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Influence of Temperature on the Physiology and Virulence of the Insect Pathogen *Serratia* sp. Strain SCBI

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The physiology of a newly recognized *Serratia* species, termed South African *Caenorhabditis briggsae* Isolate (SCBI), which is both a nematode mutualist and an insect pathogen, was investigated and compared to that of *Serratia marcescens* Db11, a broad-host-range pathogen. The two *Serratia* strains had comparable levels of virulence for *Manduca sexta* and similar cytotoxic activity patterns, but motility and lipase and hemolytic activities differed significantly between them.

Members of the genus *Serratia* are found widespread around the globe and are well known for their roles as insect pathogens (14). A newly recognized *Serratia* species, termed South African *Caenorhabditis briggsae* Isolate (SCBI), was identified following its isolation from the nematode *C. briggsae* KT0001 (2). These *C. briggsae* KT0001 nematodes were recovered from soil samples through *Galleria mellonella* bait traps in three provinces in South Africa (2). *Serratia* sp. strain SCBI is lethal to *G. mellonella*. When directly injected into the hemocoel in numbers of less than 1,000 CFU, larvae die within 72 h. A distinct characteristic of *Serratia* sp. SCBI is that it forms an apparent association with *C. briggsae* KT0001 as well as other *Caenorhabditis* nematodes, including *Caenorhabditis elegans*, which results in entomopathogenicity of the nematode (1, 2). This microbe-nematode association between *Serratia* sp. SCBI and *C. briggsae* may represent a potential emerging entomopathogenic association. Only two other *Serratia* species are known to use a nematode partner to establish an infection in an invertebrate host (40, 43).

Based on 16S phylogeny, *Serratia* sp. SCBI is closely related to *S. marcescens* Db11 (3), a reported pathogen of *C. elegans* (24, 34, 36). We have sequenced the entire *Serratia* sp. SCBI genome and have performed an analysis comparing it to the *S. marcescens* Db11 genome (F. Abebe-Akele, L. S. Tisa, V. Cooper, P. J. Hatcher, E. Abebe, and W. K. Thomas, unpublished data). *S. marcescens* Db11 is a well-known pathogen of vertebrates and invertebrates, including *Caenorhabditis* nematodes. Although their 16S RNA genes are 99% identical, genome-wide assessment (23) supports the idea that *Serratia* sp. SCBI and *S. marcescens* Db11 represent two distinct species. However, the high (about 80%) similarity at the 16S rRNA gene and within open reading frames indicates that *Serratia* sp. SCBI and *S. marcescens* Db11 share a close evolutionary relationship despite their distinct associations with *Caenorhabditis* nematodes. Since mutualistic associations have the potential to evolve from parasitic relationships (10), it is possible that *Serratia* sp. SCBI diverged from *S. marcescens*, making the leap from *Caenorhabditis* pathogen to a mutualistic relationship.

Although the virulence factors of *S. marcescens* have been well studied (6, 8, 24, 26, 33), there have been no studies on the physiological properties of *Serratia* sp. SCBI and their contribution to pathogenesis. Due to their close evolutionary history, a comparative study of *Serratia* sp. SCBI and *S. marcescens* Db11 was conducted to determine if they shared putative virulence factors such as exoenzymatic activity and cytotoxicity. In addition, the pathogenicity of these bacteria was assessed on a well-known insect model system, *Manduca sexta*. Since temperature affects virulence factor activities in *S. marcescens* Db11 (21, 25, 33), the effect of temperature on the putative virulence traits of both *Serratia* sp. was investigated at 22, 28, and 37°C, which represent soil, an intermediate, and vertebrate host temperatures, respectively.

Serratia sp. SCBI, *S. marcescens* Db11, and *Escherichia coli* EPI300 were grown in an overnight shake culture in Luria-Bertani (LB) broth at 22, 28, or 37°C for all assays. The effect of temperature on growth rate for both *Serratia* strains was determined, and results indicate that growth rate is not a major factor accounting for the differences in physiological activities investigated in this study (data not shown).

Regulatory networks linking motility and virulence have been elucidated in *S. marcescens* (26), and therefore motility was investigated in *Serratia* sp. SCBI and *S. marcescens* Db11. The plate migration assay (20) was used to investigate the swimming behavior of the *Serratia* strains. Temperature had a strong effect on swim ring formation (Fig. 1A; see also Fig. S1 in the supplemental material). For *Serratia* sp. SCBI, swim ring formation was faster (9.5 mm/h) at 37°C than at 28°C and 22°C ($P < 0.001$). A similar pattern was observed with *S. marcescens* Db11, with 37°C as the optimum temperature (6.5 mm/h; $P < 0.001$). In addition to the swimming behavior, we observed that *Serratia* sp. SCBI and *S. marcescens* Db11 exhibited swarming, or surface movement, utilizing methods described previously (31). *Serratia* sp. SCBI exhibited optimal swarming motility on medium with low agar concentrations and at elevated temperatures but was also capable of swarming movement on medium with elevated (1.25%) agar concentrations at 28°C (Fig. 1B; see also Fig. S2 in the supplemental material). *S. marcescens* Db11 similarly demonstrated optimal swarming on medium with low agar concentrations and at elevated temperatures but did not swarm on medium with agar concentrations greater than 0.85%.

Serratia spp. secrete multiple extracellular enzymes such as proteases, lipases, and chitinases that can contribute to their

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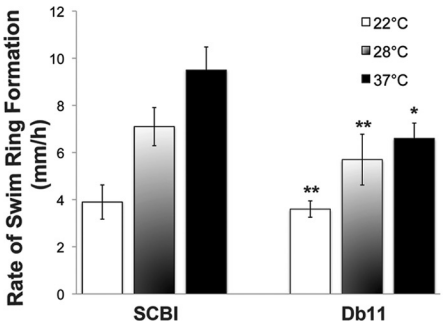
A



B

	Serratia sp. SCBI			*S. marcescens* Db11		
% Agar	22°C	28°C	37°C	22°C	28°C	37°C
0.65	49 ± 15	88 ± 0	84 ± 1	57 ± 17	80 ± 8	67 ± 17*
0.85	45 ± 16	88 ± 0	63 ± 18	19 ± 5*	52 ± 9*	14 ± 2*
1.05	19 ± 4	58 ± 13	20 ± 13	8 ± 1*	12 ± 2*	12 ± 1
1.25	9 ± 2	21 ± 7	13 ± 3	8 ± 1	11 ± 2**	13 ± 2

FIG 1 Temperature influences swimming and swarming behavior. (A) Average rates of swim ring formation for *Serratia* sp. SCBI (SCBI) and *S. marcescens* Db11 (Db11) at 22, 28, and 37°C. Results are shown as the averages of 6 measurements from 2 independent experiments, with the standard deviations indicated by error bars. (B) Swarm ring diameter measurements (expressed in millimeters) at 48 h. The maximum measurement was 88 mm (the width of the petri dish). Results are shown as the averages of 9 measurements ± standard deviations from 3 independent experiments (3 measurements each). ** ($P < 0.01$) and * ($P < 0.001$) denote significant differences from *Serratia* sp. SCBI at the same temperature.

pathogenicity (11, 15, 19, 22, 28). Protease, gelatinase, DNase, chitinase, and siderophore activities were determined using agar plate assays described previously (17, 32, 37). *Serratia* sp. SCBI secreted a wide variety of exoenzymes and had siderophore activity (see Table S1 in the supplemental material). At 28°C and 37°C, both *Serratia* sp. SCBI and *S. marcescens* Db11 exhibited gelatinase, DNase, and chitinase activities on agar test media that were higher than those seen at the lower temperature ($P < 0.01$). Protease and lipase activities were also determined by quantitative liquid assays using methods described previously (5, 42). For quantitative liquid assays, 28°C was the optimal temperature for protease and lipase production (see Fig. S3 in the supplemental material). At 37°C, both bacterial species had levels of protease and lipase activities significantly lower than those seen at 28°C ($P < 0.01$). Although *Serratia* sp. SCBI and *S. marcescens* Db11 protease activities did not differ significantly at any temperature, *Serratia* sp. SCBI showed a significantly higher level of lipase activity than *S. marcescens* Db11 at 22°C and 37°C. Furthermore, the lipase and protease activities of both *Serratia* spp. were growth phase dependent and found only with post-exponential-growth-phase cells (data not shown).

Hemolytic activity is an important virulence factor for many pathogens (4, 18). For *S. marcescens* Db11, hemolytic activity is required for *C. elegans* or *Drosophila melanogaster* pathogenesis (24) and plays a role in cytotoxic activity against the human bronchial epithelial cell line 16HBE14o- (24). Hemolytic activity was measured using a modified liquid assay (16) which measured the rate of sheep red blood cell (SRBC) lysis by bacterial culture (~4.0×10^6 cells) over 4 h. *Serratia* sp. SCBI showed a rate of hemolytic activity at 22°C that was lower than that seen at the other 2 temperatures ($P < 0.001$) (Fig. 2). Hemolytic activity occurred at similar levels at 28 and 37°C. SRBC lysis initiated after 1 h of incubation and was completed by 3 h. *S. marcescens* Db11 exhibited an optimal rate of hemolysis at 28°C compared to 22°C and 37°C ($P < 0.001$). At the 2-h time point, the hemolytic activities of *Serratia* sp. SCBI and *S. marcescens* Db11 were similar at 22°C and 28°C. However, at 37°C, *Serratia* sp. SCBI had significantly greater hemolytic activity (lysing 64% of the SRBC population) at the 2-h time point than *S. marcescens* Db11 ($P < 0.001$), which lysed ~33% of the population. In contrast to protease and lipase activities, hemolytic activity was not influenced by growth phase (data not shown).

Experiments were performed to determine the location of *Serratia* sp. SCBI hemolytic activity. Untreated culture had the highest rate of hemolysis, lysing 96% of SRBCs at 3 h, whereas bacterial cells washed in phosphate-buffered saline (PBS) lysed an average of 29% of SRBCs at 3 h (data not shown). Reduced activity of the washed cells likely resulted from removal of surface-associated hemolysins during washing. Cell lysates also showed drastically reduced activity in comparison to culture, with an average of only 15% of SRBCs lysed after 3 h. Formalin-killed *Serratia* sp. SCBI or cell-free supernatant showed no hemolysis. These results indicate the hemolysin was not secreted but might have been surface associated, requiring live cells for functionality.

Cytotoxins are well-known virulence factors for bacterial pathogens because they can cause extensive damage to host cell morphology, resulting in cell death (8, 27, 35). *S. marcescens* culture filtrates are cytotoxic to a number of different mammalian cell lines (6, 8), with a protease, a hemolysin, or a combination of factors being identified as the agents responsible for these activities (17, 29). Therefore, both *Serratia* sp. were investigated for

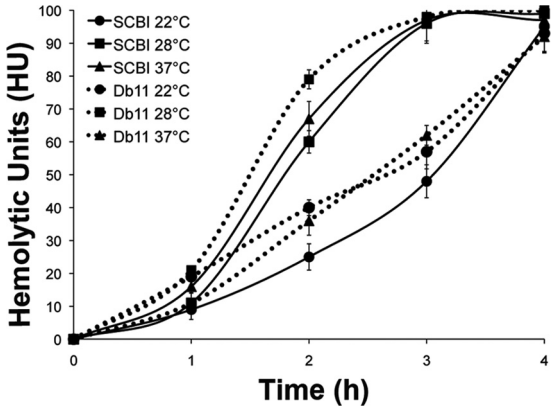


FIG 2 Comparison of the rates of hemolysis by *Serratia* sp. SCBI and *S. marcescens* Db11 at different incubation temperatures. Hemolytic activity against SRBCs was measured over 4 h of incubation. Bacterial cultures (≥4.0×10^6 CFU) were assayed for hemolytic activity at their growth temperature. Solid lines represent *Serratia* sp. SCBI, and dashed lines represent *S. marcescens* Db11. Values are the averages of 6 measurements from 2 independent experiments (3 measurements each), with the standard deviations indicated by error bars.

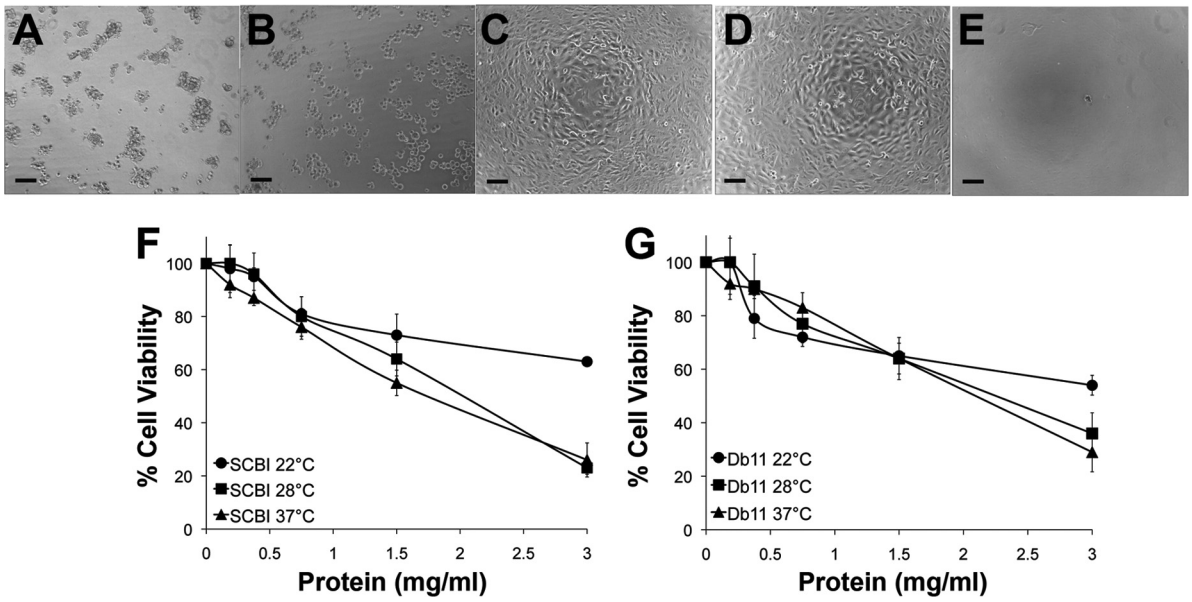


FIG 3 Cytotoxic effects of filter-sterilized bacterial supernatant fluids from *Serratia* sp. SCBI, *S. marcescens* Db11, and *E. coli* EPI300 on BGMK cells after 24 h. (A to E) Photomicrographs (100× magnification) of BGMK cells treated with supernatant fluids at a concentration of 1.5 mg/ml total protein. Bars, 100 μm. The supernatant was from bacterial cultures grown at 37°C. (A) *Serratia* sp. SCBI, (B) *S. marcescens* Db11, (C) *E. coli* EPI300, (D) no-treatment control, (E) 2% SDS. (F and G) Cell viability measurements by MTT assay after 24 h of exposure at 37°C to bacterial supernatant fluids from (F) *Serratia* sp. SCBI or (G) *S. marcescens* Db11. Values are the averages of 6 measurements from 2 independent experiments (3 measurements each), with the standard deviations indicated by error bars.

cytotoxic effects on Buffalo green monkey kidney (BGMK) cells following a 24-h exposure to bacterial supernatants grown at the 3 test temperatures. Various levels (0.18 to 3.0 mg/ml) of protein concentrations of the supernatant were also tested, and cell viability was measured using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay (29, 41). BGMK cells exposed to *Serratia* sp. SCBI and *S. marcescens* Db11 supernatant fluids from cultures grown at 37°C appeared unhealthy, rounding upward and detaching from the surface of the plates (Fig. 3A and B). There was no significant difference between *Serratia* sp. SCBI and *S. marcescens* Db11 in cytotoxic effects at 3 mg/ml protein concentration (Fig. 3F and G). Cultures grown at 28°C and 37°C were significantly more toxic than cultures grown at 22°C. Between 20% and 30% of BGMK cells remained viable after exposure to 28°C and 37°C cultures, while 60% of cells were viable when exposed to 22°C cultures. Cell lysates from both strains were also tested and produced results similar to those seen with the bacterial supernatant (data not shown).

Serratia spp. are well-known insect pathogens that infect a variety of invertebrates (12, 14, 24, 39). To assess the virulence of *Serratia* sp. SCBI and *S. marcescens* Db11, virulence was measured using the *M. sexta* model system and a modified method described previously (7). Larvae were injected through the larval head with 10 μl of bacterial suspension (500, 4.0×10^4, or 1.0×10^5 CFU) from cultures grown at the 3 test temperatures. Larvae were monitored for mortality and delays in development over 7 days. In addition, larvae were weighed on day 0 and day 7 or on the day of death. Larva injection resulted in mortality, and there were noticeable defects in insect development (Table 1; see also Fig. S4 in the supplemental material). Elevated temperatures and cell dosage increased the virulence of both *Serratia* strains. There were no significant differences observed between the bacterial strains, as they had similar mortality rates and LT_{50} values (the latter representing the average time by which 50% of the larval population had died), though *S. marcescens* Db11 had a slightly higher rate of killing. The controls, *E. coli* EPI300 or LB broth, showed no insect mortality or developmental effects. Insect larvae that survived *Serratia* infection for 7 days showed stunted development and often turned pale green and pink around the head and legs. Those larvae injected with *Serratia* most likely would have succumbed to infection with continued incubation.

Thermoregulation of virulence genes occurs in numerous bacterial pathogens, including *Yersinia*, *Shigella*, and *Aeromonas* (9, 30, 38), and the optimal temperature for their expression often reflects the host environment. The ability of *S. marcescens* Db11 to produce virulence-associated phenotypes over a wide range of temperatures is reflective of its ability to infect both vertebrate and invertebrate hosts. Our results indicate that *Serratia* sp. SCBI and *S. marcescens* Db11 share similar pathogenic and cytotoxic effects. They also shared a wide variety of virulence-associated properties and, in some cases, had similar temperature-dependent responses. These results suggest that *Serratia* sp. SCBI may potentially have the same capabilities for infecting a wide host range.

Since *Serratia* sp. SCBI and *S. marcescens* Db11 differed in their associations with *Caenorhabditis* nematodes, we expected that there would be some physiological differences between these two strains. Patterns of motility and lipase activity shown by *Serratia* sp. SCBI were different. The observed differences in surface swarming were striking and may play a role in insect colonization or nematode interactions. *Serratia* sp. SCBI bacteria were able to swarm on media with a wide range of agar concentrations (Fig. 1), suggesting that these bacteria are able to move on surfaces with different viscosities or degrees of wetness. Furthermore, we expected *Serratia* sp. SCBI to have altered hemolysis patterns since this activity is a virulence factor for *S. marcescens* Db11 against *Caenorhabditis* nematodes (24). At elevated temperatures, *Serra-*

TABLE 1 Effect of *Serratia* dosage and temperature on insect mortality and health[a]

Strain and inoculum (CFU 10 μl^{-1})	22°C			28°C			37°C		
	Mortality (%) by day 7	LT_{50} (no. of days ± SD)	Change in wt (%)	Mortality (%) by day 7	LT_{50} (no. of days ± SD)	Change in wt (%)	Mortality (%) by day 7	LT_{50} (no. of days ± SD)	Change in wt (%)
Serratia spp.									
Serratia sp. SCBI									
500	40	>7.0	+23.2	50	4.0	+51.1	70	5.6	+85.9
4.0×10^4	50	5.0 ± 1.6	+32.2	60	2.4	+25.8	100	2.8	+2.6
1.0×10^5	70	2.3 ± 1.9	+5.6	80	2.2	+9.2	100	1.0	Negative
S. marcescens Db11									
500	40	>7.0	+6.7	60	4.8	+14.7	80	3.6	+18.7
4.0×10^4	90	3.3 ± 1.9	+5.9	60	3.0	+15.1	100	1.8	Negative
1.0×10^5	90	3.3 ± 2.0	Negative	100	1.0	Negative	100	1.0	Negative
Controls									
E. coli EPI300									
1.0×10^5	0	−	+49.8	0	−	+108.9	0	−	+141.2
LB	0	−	100	0	−	100	0	−	100

[a] *M. sexta* larvae were injected with each dosage and incubated at the indicated test temperature. Insect health was monitored daily, and insect mortality was noted. Values represent the results of 2 independent experiments. Mortality values represent the total percentage of the larval population that died during the 7-day incubation period. LT_{50} values represent the average time for 50% of the larval population to die. Percent change in weight values represent the average change in weight of the population during the 7-day period (or by the day of death) and were normalized to the average weight of larvae injected with LB. Negative values indicate that there was an average decrease in larval weight following injection.

tia sp. SCBI showed significantly greater hemolytic activity than *S. marcescens* Db11 (Fig. 2), but we found several similarities between these two organisms. First, *Serratia* sp. SCBI and *S. marcescens* Db11 shared similar rates of hemolysis at lower temperatures. Second, the modes of action for hemolysis of the two bacterial strains may be similar. Cell-to-cell contact is required for full hemolytic activity by *S. marcescens* (13). Our results show that *Serratia* sp. SCBI also required contact between live bacteria and blood cells for hemolytic activity. Filter-sterilized supernatant fluids and formalin-killed cells were not hemolytic. Cell lysates had reduced hemolytic activity. However, cell lysates or supernatants from *Serratia* sp. SCBI were cytotoxic toward the BGMK cell line (Fig. 3). This result indicates that there may be two separate toxicity mechanisms for mammalian blood cells and epithelial cells.

Although only three *Serratia* species are currently known to form mutualistic relationships with nematodes (2, 40, 43), all three species share close evolutionary histories and appear to be at the nascent stages of mutualistic microbe-nematode relationships. Our study results indicate that *Serratia* sp. SCBI and *S. marcescens* Db11 could harbor similar virulence factors required for insect pathogenesis, but their relationship with *Caenorhabditis* nematodes clearly separates these two strains. Future research could shed light on how *Serratia* sp. SCBI has made the switch from *Caenorhabditis* nematode pathogen to mutualist and whether regulation of their virulence factors plays a role in altering their relationship with nematodes.

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