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Simkania negevensis, an Example of the Diversity of the Antimicrobial Susceptibility Pattern among *Chlamydiales*

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ABSTRACT In past years, several *Chlamydia*-related bacteria have been discovered, including *Simkania negevensis*, the founding member of the *Simkaniaceae* family. We evaluated the antimicrobial susceptibility patterns of this emerging intracellular bacterium and highlighted significant differences, compared with related *Chlamydiales* members. *S. negevensis* was susceptible to macrolides, clindamycin, cyclines, rifampin, and quinolones. Importantly, unlike other *Chlamydiales* members, treatment with β-lactams and vancomycin did not induce the formation of aberrant bodies, leading to a completely resistant phenotype.

KEYWORDS *Chlamydiales*, *Simkaniaceae*, intracellular bacteria

Received 28 March 2017 **Returned for modification** 19 April 2017 **Accepted** 8 May 2017

Accepted manuscript posted online 30 May 2017

Citation Vouga M, Baud D, Greub G. 2017. *Simkania negevensis*, an example of the diversity of the antimicrobial susceptibility pattern among *Chlamydiales*. Antimicrob Agents Chemother 61:e00638-17. https://doi.org/10.1128/AAC.00638-17.

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Rapid progress in diagnostic techniques has enabled the discovery of several novel *Chlamydia*-related bacteria, including *Simkania negevensis*. Mostly known for the pathogenic *Chlamydia* spp., the *Chlamydiales* order is now composed of at least 9 family-level lineages (1), each with specific biological characteristics. *S. negevensis* is the founding member of the *Simkaniaceae* family and represents an emerging pathogen previously associated with respiratory diseases, at least in the Middle East (2, 3). Infections were empirically treated with a macrolide-based regimen (4). Several differences regarding antimicrobial susceptibility have been highlighted among the different *Chlamydiales* family-level lineages (5, 6). Therefore, we investigated the antibiotic susceptibility of the *Simkaniaceae* family, which remains poorly studied, using *S. negevensis* as a model. We provide subsequent information on the evolution of antimicrobial resistance in this order, as well as potential therapeutic options.

Simkania negevensis strain Z was grown at 37°C in Vero cells in 25-cm^2 cell culture flasks (Corning, USA), in Dulbecco's modified essential medium (DMEM) (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS), with 5% CO_2. A 6- or 7-day-old coculture, diluted 1:1,000, was used to inoculate fresh A549 cells or Vero cells that had been seeded previously at 1.5×10^5 cells/ml on a 24-well plate (Corning), as described previously (7). At 2 h postinfection, the medium was changed for medium containing 2-fold serial dilutions of various antibiotics. Antibiotic-free wells served as growth controls, while uninfected cells served as negative controls. Twelve antibiotics from 8 different classes were used in this study. MICs were defined as the minimal concentrations that prevented bacterial growth at day 6, compared to a control infection performed in the absence of antibiotics. Growth at day 2 was also assessed for β-lactams, fosfomycin, and vancomycin, to ensure the absence of effects due to instability of the compounds after 48 h at 37°C. An in-house specific quantitative PCR targeting the 16S rRNA gene was used to quantify *S. negevensis* DNA, as described

TABLE 1 Antibiotic susceptibility of *Simkania negevensis*, compared to others *Chlamydiales*[a]

Drug	MIC (μg/ml)					
					Chlamydiaceae	
	Simkaniaceae, *S. negevensis* (this study)[b]	*Parachlamydiaceae*, *Parachlamydia acanthamoebae* (8)[c]	*Waddliaceae*, *W. chondrophila* (5, 11)[b]	*Criblamydiaceae*, *E. lausannensis* (6)[b]	*C. trachomatis* (10, 21–24)[b]	*Chlamydia pneumoniae* (11, 21)[b]
Cyclines						
Tetracycline	2	ND	ND	0.25	0.25–0.5	0.125–0.5
Doxycycline	0.5	2–4	0.25	0.25	0.03–0.25	0.02–0.5
Lincosamide						
Clindamycin	1	ND	2–4	ND	0.25–2	ND
Macrolides						
Erythromycin	ND	0.06	ND	ND	0.02–2	0.02–0.25
Clarithromycin	ND	<0.06	ND	ND	0.02–0.125	0.004–0.125
Azithromycin	<0.06	ND	0.25	2	0.6–2	0.02–0.5
β-Lactams						
Penicillin derivatives	>1,000	>32	>32	>32	0.25–2	5
Ceftriaxone	>1,000	>32	>32	>32	16–32	ND
Phosphonic acid derivative						
Fosfomycin	>1,000	ND	500	ND[d]	500–1,000	>1,000
Glycopeptide						
Vancomycin	>1,000	ND	ND	ND	1,000	1,000
Fluoroquinolones						
Ciprofloxacin	4	>16	>16	32	0.5–2	1–4
Ofloxacin	1	>16	>16	16	0.5–1	0.5–2
Levofloxacin	0.5	ND	ND	ND	0.12–0.5	0.25–1
Rifamycin						
Rifampin	<0.06	0.25–0.5	ND	ND	<0.125 to 1	<0.125

[a]Shown are the MICs of various antibiotics against members of the *Chlamydiales* orders (5, 6, 8, 10, 11, 21–24). This table was adapted from reference 8 with permission. ND, not done.
[b]Tested in mammalian cells.
[c]Tested in amoebae.
[d]*Criblamydiaceae* present the Cys115-to-Asp substitution in the active site of MurA, which is known to confer resistance to fosfomycin in *Chlamydia* spp.

previously (7). The absence of antibiotic toxicity toward cells was determined by examining the microplates using an inverted microscope (Zeiss Axiovert 25; Carl Zeiss). When solvents other than distilled water (i.e., dimethyl sulfoxide [DMSO], 0.1 M HCl, and 1 M NaOH) were used to suspend antibiotic solutions, the absence of effects of these solvents on *S. negevensis* growth was assessed.

Like other *Chlamydiales* species, *S. negevensis* was susceptible to macrolides, clindamycin, cyclines, and rifampin (Table 1). Interestingly, *S. negevensis* was susceptible to quinolones; while *Chlamydiaceae* are sensitive, other *Chlamydia*-related bacteria, such as *Waddlia chondrophila*, *Parachlamydia* spp., and *Estrella lausannensis*, are resistant (5, 6, 8). Previous work suggested that *S. negevensis* was resistant to ciprofloxacin (9). In that study, MICs were determined in amoebae, as the minimal concentrations that prevented amoebal lysis. The observed results might have been due to the presence of an efflux pump in amoebae and decreasing quinolone bioavailability. Although several mutations in the *gyrA* and *parC* quinolone resistance-determining regions (QRDRs) were identified, they differed from those observed in resistant *Chlamydia*-related bacteria, which may explain the observed absence of resistance (6, 9).

S. negevensis was resistant (MICs of >32 μg/ml) to three kinds of cell wall inhibitors, i.e., β-lactams, fosfomycin, and vancomycin. *Chlamydiales* members lack the traditional peptidoglycan (PG) layer. However, partial susceptibility to β-lactams is observed among *Chlamydia* spp., which are known to form aberrant bodies when treated with

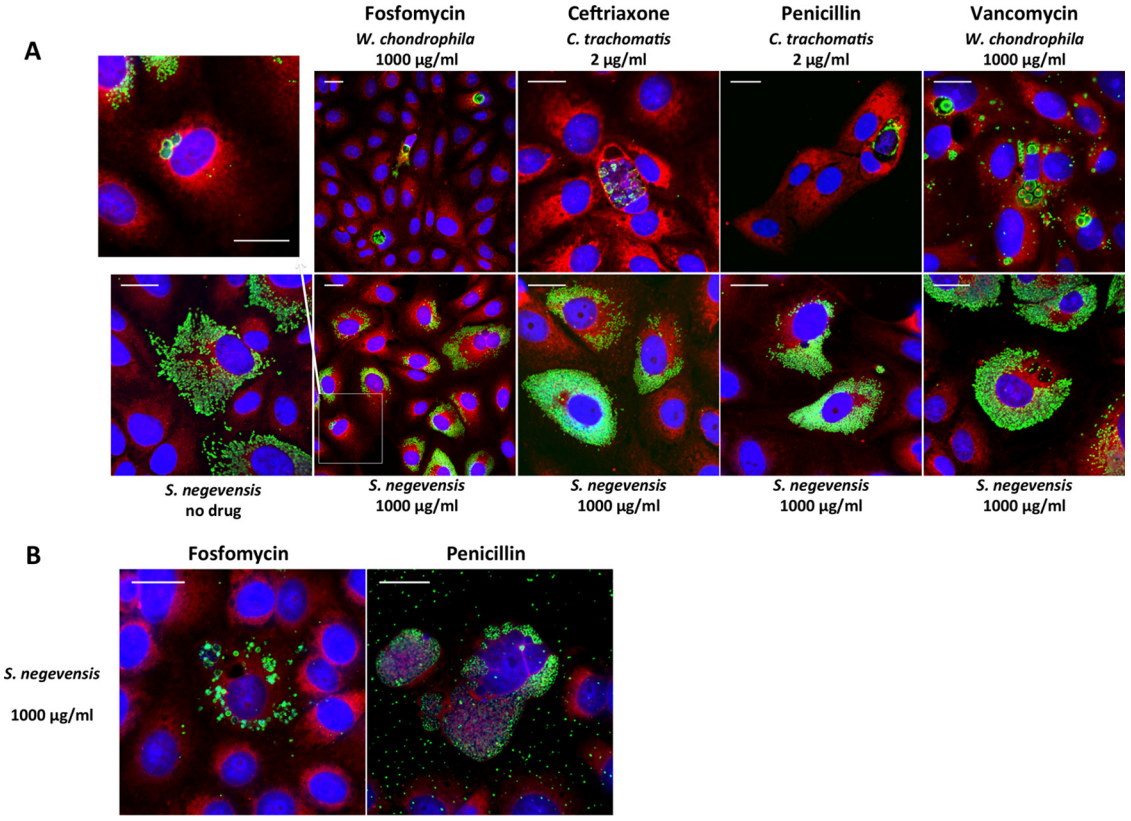

FIG 1 Effects of cell wall inhibitors on *Simkania negevensis* infection and morphology. The growth of *S. negevensis* was observed by immunofluorescence, in the presence or absence of cell wall inhibitors. (A) Effects of β-lactam, fosfomycin, and vancomycin treatment in Vero cells at 48 h postinfection. *S. negevensis*, *Chlamydia trachomatis* strain UW-3/Cx, and *Waddlia chondrophila* strain WSU 86-1044 (ATCC VR-1470) were detected using a polyclonal anti-*S. negevensis* rabbit antibody (1:2,500), a mouse anti-major outer membrane porin (MOMP) antibody (1:50) (ab20881; Abcam, Cambridge, UK), or an anti-*W. chondrophila* rabbit antibody (1:2,000), respectively (green), followed by a secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse or anti-rabbit antibody [1:500]; Molecular Probes, Thermo Fisher Scientific, Waltham, MA), mammalian cells were stained with Texas red-conjugated concanavalin A (1:50) (red), and nucleic acids were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:1,000) (blue). (B) Effects of fosfomycin and penicillin treatment in Vero cells at day 6 postinfection.

penicillin derivatives (10), while *W. chondrophila* is susceptible to high doses of fosfomycin (11). Aberrant bodies represent enlarged forms of the bacterium, due to abnormal division despite persisting DNA replication (11). Therefore, we evaluated the morphology of *S. negevensis* particles treated with β-lactams, fosfomycin, and vancomycin, in immunofluorescence assays using an in-house rabbit polyclonal anti-*S. negevensis* antibody, as described previously (7). As shown in Fig. 1A, no abnormal morphological aspects of *S. negevensis* could be observed with β-lactam treatment, even with concentrations as high as 1,000 μg/ml. This contrasted strikingly with the abnormal morphology of *Chlamydia trachomatis* observed with 2 μg/ml β-lactams, making *S. negevensis* unique among *Chlamydiales* members. Indeed, *W. chondrophila* (in the *Waddliaceae* family) and *E. lausannensis* (in the *Criblamydiaceae* family) form aberrant bodies with β-lactam treatment (500 μg/ml) (6, 12). Furthermore, unlike *W. chondrophila* (11), *S. negevensis* replication was not inhibited by high doses of β-lactams (1,000 μg/ml) (Table 1). This difference could not be explained by the slower replicative cycle, as similar observations were made at day 6 postinfection (Fig. 1B). Several β-lactamase motifs are included in the *S. negevensis* genome (13) and may contribute to the phenotype. However, *W. chondrophila* exhibits partial sensitivity to high doses of β-lactams despite having a class C β-lactamase encoded in its genome (14).

Similarly to *Chlamydia* spp. (11), *S. negevensis* replication was not inhibited by high doses of fosfomycin, which targets the enzyme MurA (implicated in the early steps of PG biosynthesis). However, a small fraction of *S. negevensis* particles, which increased by

day 6, showed abnormal morphological features consistent with aberrant bodies (Fig. 1A and B), although remaining significantly less important than observed for *W. chondrophila* (11). *Chlamydia* resistance to fosfomycin is suspected to be related to a single substitution (Cys115 to Asp) in the active site of MurA (11, 15). This mutation was not found in *S. negevensis*, supporting the observed partially sensitive phenotype. Finally, we did not observe aberrant bodies with vancomycin treatment, a drug that inhibits transpeptidation through high-affinity binding to the D-alanine precursor (Fig. 1A).

Recently, several works have demonstrated the presence of a modified version of PG, which is required for cell division (12, 16, 17), in *Chlamydiales* members, thus explaining their partial sensitivity to cell wall inhibitors. Interestingly, a recent study failed to isolate PG-like structures in *S. negevensis* (18), while such structures were identified in *Protochlamydia amoebophila* (18) and *C. trachomatis* (17). In the same work, incorporation of fluorescently labeled D-alanine could not be highlighted in *S. negevensis* (18), which correlates with the absence of vancomycin effects observed here. However, a previous work showed that, similarly to *C. trachomatis*, *S. negevensis* was susceptible to D-cycloserine, a molecule that inhibits the alanine racemase Alr and the alanine ligase Ddl, which are required for D-alanine formation (19). While a predicted Ddl enzyme is encoded in the *S. negevensis* genome, no Alr coding sequence is present, similarly to *Chlamydiaceae* (12). It is not known whether the serine hydroxymethyltransferase GlyA encoded in the *S. negevensis* genome could compensate for the absence of Alr, as described for *Chlamydiaceae* (20).

Despite the absence of PG-like structures, the activity of two PG-remodeling enzymes, NlpD and AmiA, was documented in *S. negevensis* (16), and enzymes implicated in PG biosynthesis are highly conserved among *Chlamydiales* members, including *S. negevensis*, which supports their crucial role (12). However, the different responses to different cell wall inhibitors, each targeting a specific step of PG biosynthesis, indicate that, despite the likely requirement for a modified form of PG for cell division, some significant differences exist in the PG biosynthesis pathway of *S. negevensis*, which might bring further insights into the mechanisms of *Chlamydiales* cell division.

In conclusion, in this work we highlighted several differences in the antimicrobial responses of *S. negevensis*, compared to other *Chlamydiales* members. Although the pathogenic role of *Simkania* spp. remains to be better defined, the precise knowledge of their antimicrobial susceptibility patterns provides significant information regarding the biology and evolution of the *Chlamydiales* order.

ACKNOWLEDGMENTS

This work was supported by the Swiss National Science Foundation (SNSF) (MD-PhD grant 323530-158123 and grant 310030-162603).

We do not report any potential conflicts of interest.

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