-6 and IL-8 by M-HeLa and Caco-2 cells after one-hour incubation with 50 μM nystatin, DPBS was added to control cells. Means and standard deviations are shown. Statistically significant differences between the control and treated groups are indicated by asterisks: $*p < 0.05$, $***p < 0.005$

Рис. 6. Относительная продукция цитокинов — интерлейкинов-6 и -8 клетками M-HeLa и Caco-2 после часовой инкубации с 50 мкмоль/л нистатина. К контрольным клеткам добавляли соответствующее количество фосфатно-солевого раствора Дульбекко. Показаны средние значения и стандартные отклонения. Статистически значимые различия между контрольной и обработанной группами отмечены звездочками: $*p < 0{,}05$, $***p < 0{,}005$

level in the treated cells to that in the control samples. These findings signify that TLR4, TLR2, and downstream signaling molecules do not participate actively in the invasion of the studied bacteria.

Discussion

The plasma membrane of the cell is involved in various biochemical processes, and its composition determines not only the cell structure but also its functions. Thus, alterations in the distribution of phospholipids in the lipid bilayer can be a signal for processes such as cell aggregation, adhesion, and apoptosis. Cholesterol is a key structural component of the cell membrane and is involved in maintaining its integrity and fluidity as well as compartmentalization and signaling [34].

Many bacteria capable of invasion use lipid rafts to penetrate the host cell. In this case, membrane cholesterol plays a crucial role in the host–pathogen interaction. Depletion or sequestration of cholesterol reduces the adhesion and internalization of microorganisms into eukaryotic cells. Therefore, plasma membrane cholesterol can play a dual role in bacterial invasion, namely, as a site of bacterial binding and as an integrating component of lipid rafts, providing a platform for efficient initiation of signaling cascades [35].

The ratio of phospholipids and cholesterol in the cell membrane depends on the cell type. Cholesterol depletion by methyl-β-cyclodextrin has previously been shown to promote a more effective reduction in the invasion of *S. grimesii* and *S. proteamaculans* into M-HeLa cells than into Caco-2 cells [23]. This effect could be attributed, among other things, to the fact that the cytoplasmic membrane of Caco-2 cells contains less cholesterol than HeLa cells and does not express caveolin-1, which plays an important role in cholesterol homeosta-

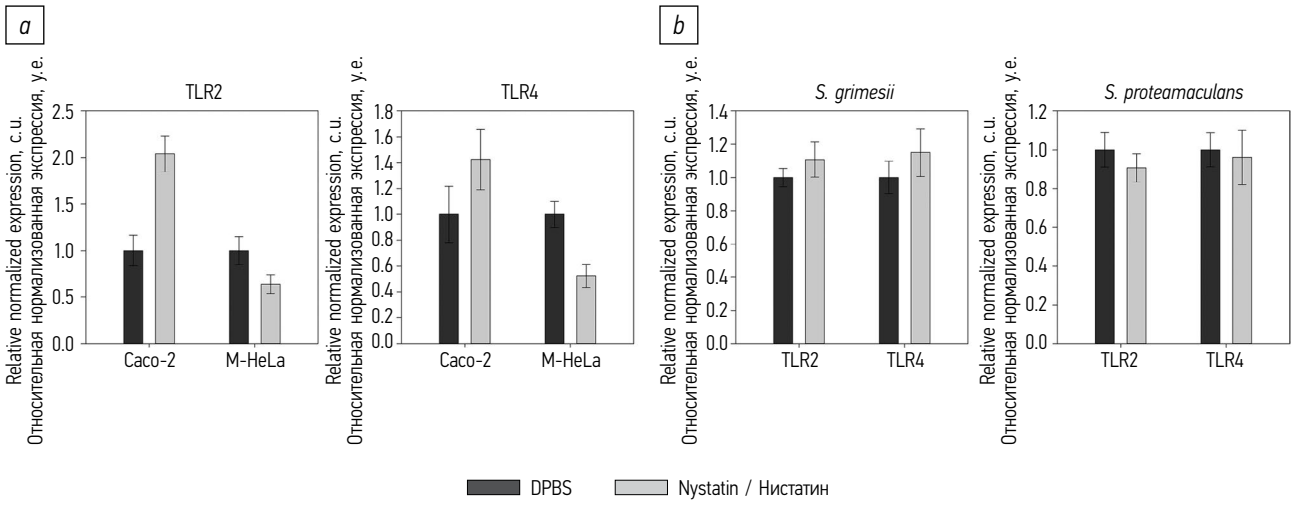

Fig. 7. Relative normalized (to β-actin) expression of *tlr2* and *tlr4* genes. Caco-2 and M-HeLa cells were incubated with 50 μM nystatin or DPBS (control) for 1 h (*a*). M-HeLa cells were incubated with 50 μM nystatin or DPBS (control) for 1 h, and then bacterial invasion was performed (MOI 100) (*b*)

Рис. 7. Относительная нормализованная (на β-актин) экспрессия генов, кодирующих TLR2 и TLR4. Клетки Caco-2 и M-HeLa инкубировали с 50 мкмоль/л нистатина или DPBS (контроль) в течение 1 ч (*a*). Клетки M-HeLa инкубировали с 50 мкмоль/л нистатина или фосфатно-солевым раствором Дульбекко (контроль) в течение 1 ч, а затем проводили бактериальную инвазию (множественность заражения 100) (*b*)

sis by binding it and transporting it to the membrane [18].

Pathogenic bacteria and viruses use various endocytic pathways and receptors to penetrate the host cell. Negatively charged particles, including bacteria, can penetrate the cell via caveolin-dependent endocytosis [36]. Nystatin is one of the inhibitors of caveolin-dependent endocytosis. Nystatin can form pores in the cell membrane, whose number is directly correlated with the concentration of the antibiotic. At the same time, low concentrations of the antibiotic (50 μmol/L) do not significantly affect the size and shape of cells [37]. Furthermore, nystatin can bind cholesterol located on the plasma membrane of eukaryotic cells and change the microstructure of lipid rafts, which play a vital role in the internalization of pathogens and the regulation of both innate and adaptive immune responses [38]. This work has shown that nystatin does not affect the distribution of cholesterol in cells but increases the production of IL-6 and IL-8 cytokines in M-HeLa and Caco-2 cells. This antibiotic induces the secretion of proinflammatory cytokines via a TLR2-dependent mechanism [32]. In addition, cytokine synthesis by epithelial cells in response to bacterial infection is mediated by signaling pathways triggered by TLRs, including TLR2 and TLR4. Signal transmission from TLR4 can activate the transcription factor NF-kB or trigger an alternative signaling pathway that increases the production of the second messenger cyclic adenosine monophosphate (cAMP). Accumulation of cAMP promotes the inactivation of the small Rho-guanosinetriphosphatase Rac1, which is involved in bacterial penetration via lipid rafts. Thus, an increase in TLR4 expression leads to a decrease in bacterial invasion [33]. Infection of nystatin-pretreated M-HeLa cells with *S. grimesii* and *S. proteamaculans* has been shown in this study to result in the increased expression of both *tlr2* and *tlr4*. However, the expression levels of these genes in antibiotic-treated infected cells did not exceed those in control samples. Therefore, we can't speak unequivocally about the participation of TLRs in the invasion of the studied bacteria.

Internalization of bacteria into eukaryotic cells relies on plasma membrane fluidity, which in turn depends on the cholesterol content in it [10]. However, some studies have indicated that a decrease in the cholesterol level in the cell membrane owing to the overexpression of caveolin-1 augments the membrane fluidity [39]. On the contrary, others have indicated that the knockdown of caveolin-1 enhances the fluidity of the plasma membrane and the mobility of membrane microdomain proteins [40]. Deficiency of caveolin-1 in eukaryotic cells can contribute to both an increase and a decrease in the number of intracellular bacteria, which is associated with the redistribution of various lipid raft components during the clustering of receptors involved in bacterial internalization. Thus, during *L. monocytogenes* invasion, caveolin-1 knockdown disrupts the recruitment of E-cadherin to bacterial attachment sites [41]. During the adhesion of *Staphylococcus aureus* to the surface of eukaryotic cells, a redistribution of membrane microdomain components and a recruitment of GM1 ganglioside to bacterial attachment sites is noted depending on the β1-integrin of the host cell. The clustering of the receptor itself does not rely on membrane microdomains. However, bacterial invasion depends on the composition of microdomains, and caveolin-1 deficiency increases the β1-integrin-mediated uptake of *S. aureus* [40]. *S. proteamaculans* has also been previously reported to recruit β1-integrin during the invasion of epithelial cells [42]. Therefore, caveolin-1 appears to limit the internalization of bacteria that bind to β1-integrin, and modulation of the mobility of membrane microdomain components is a key aspect of the pathogen–host interaction during bacterial invasion.

Conclusions

The findings from this study suggest that the decrease in the invasion of *S. grimesii* and *S. proteamaculans* into epithelial cells pretreated with nystatin is associated with a change (probably a decrease) in plasma membrane fluidity. This effect may be a characteristic of bacteria whose internalization involves β1-integrin.

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