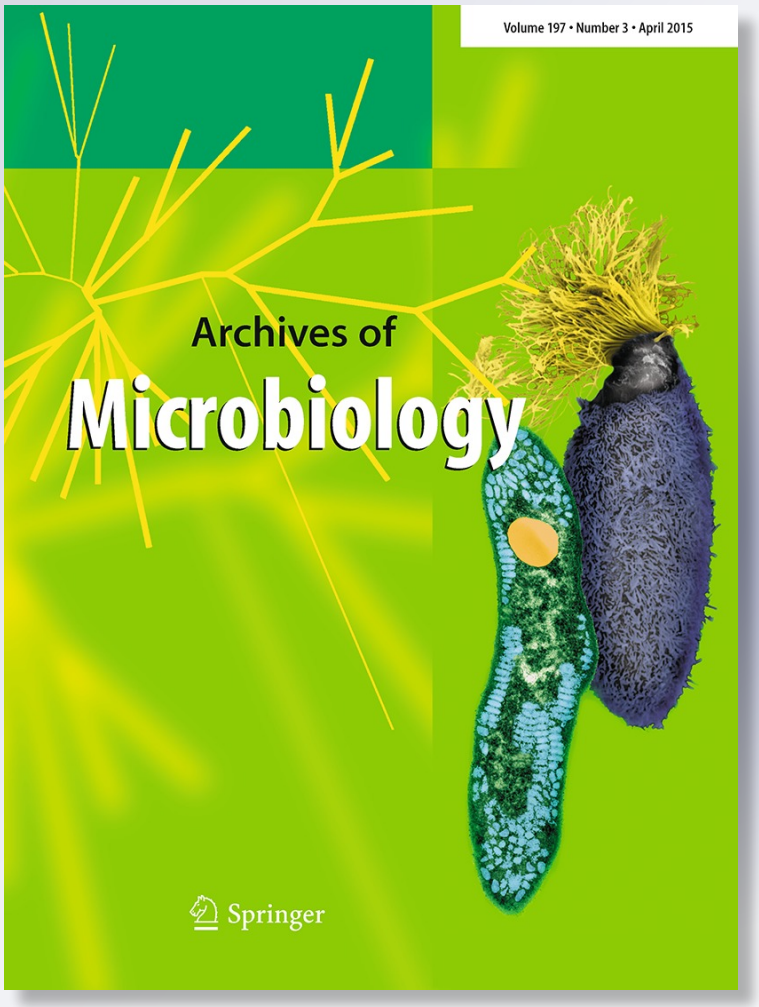
Virulence factors contributing to invasive activities of Serratia grimesii *and* Serratia proteamaculans

Olga Tsaplina, Ekaterina Bozhokina, Ayslu Mardanova & Sofia Khaitlina

Archives of Microbiology

ISSN 0302-8933
Volume 197
Number 3

Arch Microbiol (2015) 197:481-488
DOI 10.1007/s00203-014-1079-7



Springer

Your article is protected by copyright and all rights are held exclusively by Springer-Verlag Berlin Heidelberg. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".

Author's personal copy

Arch Microbiol (2015) 197:481–488
DOI 10.1007/s00203-014-1079-7

ORIGINAL PAPER

Virulence factors contributing to invasive activities of *Serratia grimesii* and *Serratia proteamaculans*

Olga Tsaplina · Ekaterina Bozhokina · Ayslu Mardanova · Sofia Khaitlina

Received: 10 December 2014 / Revised: 26 December 2014 / Accepted: 30 December 2014 / Published online: 11 January 2015
© Springer-Verlag Berlin Heidelberg 2015

Abstract Previously, we have shown that facultative pathogens *Serratia grimesii* and *Serratia proteamaculans* are capable to invade eukaryotic cells provided that they synthesize intracellular metalloprotease grimelysin or protealysin, respectively (Bozhokina et al. in Cell Biol Int 35(2):111–118, 2011). Noninvasive *Escherichia coli* transformed with grimelysin or protealysin gene became invasive, indicating that the protease is a virulence factor. Here we elucidated involvement of other virulence factors in the invasion of *S. grimesii* and *S. proteamaculans*. Under similar experimental conditions, the amount of *S. proteamaculans* internalized within human carcinoma HeLa cells was fivefold higher than that of *S. grimesii.* In accord with this, in *S. proteamaculans,* high activities of pore-forming hemolysin ShlA and extracellular metalloprotease serralysin were detected. In *S. grimesii*, activity of toxin ShlA was not detected, and the serralysin activity of the bacterial growth medium was very low. We also show that iron depletion strongly enhanced invasive activity of *S. proteamaculans*, increasing activities of hemolysin ShlA and serralysin, but did not affect *S. grimesii* properties. These results show that the invasive activity of *S. proteamaculans* is maintained, along with protealysin, by hemolysin and serralysin. On the other hand, grimelysin is so far the only known invasion factor of *S. grimesii*.

Communicated by Erko Stackebrandt.

O. Tsaplina · E. Bozhokina · S. Khaitlina (✉)
Institute of Cytology, Russian Academy of Sciences, Tikhoretsky av. 4, 194064 St. Petersburg, Russia
e-mail: skhspb@gmail.com

A. Mardanova
Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, Kazan, Russia

Keywords Bacterial invasion · Grimelysin · Protealysin · Virulence factors · Hemolysin · Serralysin

Introduction

The gram-negative bacteria *Serratia* are facultative pathogens able to cause nosocomial infections or infections in immunocompromised patients (Grimont and Grimont 2006; Mahlen 2011). The most clinically relevant strain of the genus *Serratia* is *S. marcescens* (Hejazi and Falkiner 1997; Grimont and Grimont 2006; Mahlen 2011). In addition, a number of reports describe human diseases from infections with other *Serratia* species including *S. rubidaea, S. liquefaciens, S. grimesii* and *S. proteamaculans* (Bollet et al. 1993; Ursua et al. 1996; Hertle 2005; Grimont and Grimont 2006). *Serratia* has also been associated with animal infections (Grimont and Grimont 2006). We have recently shown that, similar to pathogenic bacteria (Cossart and Sansonetti 2004) and *S. marcescens* (Hertle and Schwarz 2004), *S. grimesii* and *S. proteamaculans* can penetrate into eukaryotic cells being detected both in vacuoles and freely in cytoplasm of the infected cells (Efremova et al. 2001; Tsaplina et al. 2009; Bozhokina et al. 2011). The efficiency of the internalization of *S. grimesii* and *S. proteamaculans* is rather low, with only about 10 % of the cultured cells being invaded by the bacteria (Bozhokina et al. 2011). However, it can be enhanced under conditions that modify cell surface receptors playing a role in cell adhesion and cell–cell junctions, like incubation of eukaryotic cells with antioxidants (Gamaley et al. 2006; Bozhokina et al. 2013), i.e., by the factors that may accompany the *Serratia* nosocomial infection. Therefore, invasion of cultured mammalian cells by *S. grimesii* or *S. proteamaculans* can be a

Author's personal copy

useful model to study the involvement of various factors in the *Serratia* infections.

The invasive activities of *S. grimesii* and *S. proteamaculans* turned out to correlate with synthesis of homologous thermolysin-like metalloproteases ECP32/grimelysin or protealysin, respectively, characterized by high specificity toward actin (Khaitlina et al. 1991; Efremova et al. 2001; Demidyuk et al. 2006; Tsaplina et al. 2009; Bozhokina et al. 2011). Moreover, transformation of noninvasive *Escherichia coli* with a plasmid encoding grimelysin or protealysin confers to the bacteria invasive capacity (Bozhokina et al. 2011). However, inactivation of protealysin gene resulted in the loss of actin-hydrolyzing activity of *S. proteamaculans* extracts but did not abolish the ability of the bacteria to be internalized within the eukaryotic cells, thus indicating the presence of other factors promoting invasion of these bacteria. Among the possible virulence factors found in *Serratia* strains are a pore-forming toxin (hemolysin) ShlA known to promote invasion of *S. marcescens* (Hertle and Schwarz 2004; Hertle 2005), extracellular proteases (Marty et al. 2002), and a nuclease (Benedik and Strych 1998). The aim of the present work was to evaluate contribution of these virulence factors in invasive capacities of *S. grimesii* and *S. proteamaculans.*

Our results show that the invasive activity of *S. proteamaculans* toward human cultured cells is fourfold to fivefold higher than that of *S. grimesii*, which correlates with the high activities of the pore-forming toxin hemolysin ShlA and an extracellular gelatinase serralysin of *S. proteamaculans*. Under the invasion conditions, activity of *S. grimesii* virulence factors is very low, consistent with the relatively low invasive activity of these bacteria. These results indicate that along with protealysin, the invasive activity of *S. proteamaculans* is mediated by hemolysin and serralysin. On the other hand, grimelysin is so far the only known invasion factor of *Serratia grimesii*.

Materials and methods

Reagents

EMEM culture medium was obtained from Biolot LLC (Russia). Fetal bovine serum (FBS) was from Thermo Scientific (Thermo Fisher Scientific Inc.). Peptone and yeast extract were obtained from Difco (Franklin Lakes, NJ, USA); NEAA, PBS, Sodium deoxycholate, gelatin, 2,2′-bipyridyl, LB medium, LB-agar, Triton X100, azocasein, PMSF, *O*-phenanthroline, DNA, reagents for electrophoresis were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Bacterial strains, cell cultures and growth conditions

Human cervical carcinoma HeLa M cells were obtained from the Russian Cell Culture Collection (Institute of Cytology, St. Petersburg, Russia). Cells were grown in the antibiotic-free EMEM supplemented with 10 % fetal bovine serum and 1 % NEAA (Non-Essential Amino Acids) at 37 °C under 5 % CO_2 atmosphere, for the time required to form a monolayer (usually about 18–40 h).

Serratia grimesii strain 30063 was from the German Collection of Microorganisms and Cell Cultures (DSM 30063). *Serratia proteamaculans* strain 94 was a generous gift of Dr. Ilya Demidyuk (Institute of Molecular Genetics, RAS, Russia). The recombinant *E. coli* BL21 (DE3) (Novagen), expressing protealysin gene pProPlnHis6 was obtained as described previously (Gromova et al. 2009). Bacteria were grown either in Luria broth (LB medium) containing 1 % peptone, 0.5 % yeast extract and 1 % NaCl in the absence or presence of 0.3 mM 2,2′-bipyridyl at pH 7.0 or in M9 medium containing 0.6 % Na_2HPO_4, 0.3 % KH_2PO_4, 0.1 % NH_4Cl, 0.05 % NaCl, 2 % glucose, 1 % thiamin, 0.1 mM $CaCl_2$ in the absence or presence of 0.2 mM Fe_2SO_4. *S. grimesii* and *E. coli* BL21 (DE3) (pProPlnHis6) were grown at 37 °C with aeration. *S. proteamaculans* was grown at 30 °C with aeration, because they do not grow at 37 °C. For experiments, all bacteria were grown till the late stationary growth phase, and the aliquots of the every bacterial culture were diluted with growth medium to equal optical densities at 600 nm.

Gentamicin invasion assay

Efficiency of invasion was evaluated by gentamicin invasion assay (Prouty and Gunn 2000). Bacteria that were grown for 48 h until actinase activity on their extracts could be determined (Tsaplina et al. 2009; Bozhokina et al. 2011). Bacteria were pelleted by centrifugation at 12,000*g* for 10 min, resuspended in EMEM, and the bacteria suspension was added to HeLa M cells grown in 6-well plates containing nearly confluent monolayers of ~2 × 10^5 cells per well, to a multiplicity of infection (MOI) of approximately 100 bacteria per cell. Then the plates were centrifuged at 2,000*g* for 5 min to get bacteria to attach the cells. The plates were incubated for 2 h at 37 °C, and EMEM with bacteria was removed. After washing three times with PBS, the infected cells were suspended in 1 ml of trypsin in PBS and 400 µl of the cells suspension was incubated with 400 µl EMEM containing gentamicin (100 µg/ml) with shaking at 37 °C for 1 h, to get rid of extracellular bacteria. At the concentration used, gentamicin is impermeable to mammalian cells; therefore, it kills only extracellular rather than internalized bacteria. Then 400 µl of this cell suspension was lysed with 200 µl 4.5 % sodium deoxycholate in water, pipetted to

Author's personal copy

disperse bacterial aggregates and plated out on LB-agar to determine the number of colony forming units (CFU). The results for each experiment are the average of an assay performed in triplicate and independently repeated three times.

Hemolysis assay

To determine hemolytic activity of *S. proteamaculans* 94 and *S. grimesii* 30063, the standard hemolysis assay with erythrocytes was used (Hertle and Schwarz 2004). Horse erythrocytes were a generous gift of Maria Sergeeva (Research Institute of Influenza, Saint Petersburg, Russia). Washed erythrocytes were suspended in PBS to a final concentration of 2 %. Bacteria were grown for 48 h, and the extracts were tested for the actinase activity (Tsaplina et al. 2009; Bozhokina et al. 2011). Similar amounts of bacteria were pelleted by centrifugation at 12,000*g* for 10 min, resuspended in 100 µl PBS, and the bacteria suspension was added to 1 ml of erythrocyte suspension for 1 h at 37 °C and then centrifuged for 3 min at 2,000*g*. The absorbance of released hemoglobin was measured at 405 nm. Hemolytic activities are presented as the percentage of the total erythrocytes lysed by 4 % SDS.

Zymography with gelatin

To determine the ability of extracellular protease to cleave gelatin, zymography was performed (Hertle and Schwarz 2004). The growth media of *S. grimesii* 30063 or *S. proteamaculans* 94 was incubated with electrophoresis sample solution (62.5 mM Tris–HCl, pH 6.8, 0.1 % SDS) for 30 min and analyzed by SDS-PAGE differing from a regular SDS-PAGE by the presence of 0.3 % gelatin in the resolving gel. After electrophoresis, the gel was washed twice with 2.5 % Triton X-100 to remove SDS and incubated in 5 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.2–7.4, for 18 h at 37 °C to renature the proteases and carry out proteolysis. Finally, the gel was fixed in 25 % isopropanol with 10 % acetic acid for 30 min and stained with Coomassie brilliant blue G-250.

Proteolysis of azocasein

The reaction mixture consisted of 100 µl of azocasein (10 mg/ml) in 50 mM Tris–HCl, pH 7.2 and 50 µl of the growth media of *S. grimesii* 30063 or *S. proteamaculans* 94 (grown for 48 h) was incubated at 37 °C for 60 min. To study the effect of inhibitors, the bacteria growth media was complemented with 1 mM PMSF or 5 mM *O*-phenanthroline. The reaction was quenched by the addition of 200 µl 10 % trichloroacetic acid for 10 min at −20 °C. After centrifugation at 12,000*g* for 5 min, 250 µl of supernatant was mixed with 50 µl 4 M NaOH and the absorbance was determined at 450 nm.

Nuclease activity assay

To measure the nuclease activity, the spectrophotometric method was used. This assay detects the increase in DNA absorbance at 260 nm when the polymer is depolymerized to nucleotides or short oligonucleotides. The reaction mixture consisted of DNA (1 mg/ml) in 100 mM Tris–HCl, pH 8.5 and the growth media of *S. grimesii* 30063 or *S. proteamaculans* 94 (grown for 24 or 48 h, till the early or late postlogarithmic growth stage) was incubated at 37 °C for 45 min. The reaction was quenched by the addition of equal volume of 25 % HCl for 10 min at −20 °C. After centrifugation at 12,000*g* for 7 min, the absorbance of the supernatant was measured at 260 nm.

Statistical analysis

Data were analyzed statistically using one-way analysis of variance (ANOVA) with Excel Data Analysis Pack. A difference was considered significant at the $p < 0.05$ level.

Results

Comparison of invasive activities of *S. grimesii* and *S. proteamaculans*

Both *S. grimesii* 30063 and *S. proteamaculans* 94 display the invasive phenotype only at the postlogarithmic growth phase (Tsaplina et al. 2009; Bozhokina et al. 2011) when the ability of the bacterial lysates for limited proteolysis of actin can be detected (Usmanova and Khaitlina 1989; Bozhokina et al. 2008; Tsaplina et al. 2009; Bozhokina et al. 2011). Therefore, to quantitatively evaluate invasive activities of *S. grimesii* and *S. proteamaculans*, human carcinoma HeLa cells were infected by the bacteria grown till the late postlogarithmic growth phase and the amount of the intracellular bacteria was determined using the gentamicin invasion assay (Prouty and Gunn 2000). The number of intracellular *S. grimesii* 30063 and *S. proteamaculans* 94 in HeLa cells incubated with bacteria for 2 h is presented in Fig. 1a. The data show that the invasive capability of *S. proteamaculans* 94 is about fivefold higher than that of *S. grimesii* 30063, suggesting that these closely related bacteria contain either a different repertoire of virulence factors or their similar virulence factors have different activities.

Is hemolysin ShlA a virulence factor of *S. grimesii* and *S. proteamaculans*?

The development of *S. marcescens* infections is known to be associated with the pore-forming toxin ShlA that is

Author's personal copy

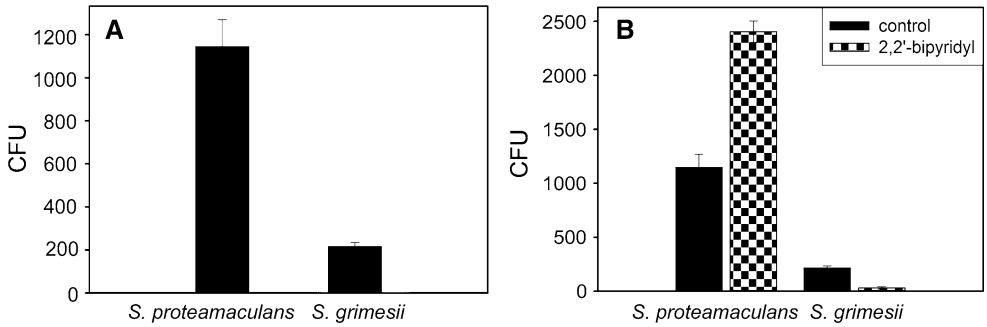


Fig. 1 Quantitative evaluation of the susceptibility of HeLa cells to invasion by *S. proteamaculans* 94 and *S. grimesii* 30063. **a** HeLa M cells were infected by the bacteria grown for 48 h. **b** *S. proteamaculans* 94 and *S. grimesii* 30063 were grown in the absence and presence of 0.3 mM 2,2′-bipyridyl for 48 h and incubated with HeLa M cells. The invasive activity was determined by the gentamicin assay as described in "Materials and methods"

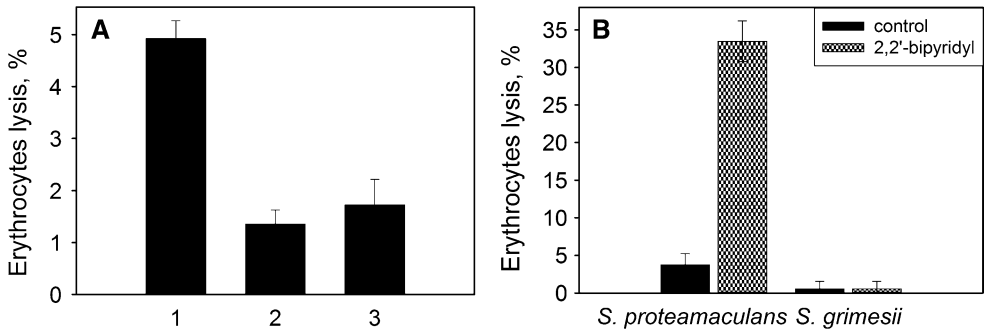


Fig. 2 Evaluation of hemolytic activity of *S. proteamaculans* 94 and *S. grimesii* 30063. **a** Hemolytic activity of *S. proteamaculans* 94 (*1*) and *S. grimesii* 30063 (*2*) was determined by a standard hemolysis assay with erythrocytes as described in "Materials and methods." (*3*) Hemolytic activity of control *E. coli* BL21 (DE3) (pProPlnHis$_6$). **b** Hemolytic activity of *S. proteamaculans* 94 and *S. grimesii* 30063 grown in the absence and presence of 0.3 mM 2,2′-bipyridyl

not absolutely necessary for internalization of *S. marcescens* within transformed eukaryotic cells but strongly mediates the internalization (Hertle and Schwarz 2004). The genome sequences of *S. proteamaculans* 568 [GenBank CP000826.1] and *S. grimesii* A2 [GenBank JGVP00000000.1] both contain ShlA gene.

We examined hemolytic activity of S. *proteamaculans* 94 and *S. grimesii* 30063 using a standard hemolysis assay with erythrocytes (Hertle and Schwarz 2004). To exclude cytolytic effects of other virulence factors, recombinant *E. coli* encoding protealysin and capable of invasion but lacking ShlA was taken as a control. Incubation of erythrocytes with *S. proteamaculans* 94 resulted in the lysis of 5 % of erythrocytes (Fig. 2a). *S. grimesii* 30063 induced the lysis of only 1.5 % of erythrocytes, which was similar to the effect produced by the control bacteria (Fig. 2a), evidencing a fourfold higher activity of ShlA in *S. proteamaculans* than in *S. grimesii* 30063 or the control *E. coli*.

It is known that the hemolytic activity of *S. marcescens* depends on iron availability (Hertle 2005). Synthesis of hemolysin is repressed by iron, while restriction of iron by chelator 2,2′-bipyridyl produced a marked increase in hemolytic activity (Poole and Braun 1988b). Therefore, to increase sensitivity of the test revealing the hemolytic effects of ShlA, we compared hemolytic activity of *S. proteamaculans* 94 and *S. grimesii* 30063 in the presence of 2,2′-bipyridyl. Figure 2b illustrates at least a sevenfold increase in hemolytic activity of *S. proteamaculans* produced by the iron limitation. At the same time, no effect of 2,2′-bipyridyl on the hemolytic activity of *S. grimesii* 30063 was observed (Fig. 2b).

Extracellular protease serralysin as a virulence factor of *S. proteamaculans* and *S. grimesii*

S. marcescens produces extracellular metalloprotease serralysin, which is a virulence factor playing an important role in pathogenesis of infections (Maeda and Morihara 1995; Kida et al. 2007; Ishii et al. 2014). Serralysin was also isolated from *S. proteamaculans* (Kwak et al. 2007), but whether it is produced by *S. grimesii* is still unknown. To address this issue, we compared the 48-h growth media

Author's personal copy

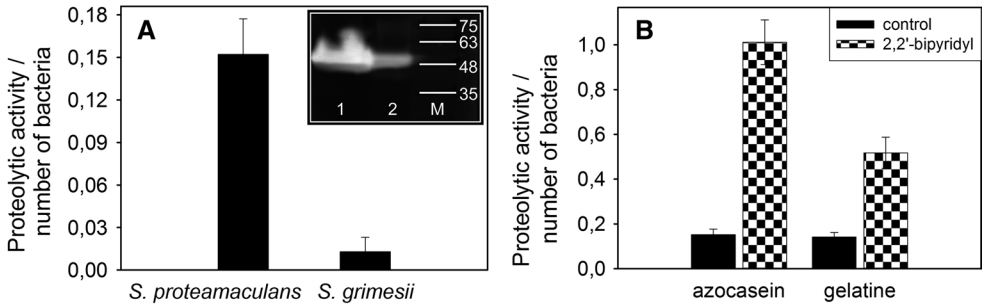


Fig. 3 Evaluation of serralysin activity in *S. grimesii* 30063 and *S. proteamaculans* 94 growth medium. **a** Protealytic activity in the growth media of *S. proteamaculans* 94 (*1*) and *S. grimesii* 30063 (*2*) was determined by the azocasein assay and zymography (*inset*). **b** The effect of 2,2′-bipyridyl on the proteolytic activity in the growth media of *S. proteamaculans* 94. *S. proteamaculans* 94 were grown for 48 h both in the absence and presence of 2,2′-bipyridyl

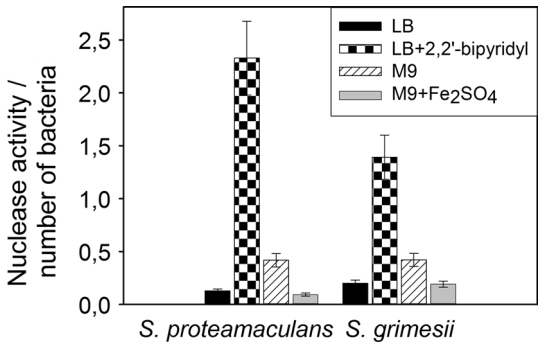


Fig. 4 Evaluation of nuclease activity in the *S. grimesii* 30063 and *S. proteamaculans* 94 growth medium. *S. grimesii* 30063 and *S. proteamaculans* 94 were grown in LB medium in the absence or presence of 0.3 mM 2,2′-bipyridyl or in M9 medium in the absence or presence of 0.2 mM Fe_2SO_4 for 24 h. The ability of the growth medium to cleave DNA was determined as described in the "Materials and methods" section

of *S. grimesii* 30063 and *S. proteamaculans* 94 for their ability to hydrolyze azocasein. Figure 3a shows that the medium of *S. grimesii* 30063 displayed a proteolytic activity, but this activity was more than tenfold lower than that of *S. proteamaculans* 94. The proteolysis of azocasein was not inhibited by an inhibitor of serine proteases PMSF, whereas strong inhibition of the enzyme by an inhibitor of metalloproteases *O*-phenanthroline was observed.

To identify the protease(s) providing azocasein cleavage in the growth media of *S. grimesii* 30063 and *S. proteamaculans* 94, we used zymography with gelatin. The method is based on the ability of a protease in question to renature after SDS electrophoresis, which results in the cleavage of gelatin in the area of the gel where the protease is present. In this way, an extracellular protease with molecular mass of about 56 kDa was detected in the *S. proteamaculans* 94 medium (Fig. 3a, inset). Both the molecular weight of the protease and its ability to hydrolyze gelatin characterize this protease as serralysin. Serralysin was also detected in the growth medium of *S. grimesii* 30063 (Fig. 3a, inset). However, despite a twofold higher amount of *S. grimesii* 30063 grown in the medium, the yield of *S. grimesii* 30063 serralysin was much lower than that of *S. proteamaculans* 94 (Fig. 3a, inset). As shown in Fig. 3b, a strong increase in *S. proteamaculans* serralysin activity upon iron depletion was registered both by the azocasein assay and zymography. In contrast, serralysin activity of *S. grimesii* was not increased by iron depletion (data not shown).

Extracellular nuclease activity of *S. grimesii* and *S. proteamaculans*

Several bacterial pathogens secrete nucleases that may be involved in bacterial infections (Benedik and Strych 1998; Olson et al. 2013). Specifically, nuclease Nuc of *Staphylococcus aureus* is well known as a virulence factor playing important role in combating the host immune response (Berends et al. 2010; Olson et al. 2013). *S. marcescens* produces a nuclease that is released into the surrounding medium. The enzyme is widely used in studies on gene regulation and protein properties, applied in biotechnology (Benedik and Strych 1998) and is considered (but not shown) to be a virulence factor of *S. marcescens* (Hejazi and Falkiner 1997). Therefore, we have tested *S. grimesii* 30063 and *S. proteamaculans* 94 for an extracellular nuclease activity determined in vitro as a capability of bacterial culture medium to degrade DNA. Figure 4 shows that the nuclease activity of the 24-h LB growth medium of either *S. grimesii* 30063 or *S. proteamaculans* 94 was rather low, but it was strongly increased in the presence of 2,2′-bipyridyl. Consistently, the nuclease activity of synthetic M9 growth medium was higher than that of the corresponding LB medium, and introduction of iron ions to the medium decreased the nuclease activity (Fig. 4). Similar results were obtained for the 48-h growth media (not shown).

The observed effects of iron depletion on the nuclease activity were similar for *S. grimesii* 30063 and *S.*

Author's personal copy

proteamaculans 94, suggesting that the extracellular nuclease activity is not contributed to the differences in the invasive activity of these bacteria. However, a strong dependence of the nuclease activity on iron concentration supports a possible involvement of the nuclease in the regulation of *Serratia* spp. virulence.

Effects of iron depletion on the invasive activity of *S. grimesii* and *S. proteamaculans*

Consistent with the enhanced activities of hemolysin and serralysin in *S. proteamaculans*, the presence of 2,2′-bipyridyl in the growth medium produced a twofold enhancement of the invasive activity of *S. proteamaculans* 94 (Fig. 1b). In contrast, the invasive activity of *S. grimesii* 30063 grown under similar conditions was inhibited (Fig. 1b).

Discussion

Serratia grimesii and *S. proteamaculans* are closely related members of the *S. liquefaciens* group. Therefore, a striking difference in invasive properties of these bacteria demonstrated in our work has been rather surprising. Our results show that penetration of *S. proteamaculans* in eukaryotic cells is several times more efficient than that of *S. grimesii*. Similar to *S. marcescens*, a most virulent strain of the genus *Serratia, S. proteamaculans* produces such virulence factors as hemolysin ShlA and serralysin. In contrast, hemolysin ShlA was not detected in *S. grimesii* by the hemolysis assay. Moreover, in *S. proteamaculans,* production of these virulence factors was strongly enhanced by iron depletion, whereas in *S. grimesii,* the iron-associated regulation of hemolysin and serralysin activity was hardly detectable. These data show that the relatively high invasive activity of *S. proteamaculans* may be due to the contribution of such factors as hemolysin and serralysin whose activity in *S. grimesii* appears to be very low. On the other hand, our results emphasize the role of grimelysin (Bozhokina et al. 2011) which is so far the only known invasion factor of *S. grimesii*. Moreover, hemolysin ShlA of *S. marcescens* is known to be activated during secretion by the ShlB protein (Walker et al. 2004; Pramanik et al. 2014). Both *S. proteamaculans* and *S. grimesii* have ShlB gene. However, a BLAST search revealed that ShlB of *S. proteamaculans* is more closely related to ShlB of *S. marcescens* than ShlB of *S. grimesii*, with 76 and 32 % identity, respectively. These variations may modify the level of hemolysin ShlA activation and thus the virulence of the *Serratia* strains.

It is important to emphasize, however, that a direct comparison of either invasive activities of *S. grimesii and S. proteamaculans* or their virulence factors is not correct because optimal temperatures for the growth of these bacteria are different, being 37 and 30 °C for *S. grimesii and S. proteamaculans,* respectively. It is known that accumulation of enzymes can be increased with the decreasing of temperature (Poole and Braun 1988a; Petersen and Tisa 2012). One more limitation is due to the fact that cultivation of eukaryotic cells and invasion assay are restricted to 37 °C. As *S. grimesii* and *S. proteamaculans* are closely related and their genomes contain hemolysin and serralysin genes, we cannot exclude that the higher invasive potential of *S. proteamaculans* corresponds to stronger accumulation of hemolysin and serralysin at the lower temperature of the bacteria growth. On the other hand, the temperature-independent difference in the regulation of gene expression or protein folding may contribute to the difference in invasive capabilities of these bacteria.

The pore-forming hemolysin ShlA has been shown to trigger invasion of *S. marcescens* in epithelial cells by enhancing vacuolization of the host cells and their lysis, thus playing a major role in the development of *S. marcescens* infections (Hertle and Schwarz 2004). However, the hemolytic-negative derivative of a wild-type *S. marcescens* strain defective in hemolysin production and cytotoxicity still remained invasive, although entered RT112 epithelial cells nearly 90-fold less efficiently than the parental strain (Hertle and Schwarz 2004). It is interesting that the low invasive activity of the mutant *S. marcescens* is comparable with that of the toxin-lacking *S. grimesii* in our experiments. Moreover, the *S. marcescens* genome contains a gene homologous to the grimelysin/protealysin gene. This suggests that the homologous protease may be involved in the *S. marcescens* invasion.

Along with hemolysin, serralysin secreted by *S. marcescens* was shown to be cytotoxic to mammalian cells (Marty et al. 2002). The mutant strains deficient in serralysin production significantly lost cytotoxicity, whereas *E. coli* strains expressing and secreting the recombinant serralysin confer a cytotoxic phenotype toward these cells (Marty et al. 2002). In addition, similarity of serralysin substrate specificity to that of serine proteases allows it to activate transmembrane receptors or to be internalized via the receptor on the host cell membrane, producing a wide variety of intracellular pathological actions (Maeda and Morihara 1995; Miyoshi and Shinoda 2000; Kida et al. 2007). We believe that these serralysin properties promote internalization of *Serratia* within HeLa cells. Moreover, combination of a high proteolytic activity of *S. proteamaculans* serralysin with the relatively low invasive capacity of these facultative pathogenes provides a good model to study intracellular responses of host cells to serralysin.

ECP32/grimelysin and protealysin are thermolysin-like proteases characterized by the specific structural organization of precursor and of the substrate binding site (Demidyuk et al. 2008, 2010). This specificity may

Author's personal copy

be responsible for the narrow substrate specificity of the enzyme toward native proteins (Matveyev et al. 1996). In addition to actin, only histones, melittin and bacterial heat shock protein DnaK have been found to be substrates for ECP32/grimelysin among more than 30 native proteins tested (Matveyev et al. 1996; Morozova et al. 2011). Also, protealysin has been shown to hydrolyze extracellular matrix metalloprotease MMP2 (Tsaplina et al. 2009). Bacterial actin analogs do not contain the specific sequence that is cleaved in actin (van den Ent et al. 2001, 2002). It is plausible therefore that grimelysin and protealysin promote invasion being injected into host cell, which was confirmed by revealing protealysin in the cytoplasm of the infected cells (Tsaplina et al. 2012). It is clear, however, that the initial steps of bacterial internalization should be promoted by other factors. We show here that the increased activities of hemolysin and serralysin in the *S. proteamaculans* growth medium under the iron depletion correlate with the increased invasive activity of these bacteria. These results allow us to conclude that the invasion of *S. proteamaculans* is determined by a combination of extracellular hemolysin and serralysin with intracellular protealysin. Further work is needed to reveal analogous partners of grimelysin in invasive activity of *S. grimesii*.

Acknowledgments The work was supported by the Russian Foundation for Basic Research (Grant Numbers 14-04-00316, 14-04-31085 and 13-04-90799) and by the Program for Molecular and Cell Biology of the Russian Academy of Sciences.

References

Benedik MJ, Strych U (1998) *Serratia marcescens* and its extracellular nuclease. FEMS Microbiol Lett 165(1):1–13

Berends ET, Horswill AR, Haste NM, Monestier M, Nizet V, von Köckritz-Blickwede M (2010) Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps. J Innate Immun 2(6):576–586. doi:10.1159/000319909

Bollet C, Grimont P, Gainnier M, Geissler A, Sainty JM, De Micco P (1993) Fatal pneumonia due to *Serratia proteamaculans* subsp. quinovora. J Clin Microbiol 31(2):444–445

Bozhokina E, Khaitlina S, Adam T (2008) Grimelysin, a novel metalloprotease from *Serratia grimesii*, is similar to ECP32. Biochem Biophys Res Commun 367(4):888–892. doi:10.1016/j.bbrc.2008.01.003

Bozhokina ES, Tsaplina OA, Efremova TN, Kever LV, Demidyuk IV, Kostrov SV, Adam T, Komissarchik YY, Khaitlina SY (2011) Bacterial invasion of eukaryotic cells can be mediated by actin-hydrolysing metalloproteases grimelysin and protealysin. Cell Biol Int 35(2):111–118. doi:10.1042/CBI20100314

Bozhokina E, Vakhromova E, Gamaley I, Khaitlina S (2013) *N*-acetylcysteine increases susceptibility of HeLa cells to bacterial invasion. J Cell Biochem 114(7):1568–1574. doi:10.1002/jcb.24498

Cossart P, Sansonetti PJ (2004) Bacterial invasion: the paradigms of enteroinvasive pathogens. Science 304(5668):242–248

Demidyuk IV, Kalashnikov AE, Gromova TY, Gasanov EV, Safina DR, Zabolotskaya MV, Rudenskaya GN, Kostrov SV (2006) Cloning, sequencing, expression, and characterization of protealysin, a novel neutral proteinase from *Serratia proteamaculans* representing a new group of thermolysin-like proteases with short N-terminal region of precursor. Protein Expr Purif 47(2):551–561

Demidyuk IV, Gasanov EV, Safina DR, Kostrov SV (2008) Structural organization of precursors of thermolysin-like proteinases. Protein J 27(6):343–354. doi:10.1007/s10930-008-9143-2

Demidyuk IV, Gromova TY, Polyakov KM, Melik-Adamyan WR, Kuranova IP, Kostrov SV (2010) Crystal structure of the protealysin precursor: insights into propeptide function. J Biol Chem 285(3):2003–2013. doi:10.1074/jbc.M109.015396

Efremova T, Ender N, Brudnaja M, Komissarchik Y, Khaitlina S (2001) Specific invasion of transformed cells by *Escherichia coli* A2 strain. Cell Biol Int 25(6):557–561

Gamaley I, Efremova T, Kirpichnikova K, Kever L, Komissarchik Y, Polozov Y, Khaitlina S (2006) *N*-acetylcysteine-induced changes in susceptibility of transformed eukaryotic cells to bacterial invasion. Cell Biol Int 30(4):319–325

Grimont F, Grimont PAD (2006) The genus Serratia. Prokaryotes 6:219–244. doi:10.1007/0-387-30746-X_11

Gromova TY, Demidyuk IV, Kozlovskiy VI, Kuranova IP, Kostrov SV (2009) Processing of protealysin precursor. Biochimie 91(5):639–645. doi:10.1016/j.biochi.2009.03.008

Hejazi A, Falkiner FR (1997) *Serratia marcescens*. J Med Microbiol 46(11):903–912

Hertle R (2005) The family of *Serratia* type pore forming toxins. Curr Protein Pept Sci 6(4):313–325

Hertle R, Schwarz H (2004) *Serratia marcescens* internalization and replication in human bladder epithelial cells. BMC Infect Dis 4:16

Ishii K, Adachi T, Hamamoto H, Sekimizu K (2014) *Serratia marcescens* suppresses host cellular immunity via the production of an adhesion-inhibitory factor against immunosurveillance cells. J Biol Chem 289(9):5876–5888. doi:10.1074/jbc.M113.544536

Khaitlina SY, Collins JH, Kuznetsova IM, Pershina VP, Synakevich IG, Turoverov KK, Usmanova AM (1991) Physico-chemical properties of actin cleaved with bacterial protease from *E. coli* A2 strain. FEBS Lett 279(1):49–51

Kida Y, Inoue H, Shimizu T, Kuwano K (2007) *Serratia marcescens* serralysin induces inflammatory responses through protease-activated receptor 2. Infect Immun 75(1):164–174

Kwak J, Lee K, Shin DH, Maeng JS, Park DS, Oh HW, Son KH, Bae KS, Park HY (2007) Biochemical and genetic characterization of arazyme, an extracellular metalloprotease produced from *Serratia proteamaculans* HY-3. J Microbiol Biotechnol 17(5):761–768

Maeda H, Morihara K (1995) Serralysin and related bacterial proteinases. Methods Enzymol 248:395–413

Mahlen SD (2011) *Serratia* infections: from military experiments to current practice. Clin Microbiol Rev 24(4):755–791. doi:10.1128/CMR.00017-11

Marty KB, Williams CL, Guynn LJ, Benedik MJ, Blanke SR (2002) Characterization of a cytotoxic factor in culture filtrates of *Serratia marcescens*. Infect Immun 70(3):1121–1128

Matveyev VV, Usmanova AM, Morozova AV, Collins JH, Khaitlina SY (1996) Purification and characterization of the proteinase ECP 32 from *Escherichia coli* A2 strain. Biochim Biophys Acta 1296(1):55–62

Miyoshi S, Shinoda S (2000) Microbial metalloproteases and pathogenesis. Microbes Infect 2(1):91–98

Morozova AV, Khaitlina SY, Malinin AY (2011) Heat shock protein DnaK—substrate of actin-specific bacterial protease ECP32. Biochemistry (Mosc) 76(4):455–461

Olson ME, Nygaard TK, Ackermann L, Watkins RL, Zurek OW, Pallister KB, Griffith S, Kiedrowski MR, Flack CE, Kavanaugh JS, Kreiswirth BN, Horswill AR, Voyich JM (2013) *Staphylococcus aureus* nuclease is an SaeRS-dependent virulence factor. Infect Immun 81(4):1316–1324. doi:10.1128/IAI.01242-12

Author's personal copy

Petersen LM, Tisa LS (2012) Influence of temperature on the physiology and virulence of the insect pathogen *Serratia* sp. Strain SCBI. Appl Environ Microbiol 78(24):8840–8844. doi:10.1128/AEM.02580-12

Poole K, Braun V (1988a) Influence of growth temperature and lipopolysaccharide on hemolytic activity of *Serratia marcescens*. J Bacteriol 170(11):5146–5152

Poole K, Braun V (1988b) Iron regulation of *Serratia marcescens* hemolysin gene expression. Infect Immun 56(11):2967–2971

Pramanik A, Könninger U, Selvam A, Braun V (2014) Secretion and activation of the *Serratia marcescens* hemolysin by structurally defined ShlB mutants. Int J Med Microbiol 304(3–4):351–359. doi:10.1016/j.ijmm.2013.11.021

Prouty AM, Gunn JS (2000) *Salmonella enterica* serovar typhimurium invasion is repressed in the presence of bile. Infect Immun 68(12):6763–6769

Tsaplina OA, Efremova TN, Kever LV, Komissarchik YY, Demidyuk IV, Kostrov SV, Khaitlina SY (2009) Probing for actinase activity of protealysin. Biochemistry (Mosc) 74(6):648–654

Tsaplina O, Efremova T, Demidyuk I, Khaitlina S (2012) Filamentous actin is a substrate for protealysin, a metalloprotease of invasive *Serratia proteamaculans*. FEBS 279(2):264–274. doi:10.1111/j.1742-4658.2011.08420.x

Ursua PR, Unzaga MJ, Melero P, Iturburu I, Ezpeleta C, Cisterna R (1996) *Serratia rubidaea* as an invasive pathogen. J Clin Microbiol 34(1):216–217

Usmanova AM, Khaĭtlina SI (1989) Protease from a strain of bacteria *E. coli* A2, specifically cleaving actin. Biokhimiia 54(8):1308–1314

van den Ent F, Amos LA, Löwe J (2001) Prokaryotic origin of the actin cytoskeleton. Nature 413(6851):39–44

van den Ent F, Møller-Jensen J, Amos LA, Gerdes K, Löwe J (2002) F-actin-like filaments formed by plasmid segregation protein ParM. EMBO J 21(24):6935–6943

Walker G, Hertle R, Braun V (2004) Activation of *Serratia marcescens* hemolysin through a conformational change. Infect Immun 72(1):611–614