Med Microbiol Immunol (1999) 188: 91–97

ORIGINAL INVESTIGATION

Susanne Häußler · Manfred Rohde · Ivo Steinmetz

Highly resistant *Burkholderia pseudomallei* small colony variants isolated in vitro and in experimental melioidosis

Received: 10 September 1998

Abstract *Burkholderia pseudomallei* is the causative agent of melioidosis, a disease in which treatment failures and relapses are common. This study reports on slow growing *B. pseudomallei* 'small colony variants' (SCVs), isolated either in vitro after exposure to ceftazidime, ciprofloxacin or gentamicin or from the spleen and liver in a mouse model of melioidosis after treatment with ceftazidime. Interestingly, SCVs isolated by either method or antimicrobial agent showed a significant increase in the minimal inhibitory concentrations of various unrelated classes of antimicrobial agents. *B. pseudomallei* SCVs did not differ from their parental strains in standard biochemical profiles, nor by pulsed field gel electrophoresis or electron microscopy. Although the SCV phenotype was stable throughout numerous passages on antibiotic-free solid media, revertants with the parental colony morphology and, most importantly, with the parental susceptibility pattern occurred. These revertants led to rapid overgrowth of SCVs in liquid media without added antibiotics. Future studies will have to determine the clinical relevance of *B. pseudomallei* SCVs especially in treatment failure and relapse of infection.

Key words *Burkholderia pseudomallei* · Small colony variants

Introduction

The gram-negative bacterium *Burkholderia pseudomallei* is the causative agent of melioidosis, a tropical disease of man and animals. Melioidosis is endemic primarily in Southeast Asia and Northern Australia, where it is a major cause of morbidity and mortality [5, 10]. Since the definitive diagnosis of melioidosis requires microbiological facilities, it seems evident that the disease is greatly underdiagnosed in many areas of the tropics [10]. Infection occurs by contact with environmental organisms ubiquitous in the soil and surface water in endemic areas [8, 9, 17]. The clinical manifestations show extreme variations ranging from inapparent infections to localized subacute or chronic infections and septicemias with abscesses in multiple organs [8, 17, 36]. Severe septicemia is the most common clinical presentation of melioidosis. In parts of Northeastern Thailand it accounts for about 20% of community acquired septicemia [5]. The introduction of high-dose parenteral ceftazidime and amoxicillin/clavulanate in the treatment of severe melioidosis has significantly improved the clinical outcome but mortality still remains at about 40% [32, 35]. Despite the in vitro susceptibility of *B. pseudomallei* to a variety of antibiotics, many melioidosis patients remain febrile and culture positive even after treatment for prolonged periods [32]. Moreover, a characteristic feature of the disease are relapses in up to 23% of patients [6], which might even occur after extended disease-free intervals. It has been demonstrated by molecular typing methods that recurrent infections are indeed the result of reactivation of an endogenous focus of *B. pseudomallei* in most melioidosis patients [13]. The risk of relapse can be reduced if 20 weeks of maintenance antibiotic therapy are given [26].

The isolation of slow growing subpopulations – termed 'small colony variants' (SCVs) – in different gram-positive and gram-negative bacteria after exposure to gentamicin has been known for many years [4, 20, 21]. However, recently attention has again been focused on SCVs, following reports of an association between the detection of *Staphylococcus aureus* SCVs and persistent and relapsing infection after intensive antimicrobial therapy [24, 25]. It has been demonstrated that these SCVs are not only more resistant against aminoglycosides but also persist inside cultured endothelial cells [14, 33].

S. Häußler · I. Steinmetz (✉)
Institute of Medical Microbiology,
Hannover Medical School, Carl-Neuberg-Strasse 1,
D-30625 Hannover, Germany
e-mail: Steinmetz.Ivo@MH-Hannover.de,
Tel.: +49-511-5324352; Fax: +49-511-5324366

M. Rohde
GBF, Gesellschaft für Biotechnologische Forschung mbH,
D-38124 Braunschweig, Germany

The main purpose of the present study was to investigate whether *B. pseudomallei* is capable of generating SCVs under exposure to antibiotics used for the treatment of melioidosis. We report the in vitro and in vivo isolation of slow growing *B. pseudomallei* SCVs, exhibiting high-level cross-resistance to various classes of antibiotics proposed for treatment of *B. pseudomallei* infection. Revertants with the parental growth rate, phenotype and susceptibility pattern occurred in the SCV populations at high frequencies, and this might impede the detection of *B. pseudomallei* SCVs in clinical material.

Materials and methods

Bacteria and culture conditions

The *B. pseudomallei* isolates used in this study were obtained from the National Collection of Type Cultures (NCTC). The strains were stored until use in Luria broth (LB) supplemented with 20% (vol/vol) glycerin at −70 °C. Agar cultures were grown on Columbia blood agar at 37 °C unless otherwise stated. Broth cultures were grown in brain-heart-infusion broth (BHI) or LB at 37 °C with shaking at 200 rpm.

Antibiotic susceptibility testing

In vitro susceptibility testing was performed by a standard broth microdilution procedure (according to DIN 58 940) using direct colony suspension. Briefly, overnight grown colonies were suspended in broth medium and adjusted to the turbidity of a 0.5 McFarland standard. Microtiter plates containing twofold dilutions of antibiotics in Mueller-Hinton broth (MHB) were inoculated with the appropriate dilution of the bacterial suspension. The trays with the inoculated parent strains were incubated at 37 °C for 18–24 h, while incubation of SCVs was carried out for 36–48 h as described for minimal inhibitory concentration (MIC) determination of SCVs of *S. aureus* [14]. The MIC was defined as the lowest concentration of antibiotics that prevented visible growth.

In vitro isolation of *B. pseudomallei* SCVs

The different *B. pseudomallei* strains were grown overnight in BHI and 0.1 ml was used to inoculate 5 ml BHI supplemented with either gentamicin (10, 30 and 150 μg/ml), ciprofloxacin (1 μg/ml) or ceftazidime (2 μg/ml). When visible turbidity occurred after 24–72 h, a 0.1-ml aliquot of the bacterial suspension was again inoculated in 5 ml BHI supplemented with the same antimicrobial agents in the same concentration as before. In parallel an aliquot (10 μl) was spread on a Columbia blood agar without added antibiotics and examined for the appearance of SCVs after 48 h of incubation. This process was repeated until microcolonies could be detected. Small colonies which appeared were termed SCVs if they maintained their phenotype in at least two subcultures.

In vivo isolation of *B. pseudomallei* SCVs

Two 8- to 10-week-old BALB/c mice were infected intravenously with 10^5 colony-forming units (CFU) *B. pseudomallei* NCTC 7431 as described previously [15]. Immediately after infection mice were subcutaneously treated with ceftazidime [18]. The animals received 60 mg ceftazidime/kg body weight in a volume of 200 μl of buffer A (0.01 M potassium phosphate buffer made isotonic with saline; pH 7.5) twice daily for 6 days. Antibiotic treatment was discontinued until day 13 post infection (p.i.). One animal was killed and one animal was treated again with ceftazidime (60 mg ceftazidime/kg body weight once daily) until day 18 and killed on day 19 p.i. Determination of CFU in organ homogenates of the spleen and liver of sacrificed animals was performed as described previously [15]. The frequency of SCVs among the *B. pseudomallei* CFU of spleen and liver was determined on antibiotic-free agar using homogenates at dilutions in the range of 1:1000–1:1,000,000.

Growth characteristics

To determine the potential of revertants to overgrow SCVs in liquid medium, 1 ml of an overnight culture of SCVs (grown in LB supplemented with 150 μg/ml gentamicin) was added to 100 ml of antibiotic-free LB. At given time intervals 1-ml aliquots were removed and serial dilutions were plated on antibiotic-free agar plates to determine the CFU of SCVs and revertants. To determine growth of SCVs and revertants under different culture conditions, bacteria were grown on an amino acid-free modified Vogel-Bonner agar (3.3 mM $MgSO_4$, 10 mM citric acid, 28 mM $NaNH_4HPO_4$, 37 mM K_2HPO_4, 214 mM D-gluconic acid, 1% agar, medium pH 7.4), on modified Vogel-Bonner agar supplemented with 5 g/l acid-hydrolyzed casein (casamino acids; Difco, Detroit), 0.53 mM diamino-pimelic acid (Sigma, St. Louis), 0.1 mM tryptophan (Sigma, St. Louis), on Mueller-Hinton agar, on chocolate agar, on Ashdown agar [12] and on Columbia blood agar with 5% CO_2 or at 42 °C. Auxotrophy for hemin and menadione was tested as described [2] using Mueller-Hinton agar supplemented with 2 μg/ml and 20 μg/ml hemin (Sigma, St. Louis) or menadione (Fluka, Buchs, Switzerland), respectively. A site-directed *hemB* mutant of *S. aureus* [14] and an *S. aureus* mutant auxotrophic for menadione were kindly provided by Christof v. Eiff (Medical Microbiology, University of Muenster, Germany) and served as positive controls.

Biochemical characterization

The API NE system (bioMerieux) was used for comparative testing of biochemical reactions [12] of the parental strains and their SCVs. For the biochemical characterization of SCVs the API tubes were inoculated with SCVs diluted in NaCl supplemented with gentamicin at a final concentration of 150 μg/ml to prevent overgrowth of revertants. After 48 h of incubation reactions were read and a subculture was performed to confirm the growth of SCVs in the API NE system. The presence of catalase was tested by emulsifying a small portion of a colony in one drop of 30% (vol/vol) hydrogen peroxide on a glass slide. The production of the *B. pseudomallei* exopolysaccharide was tested using the immunoglobulin G1 monoclonal antibody 3015 specific for the exopolysaccharide in a latex agglutination test as described previously [31]. Testing of cytochrome oxidase was performed with Bacident oxidase test strips (Merck, Darmstadt, Germany).

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) was performed as described previously [27]. Briefly, chromosomal DNA of the parental strain and the SCVs were isolated from overnight-grown colonies and *Spe*I restriction fragments were resolved in 1% agarose gel by PFGE in 0.5 × TRIS-borate-EDTA buffer. A CHEF RP II apparatus was used at 200 V and 14 °C for 28 h with pulse times of 5–30 s. The gels were photographed over ultraviolet light after being stained with ethidium bromide.

Ruthenium-red treatment and embedding for transmission electron microscopy

Bacteria were fixed in glutaraldehyde-ruthenium red-osmium according to Luft [19]. Samples were dehydrated with acetone and embedded in epoxy resin following a described protocol [30]. Samples were examined in a Zeiss TEM 910.

Scanning electron microscopy

Bacteria were fixed in 0.2 M cacodylate buffer containing 3% glutaraldehyde and 5% formaldehyde pH 7.0 for 1 h on ice. After washing in cacodylate buffer samples were dehydrated with a graded series of acetone, dried at the critical point in liquid CO_2, sputter-coated with an approximately 10-nm-thick gold film, and examined in a Zeiss DSM 982 Gemini.

Results

Isolation of *B. pseudomallei* SCVs exposed to various antibiotics in vitro

The general ability of *B. pseudomallei* to generate SCVs in liquid media when exposed to different antimicrobial agents was tested with four strains (NCTC 7431, 10276, 10274 and 11642). Although gentamicin is not used in the treatment of *B. pseudomallei* infections due to natural resistance, we first examined the potential of this agent to select *B. pseudomallei* SCVs, because gentamicin is known to generate SCVs of a variety of gram-negative bacteria [21, 29]. Serial passages of the *B. pseudomallei* strains in broth culture supplemented with gentamicin in the range of the MICs (30 μg/ml) repeatedly led to the isolation of SCVs in subcultures on solid agar (Fig. 1). In most experiments isolation was achieved within 4–5 days (two to three passages). When gentamicin was used in higher concentrations (150 μg/ml), SCVs could be isolated after a single passage after 4 days of incubation. In broth with gentamicin concentrations below the MIC (10 μg/ml) no SCVs were isolated even after 25 days of incubation and daily passages. We then examined the potential of antimicrobial agents used for the treatment of melioidosis to select SCVs. Serial passages of *B. pseudomallei* in broth supplemented with either ciprofloxacin or ceftazidime in the range of the MICs (1 μg and 2 μg/ml, respectively) also repeatedly led to the isolation of SCVs within the same time as the gentamicin-selected SCVs. The phenotype of these SCVs was identical to the gentamicin-selected SCVs shown in Fig. 1.

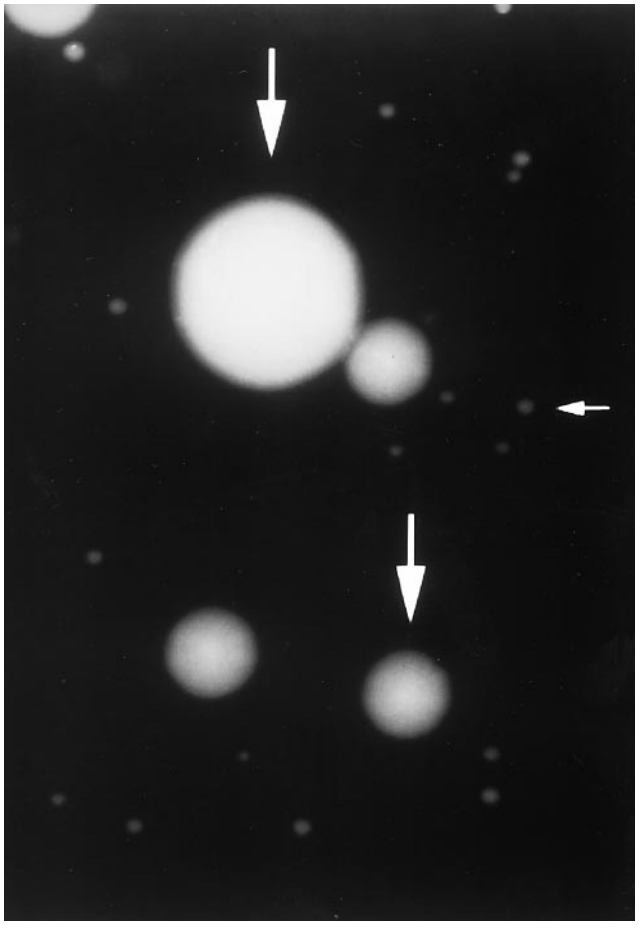

Fig. 1 Colony morphology of *Burkholderia pseudomallei* NCTC 7431 SCVs (←) and their revertants (↓) on antibiotic-free LB-agar (*SCV* small colony variant, *LB* Luria broth)

A striking feature of the *B. pseudomallei* SCVs was the appearance of revertants with the parental colony morphology in the inoculum streak when single microcolonies were subcultured in media without added antibiotics. The frequency of these revertants was estimated to be in the range of 10^{-2}–10^{-3}. No differences were observed in the frequency of revertants selected by either of the antimicrobial agents. However, SCVs could easily be maintained by subculturing on solid antibiotic-free agar for several months. Generally, continuous subculturing of SCVs on antibiotic free agar led to a decreased frequency of revertants, e.g. in a frequency of revertants of less than 10^{-4} with the SCV isolated after exposure to ciprofloxacin.

Isolation of *B. pseudomallei* SCVs after treatment of infected mice with ceftazidime

Two BALB/c mice were challenged with 10^5 CFU *B. pseudomallei* NCTC 7431. This infection dose corresponds to 100 times the LD_{50} of non-mouse-passaged *B. pseudomallei* NCTC 7431 in this mouse strain [15], leading to death of animals within 48 h without antibiotic treatment. After treatment with ceftazidime for 6 days and further 6 days without treatment one animal showed no clinical signs of illness, whereas the other mouse was clinically compromised. No bacteria were detectable in the spleen and liver of the apparently clinically well animal on day 13 p.i. The clinically compromised animal was further treated for 5 days before sacrifice. In this animal SCVs were detected in the liver, and accounted for approximately 30% of the 5×10^3 CFU in this organ at this time point. In the spleen about 5% of 6×10^6 CFU exhibited the SCV phenotype. In a previous study [15] no SCVs could be detected among comparable CFU counts in the spleen and liver of *B. pseudomallei*-infected mice without antibiotic treatment. During the first subcultures of SCVs on antibiotic-free agar reversion occurred at very high frequencies, but as observed with the SCV isolated in vitro several passages led to stable SCVs with reversion frequencies of less than 10^{-4}.

Growth characteristics of SCVs

When *B. pseudomallei* SCVs were grown in liquid medium without added antibiotics revertants with the parental phenotype exceeded the number of SCVs within several hours of incubation (Fig. 2). We further investigated if different culture conditions influence the phenotype of *B. pseudomallei* SCVs. Growth on various media, such as Chocolate agar, Mueller-Hinton agar and modified Ashdown agar, the latter being used for the isolation of *B. pseudomallei* from clinical material, did not alter the small phenotype of SCVs, while the revertants exhibited their larger phenotype on these media. Furthermore, neither an atmosphere containing 5% CO_2 nor growth at a temperature of 42 °C led to any change in the small phenotype of SCVs in comparison to the large phenotype of the revertants. SCVs retained their phenotype on Mueller-Hinton agar supplemented with hemin or menadione. Moreover, SCVs and their corresponding revertants could be grown on amino acid-free modified Vogel-Bonner agar and did not change their phenotype when grown on the same agar supplemented with amino acids.

Biochemical reactions, DNA restriction profiles and ultrastructure of SCVs

Comparison of biochemical reactions in the API 20 NE system of strains NCTC 7431 and NCTC 10274 and their respective SCVs isolated either in vivo or in vitro revealed no differences. SCVs and parental strains exhibited catalase activity and were positive for cytochrome oxidase. Genotypic analysis by PFGE exhibited indistinguishable *Spe*I restriction profiles of the chromosomal DNA from SCVs and parental strains (data not shown), indicating clonal identity. Scanning and transmission electron microscopy revealed no significant difference in cell size or morphology of SCVs compared to parental cells. Furthermore, using a latex agglutination test the mAb 3015, specific for a *B. pseudomallei* exopolysaccharide [31] consisting of galactose and 3-deoxy-D-manno-2-octulosonic acid (Kdo) [23], also showed reactivity with SCVs.

Antibiotic resistance profiles of SCVs

The susceptibility of SCVs from strains NCTC 7431 (Table 1) and NCTC 10274 (Table 2) isolated by exposure to either gentamicin, ciprofloxacin or ceftazidime in vitro, and of the SCV (NCTC 7431, Table 1) isolated from the liver of an infected mouse after treatment with ceftazidime to a variety of antimicrobial agents, was tested. Interestingly, SCVs selected from either strain exhibited similar patterns of increased MICs to various antibiotics, independently of the agent and method used for their selection. The most prominent increase in MIC was observed for the quinolones ciprofloxacin and ofloxacin and for doxicyclin (up to 32-fold for all three substances). Moreover, SCVs exhibited an increase (2- to 8-fold) in the MIC of meropenem, ceftazidime, piperacillin, chloramphenicol and amoxicillin/clavulanate. Although *B. pseudomallei* shows natural aminoglycoside resistance, the MICs of SCVs for gentamicin and

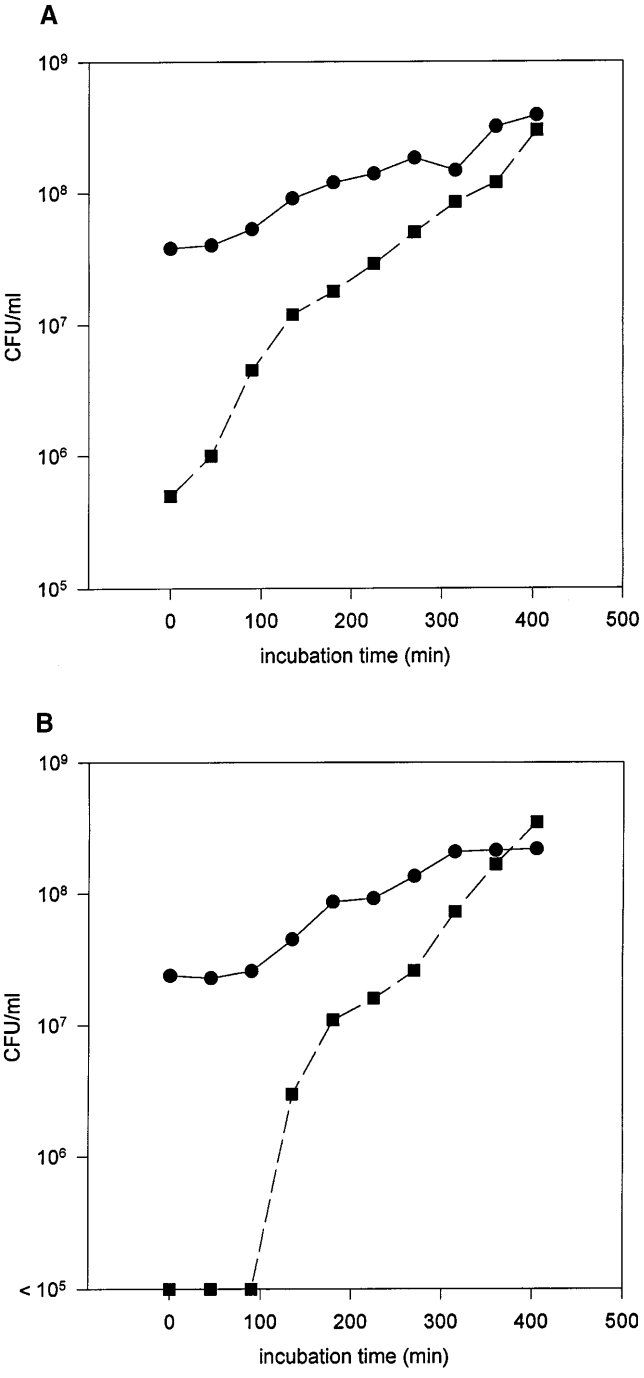

Fig. 2A, B. Growth curves of gentamicin-selected *B. pseudomallei* SCVs (●) and the corresponding revertants (■) in antibiotic-free broth. *B. pseudomallei* NCTC 7431 (**A**) and NCTC 10274 (**B**). Of an overnight culture of SCVs (grown in LB supplemented with 150 μg/ml gentamicin) 1 ml was added to 100 ml of antibiotic-free LB. CFU of SCVs and revertants were determined at different time points (*CFU* colony-forming units)

Table 1 Susceptibility testing of the parental strain *Burkholderia pseudomallei* NCTC 7431, the corresponding SCVs isolated in vitro or from the liver of an infected mouse after antibiotic treatment and the revertants derived from each SCV. Minimal inhibitory concentrations (μg/ml) of different antimicrobial agents are shown (*SCV* small colony variant, *SCV GM* in vitro exposure to gentamicin, *SCV CI* in vitro exposure to ciprofloxacin, *SCV TZ* in vitro exposure to ceftazidime, *SCV TZ in vivo* isolated after treatment with ceftazidime, *REV* revertants)

	Parental strain	SCV GM	REV GM	SCV CI	REV CI	SCV TZ	REV TZ	SCV TZ in vivo	REV TZ
B. pseudomallei NCTC 7431									
Gentamicin	64	> 256	64	> 256	64	> 256	64	> 256	64
Kanamycin	32	> 256	32	128	64	128	32	> 256	32
Ciprofloxacin	1	16	1	32	1	32	1	> 32	4
Ofloxacin	2	16	4	> 32	4	> 32	2	> 32	4
Doxicyclin	0,5	2	1	16	1	16	0,5	> 16	4
Ceftazidime	4	8	4	16	8	16	4	8	4
Piperacillin	4	16	4	16	8	16	4	16	8
Meropenem	1	2	1	4	2	4	1	4	1
Chloramphenicol	8	16	8	16	8	> 16	8	> 16	16
Amoxicillin/clavulanate	2/2	8/2	4/2	8/2	8/2	16/2	4/2	8/2	4/2

Table 2 Susceptibility testing of the parental strain *B. pseudomallei* NCTC 10274, the corresponding SCVs isolated in vitro and the revertants derived from each SCV. Minimal inhibitory concentrations (μg/ml) of different antimicrobial agents are shown (*SCV GM* in vitro exposure to gentamicin, *SCV CI* in vitro exposure to ciprofloxacin, *SCV TZ* in vitro exposure to ceftazidime, *REV* revertants)

	Parental strain	SCV GM	REV GM	SCV CI	REV CI	SCV TZ	REV TZ
B. pseudomallei NCTC 10274							
Gentamicin	32	256	64	256	64	128	32
Kanamycin	32	128	32	64	32	64	32
Ciprofloxacin	0,5	32	1	32	1	32	1
Ofloxacin	2	> 32	1	> 32	4	32	2
Doxicyclin	0,25	16	1	16	0,5	16	0,5
Ceftazidime	2	16	16	16	4	8	2
Piperacillin	4	8	8	8	4	8	4
Meropenem	0,5	4	2	4	1	4	0,5
Chloramphenicol	8	32	8	32	8	64	8
Amoxicillin/clavulanate	2/2	8/2	8/2	8/2	4/2	8/2	2/2

kanamycin were also significantly increased (2- to > 8-fold) compared to the parental strains. We then performed susceptibility testing of revertants of SCVs. Generally, the revertants lost their high level cross-resistance (Tables 1, 2) and exhibited susceptibility profiles almost identical to the parental strains.

Discussion

The cause for frequent relapses in melioidosis and prolonged clinical courses despite appropriate use of antibiotics is not well understood. Although intensive antimicrobial chemotherapy has greatly improved the prognosis of severe melioidosis, mortality is still high and blood cultures, wound swabs and sputa often remain culture positive even after 2 weeks of parenteral treatment [32]. For the past few years, new interest has been paid to SCVs of bacterial pathogens because of an association found between the presence of *S. aureus* SCVs in human clinical material and persistent and relapsing infections after antibiotic therapy [25]. Due to a reduced production of exotoxins, *S. aureus* SCVs are thought to be able to persist intracellulary in non-professional phagocytes and thus evade host defense mechanisms [2, 14, 33]. The occurrence of SCVs in various gram-negative bacteria has been described previously; however, those SCVs had been exclusively selected under exposure to aminoglycosides in vitro [21] and in vivo [1, 29].

In this study highly resistant *B. pseudomallei* SCVs were isolated in vitro under exposure not only to gentamicin but to ciprofloxacin or ceftazidime. Moreover, SCVs could be isolated from the spleen and liver of an infected BALB/c mouse after antibiotic treatment with ceftazidime. Further studies will have to systematically elucidate the role of different treatment regimes in experimental models in generating *B. pseudomallei* SCVs. A distinctive feature of the *B. pseudomallei* SCVs described in this study is their decreased susceptibility to various antimicrobial agents. SCVs selected with either gentamicin, ciprofloxacin or ceftazidime in vitro and in vivo exhibited a similarly increased resistance profile against a variety of antimicrobial agents, including the two parenteral antimicrobial agents amoxicillin/clavulanic acid and ceftazidime currently recommended for

the treatment of severe melioidosis [32, 34, 35]. Most striking is the 32-fold MIC increase of the quinolones ciprofloxacin and ofloxacin, and of doxyciclin: two classes of antimicrobial agents also used for the chemotherapy of melioidosis [7, 11, 26]. Interestingly, the revertants regain antibiotic susceptibility and their resistance profile resembles the profile of the parental strains. The simultaneous acquisition of resistance of SCVs selected with either gentamicin, ciprofloxacin or ceftazidime to structurally unrelated antimicrobial agents suggests an altered drug permeation and/or efflux mechanism [22]. Altered drug permeation as a cause for *Pseudomonas aeruginosa* variants that are resistant to aminoglycosides has been postulated previously [3, 16]. The fact that *B. pseudomallei* SCVs and their parental strains exhibit the same standard biochemical profile is in accordance with previously described SCVs of various enterobacteriaceae [21].

In contrast to *S. aureus* SCVs [24] and an *Escherichia coli* SCV [28], both associated with chronic infections, supplementation of the *B. pseudomallei* SCVs by either hemin or menadione did not result in a wild-type colony morphology. In addition, SCVs and their revertants grew on amino acid-free agar and did not change their phenotype on agar supplemented with amino acids. Therefore, auxotrophy for hemin, menadione or amino acids seems to be unlikely.

Further studies are needed to determine the frequency of *B. pseudomallei* SCVs in human clinical material and to investigate if more resistant *B. pseudomallei* SCVs are selected under currently used antimicrobial therapies. Because of the slow growth rate of the SCVs and their frequent reversions to the parental phenotype in antibiotic-free medium, it seems likely that an in vivo selection of *B. pseudomallei* SCVs under antibiotic treatment is difficult to detect in clinical specimens, especially when enrichment cultures are used. It remains to be determined whether fast-growing revertants, originating from SCVs when antimicrobial pressure is discontinued, contribute to the frequently observed relapses in melioidosis.

Acknowledgement We are grateful to D. Bitter-Suermann for his continuous encouragement.

References

1. Badenoch PR, Coster DJ (1989) Selection of gentamicin-resistant variants of *Pseudomonas aeruginosa* in the rat cornea. J Ocul Pharmacol 5:19–25
2. Balwit JM, Langevelde P van, Vann JM, Proctor RA (1994) Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. J Infect Dis 170:1033–1037
3. Bayer AS, Norman DC, Sik Kim K (1987) Characterization of impermeability variants of *Pseudomonas aeruginosa* isolated during unsuccessful therapy of experimental endocarditis. Antimicrob Agents Chemother 31:70–75
4. Bulger RJ (1969) In vitro studies on highly resistant small colony variants *of Staphylococcus aureus* resistant to methicillin. J Infect Dis 120:491–494
5. Chaowagul W, White NJ, Dance DAB, Wattanagoon Y, Naigowit P, Davis TME, Looareesuwan S, Pitakwatchara N (1989) Melioidosis: a major course of community acquired septicemia in Northeastern Thailand. J Infect Dis 159:890–899
6. Chaowagul W, Suputtamongkol Y, Dance DAB, Rajchanuwong A, Pattara-arechachai J, White NJ (1993) Relapse in melioidosis: incidence and risk factors. J Infect Dis 168:1181–1185
7. Chaowagul W, Suputtamongkul Y, Smith MD, White NJ (1997) Oral fluoroquinolones for maintenance treatment of melioidosis. Trans R Soc Trop Med Hyg 91:599–601
8. Dance DAB (1990) Melioidosis. Rev Med Microbiol 1:143–150
9. Dance DAB (1991) *Pseudomonas pseudomallei*: danger in the paddy fields. Trans R Soc Trop Med Hyg 85:1–3
10. Dance DAB (1991) Melioidosis: the tip of the iceberg? Clin Microbiol Rev 4:52–60
11. Dance DAB, Wuthiekanun V, Chaowagul W, White NJ (1989) The antimicrobial suspectibility *of Pseudomonas pseudomallei*. Emerge of resistance in vitro and during treatment. J Antimicrob Chemother 24:295–309
12. Dance DAB, Wuthiekanun V, Naigowit P, White NJ (1989) Identification of *Pseudomonas pseudomallei* in clinical practice: use of simple screening tests and API 20NE. J Clin Pathol 42:645–648
13. Desmarchelier PM, Dance DAB, Chaowagul W, Suputtamongkol Y, White NJ, Pitt TL (1993) Relationships among *Pseudomonas pseudomallei* isolates from patients with recurrent melioidosis. J Clin Microbiol 31:1592–1596
14. Eiff C von, Heilmann C, Proctor RA, Woltz C, Peters G, Götz F (1997) A site-directed *Staphylococcus aureus* hemB mutant is a small-colony variant which persists intracellularly. J Bacteriol 179:4706–4712
15. Hoppe I, Brenneke B, Rohde M, Kreft A, Häußler S, Reganzerowski A, Steinmetz I (1999) Characterization of a murine model of melioidosis: comparison of different strains of mice. Infect Immun 67:2891–2900
16. Langford PR, Anwar H, Gonda I, Brown MRW (1989) Outer membrane proteins of gentamicin induced small colony variants of *Pseudomonas aeruginosa*. FEMS Microbiol Lett 61:33–36
17. Leelarasmee A, Bovornkitti S (1989) Melioidosis: review and update. Rev Infect Dis 11:413–425
18. Leggett JE, Fantin B, Ebert S, Totsuka K, Vogelman B, Calame W, Mattie H, Craig WA (1989) Comparative antibiotic dose-effect relations at several dosing intervals in murine pneumonitis and thigh-infection models. J Infect Dis 159:281–292
19. Luft JH (1971) Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anat Rec 171:347–368
20. Morris JF, Sellers TF, Brown AW (1941) The primary isolation of small colony strains of *Eberthella typhosa* from blood, feces, urine and sputum. J Infect Dis 68:117
21. Musher DM, Baughn RE, Merrell GL (1979) Selection of small colony variants of enterobacteriaceae by in vitro exposure to aminoglycosides: pathogenicity for experimental animals. J Infect Dis 140:209–214
22. Nikaido H (1994) Prevention of drug access to bacterial tagets: permeability barriers and active efflux. Science 264:382–388
23. Nimtz M, Wray V, Domke T, Brenneke B, Häußler S, Steinmetz I (1997) Structure of an acidic exopolysaccharide of *Burkholderia pseudomallei*. Eur J Biochem 250:608–616
24. Proctor RA, Balwit JM, Vesga O (1994) Variant subpopulations of *Staphylococcus aureus* as cause of persistent and recurrent infections. Infect Agents Dis 3:302–312
25. Proctor RA, Langevelde P van, Kristrjansson M, Maslow JN, Arbeit RD (1995) Persistant and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. Clin Infect Dis 20:95–102
26. Rajchanuvong A, Chaowagul W, Suputtamongkol Y, Smith MD, Dance DAB, White NJ (1995) A prospective comparison of co-amoxiclav and the combination of chloramphenicol, doxycicline, and co-trimoxazole for the oral maintenance

treatment of melioidosis. Trans R Soc Trop Med Hyg 89:546–549
27. Römling U, Fiedler B, Boßhammer J, Grothues D, Greipt J, Tümmler B (1994) Epidemiology of the chronic *Pseudomonas aeruginosa* infection in cystic fibrosis. J Infect Dis 170:1616–1621
28. Roggenkamp A, Sing A, Hornef M, Brunner U, Autenrieth IB, Heesemann J (1998) Chronic prostetic hip infection caused by a small-colony variant of *Escherichia coli*. J Clin Microbiol 36:2530–2534
29. Rusthoven JJ, Davies TA, Lerner SA (1979) Clinical isolation and characterisation of aminoglycoside-resistant small colony variants of *Enterobacter aerogenes*. Am J Med 67:702–706
30. Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26:31–43
31. Steinmetz I, Reganzerowski A, Brenneke B, Häussler S, Simpson A, White NJ (1999) Rapid identification of *Burkholderia pseudomallei* by latex agglutination based on an exopolysaccharide-specific monoclonal antibody. J Clin Microbiol 37:225–228
32. Suputtamongkol Y, Rajchanuwong A, Chaowagul W, Dance DAB, Smith MD, Wuthiekanun V, Walsh AL, Pukrittayakamee S, White NJ (1994) Ceftazidime vs. amoxicillin/clavulanate in the treatment of severe melioidosis. Clin Infect Dis 19:846–853
33. Vesga O, Groeschel MC, Otten MF, Brar DW, Vann JM, Proctor RA (1996) *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. J Infect Dis 173:739–742
34. Walsh AL, Smith MD, Wuthiekanun V, White NJ (1995) Postantibiotic effects and *Burkholderia* (*Pseudomonas*) *pseudomallei*: evaluation of current treatment. Antimicrob Agents Chemother 39:2356–2588
35. White NJ, Dance DAB, Chaowagul W, Wattanagoon Y, Wuthiekanun V, Pitakwatchara N (1989) Halving mortality of severe melioidosis by ceftazidime. Lancet II:697–701
36. Yabuuchi E, Arakawa M (1993) *Burkholderia pseudomallei* and melioidosis: be aware in temperate area. Microbiol Immunol 37:832–836