RESEARCH ARTICLE

Isolation and Characterization of a Bacteriocin with Anti-MRSA Activity from *Bacillus* sp. Strain WASM9-25M

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Abstract

The infection with methicillin resistant *Staphylococcus aureus* or MRSA has become a serious problem worldwide. The use of a bacteriocin as an alternative compound to overcome this problem is promising. The partially purified bacteriocin or PPB obtained from strain WASM9-25M showed anti-MRSA activity. The PPB was heat stable up to 100°C for 30 min and active within the pH range of 3-9. The inactivation after treatment with all proteolytic enzymes (trypsin, α-chymotrypsin and proteinase K) reveals its antimicrobial peptides. Therefore, this isolated strain WASM9-25M can be regarded as a bacteriocin producing bacteria. It was identified as *Bacillus* sp. by the 16S rRNA gene sequence. Tris-Tricine SDS-PAGE analysis revealed that bacteriocin from strain WASM9-25M had an apparent molecular weight of 3.14 kDa. PCR analysis showed the presence of iturin A, lichenysin synthetase C and bacillomycin D. The bioactive peptide from *Bacillus* sp. strain WASM9-25M has a potential for use as an alternative antibacterial agent for the treatment of infection with MRSA in the future.

Keywords: bacteriocin, anti-MRSA, *Bacillus* sp., WASM9-25M

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การคัดเลือกและการศึกษาคุณสมบัติของแบคเทอริโอซินจากเชื้อแบคทีเรีย *Bacillus* sp. สายพันธุ์ WASM9-25M ที่มีฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคดื้อยาสายพันธุ์ MRSA

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บทคัดย่อ

การติดเชื้อแบคทีเรียก่อโรคดื้อยาสายพันธุ์ methicillin resistant *Staphylococcus aureus* (MRSA) ได้กลายเป็นปัญหาสำคัญทั่วโลก การใช้เปปไทด์ชนิดแบคเทอริโอซินถือได้ว่าเป็นอีกหนึ่งทางเลือกที่น่าสนใจ สารสกัดแบคเทอริโอซินกึ่งบริสุทธิ์ (partially purified bacteriocin, PPB) ของเชื้อแบคทีเรียสายพันธุ์ WASM9-25M มีฤทธิ์ในการต่อต้านเชื้อแบคทีเรียก่อโรคดื้อยาสายพันธุ์ MRSA สารสกัดแบคเทอริโอซินกึ่งบริสุทธิ์สามารถทนทานต่อความร้อนได้สูงสุดที่อุณหภูมิ 100°C นาน 30 นาที และมีความสามารถในการทนต่อความเป็นกรด-ด่างได้ในช่วง pH 3-9 สารสกัดแบคเทอริโอซินกึ่งบริสุทธิ์ของเชื้อแบคทีเรียสายพันธุ์ WASM9-25M หมดสภาพเมื่อย่อยด้วยเอนไซม์ที่ย่อยโปรตีนคือ โปรติเนสเค แอลฟาไคโมทริปซินและทริปซิน ซึ่งแสดงให้เห็นว่าสารสกัดแบคเทอริโอซินกึ่งบริสุทธิ์ของเชื้อแบคทีเรียสายพันธุ์ WASM9-25M มีคุณสมบัติเป็นโปรตีนหรือเปปไทด์ชนิดแบคเทอริโอซินนั่นเอง สายพันธุ์ WASM9-25M เป็นเชื้อแบคทีเรีย *Bacillus* sp. ตามการวิเคราะห์ลำดับเบสของยีน 16S rRNA จากผลการศึกษาขนาดของเปปไทด์ชนิดแบคเทอริโอซินด้วยเทคนิค Tris-Tricine SDS-PAGE พบว่าแบคเทอริโอซินจากเชื้อแบคทีเรีย *Bacillus* sp. สายพันธุ์ WASM9-25M มีขนาดประมาณ 3.14 kDa ในสายพันธุ์นี้มียีนที่สร้างเปปไทด์จำนวน 3 ชนิดด้วยกันคืออิทูรินเอ ไลเคนไนซินซี และบาซิลโลมัยซินซี จากผลการวิจัยในครั้งนี้สรุปได้ว่า เปปไทด์ชนิดแบคเทอริโอซินของเชื้อแบคทีเรียสายพันธุ์ WASM9-25M อาจจะนำไปประยุกต์ใช้เป็นยารักษาการติดเชื้อ MRSA ได้ในอนาคต

คำสำคัญ: แบคเทอริโอซิน, ฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคดื้อยาสายพันธุ์ MRSA, *Bacillus* sp., WASM9-25M

Introduction

The emergence of antibiotic resistance pathogen such as methicillin resistant *Staphylococcus aureus* (MRSA) becomes an increasing serious problem in the public health worldwide notably in Asia. Asia is among the regions with the highest prevalence rates of multidrug-resistant MRSA with estimated proportion from 28% (in Hong Kong and Indonesia) to >70% (in Korea) among all clinical *S. aureus* isolates in the early 2010s.[1] A retrospective study at Chiang Mai University Hospital has shown that the prevalence of MRSA among *S. aureus* bloodstream infections increased from 23% in 2007 to 43% in 2011.[2] Moreover, MRSA strains tend to accumulate additional new antibiotic resistance such as mupirocin.[3] These problems become serious over the world and new strategies for controlling MRSA are urgently needed. It is promising to use antibacterial compound known as bacteriocins to replace currently used antibiotics. Bacteriocins are ribosomally synthesized antimicrobial peptides that are produced by bacteria that usually inhibit closely related species.[4] The potential application of bacteriocin is in food industry as natural and safe food preservatives while less research has been conducted on the therapeutic applications as antimicrobial agent. The 6.5 kDa heat stable bacteriocin KU24 produced by *Lactococcus lactis* KU24 showed an inhibitory effect against MRSA.[5] Bacteriocin production is common among many gram-positive bacteria[6] especially soil bacteria in the genus *Bacillus*. Like Lactic acid bacteria, the genus *Bacillus* includes a variety of industrially species which has been granted Generally Recognized as Safe (GRAS) status by the Food and Drug Administration, USA.[7] In a previous research, strain WASM9-25M showing high antibacterial activity against MRSA was isolated.[8] The objective of this study was to characterize the bacteriocin from isolated strain WASM9-25M. This strain is not hazardous and might be suitable for using as an alternative source of peptide antibiotic to control many important antibiotic resistant pathogenic bacteria in the future.

Materials and Methods

Bacterial strain identification

The strain WASM9-25M was isolated from water in Samut Songkhram province of Thailand.[8] The bacteriocin-producing strains against the indicator strain, methicillin resistant *Staphylococcus aureus* (MRSA, DMST5199), were isolated by co-culture method as previously described.[9] It was identified according to its 16S rRNA gene sequence using the universal primers 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3').[10] The nucleotide sequence of PCR product (1,484 bp) was compared with nucleotide database using Blastn search (http://www.ncbi.nlm.nih.gov/blastn) in order to identify the genus of this isolated strain.

Determination of bacteriocin activity

The antibacterial activity of bacteriocin was detected by an agar-well diffusion method.[11] Briefly, the cell suspension of the indicator strain (MRSA, DMST5199) at a final concentration of ca. 10^5 CFU/mL was prepared in a soft agar

(1.2% agar). Six mm diameter wells were punched and 20 µL of cell-free culture supernatant (CFS) obtained through centrifugation at 8,000 × g for 20 min (Sorvall Biofuge, Mandel Scientific, Canada) and sterile by filtration was dispensed into the wells. The plates were then incubated at 37°C for 16-18 h and the appearance of clear zones showing the antibacterial activity was observed. The assay for each sample was done in triplicate. Equal volume of sterile media and 50 µg chloramphenicol was used as a negative and positive control, respectively.

Preparation of partially purified bacteriocin (PPB)

A 200 mL Tryptic soy broth (TSB) was inoculated with 10^6 CFU/mL of an overnight culture of strain WASM9-25M. The cultures were incubated at 37°C for 18 h with shaking. The cell-free culture supernatant following cultivation was obtained through centrifugation. Ammonium sulfate was added to the supernatant with stirring to reach 80% saturation and left overnight at 4°C. The sample was centrifuged at 10,000 × g for 40 min (Sorvall Biofuge, Mandel Scientific, Canada) and the precipitate was dissolved in 5 mL of sterile distilled water and dialyzed against 1.5 L of sterile distilled water for 24 h. The active supernatant was designated as partially purified bacteriocin or PPB.

Enzyme sensitivity, heat and pH stability

The PPB was treated at 37°C for 1 h with 1 mg/mL of the following enzymes: trypsin, α-chymotrypsin and proteinase K (Sigma-Aldrich, USA). After incubation, the reaction mixtures were boiled for 10 min to inactivate the enzymes and the residual antibacterial activity was measured by agar-well diffusion. Thermal stability of bacteriocin was investigated by determination of the residual antibacterial activity after incubation of PPB at different temperature ranging from 40-100°C for 30 and 60 min, and at 121°C for 15 min. To investigate the effect of pH, antibacterial activity was measured following the pH adjustment of the bacteriocin with 0.1 N NaOH or 0.1 N HCl and incubation at 4°C for 1 h.

PCR detection of antimicrobial peptide encoding gene

The genomic DNA of strain WASM9-25SM was extracted with E.Z.N.A Bacterial DNA kit (Omega Biotek, USA). Nine pairs of primers used for amplification of genes involved in antimicrobial peptide synthesis are listed in Table 1. The PCR reaction (50 µL) composed of 5x PCR Master mix (Promega, USA), 1.5 µL of each primer (10 pmol/µL), 5 µL of dNTP (2 mM), 4 µL of $MgCl_2$ (25 mM) and 5 µL of DNA (100 ng/µL). PCR product was amplified using the appropriate conditions as indicated in the reference. Equal volume of water was used as a negative control. PCR products were purified by E.Z.N.A. Gel Extraction Kit (Omega Biotek, USA) and ligated with TA cloning vector (Vivantis, Malaysia). The nucleotide sequences were determined using M13F forward primer (1^{st} Base, Singapore) and compared with GenBank nucleotide database using Blastn search (http://www.ncbi.nlm.nih.gov/blastn).

Table 1. PCR primers used in this study.

Primer name	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
BACCF1F BACC1R	GAAGGACACGGCAGAGAGTC CGCTGATGACTGTTCATGCT	Bacillomycin D	875	16
FEND1F FEND1R	TTTGGCAGCAGGAGAAGTTT GCTGTCCGTTCTGCTTTTTC	Fengycin	964	16
ituDF ituDR	ATGAACAATCTTGCCTTTTTA TTATTTTAAAATCCGCAATT	Iturin A	1,203	17
LicAF LicAR	GTGCCTGATGTAACGAATG CACTTCCTGCCATATACC	Lichenysin synthetase A *lchAA*	735	18
LicB2F LicB2R	TGATCAGCCGGCCGTTGTCT GGCGAATTGTCCGATCATGTCC	Lichenysin synthetase B *lchAB*	904	18
LicCF LicCR	GCCTATCTGCCGATTGAC TATATGCATCCGGCACCA	Lichenysin synthetase C *lchAC*	1,195	18
sfpF sfpR	ATGAAGATTTACGGAATTTA TTATAAAAGCTCTTCGTACG	Surfactin synthetase	675	19
subF subtilinR	CAAAGTTCGATGATTTCGATTT GGATGT GCAGTTACAAGTTAGTGTTTGA AGGAA	Subtilin	566	20
subsinF subsinR	CGCGCAAGTAGTCGATTTCTAACA CGCGCAAGTAGTCGATTTCTAACA	Subtilosin	734	21

Molecular weight determination

The molecular weight of bacteriocin from isolated strain WASM9-25M were determined by the Tris-Tricine SDS–PAGE with 5% stacking gel and 16% separating gel.[12] The first half of the gel or protein gel was stained with PageBlue Protein staining solution (Fermentas, USA) whereas another half of the gel or activity gel was washed in sterile distilled water for 30 min and overlaid with TSA (0.8% agar) seeded with 1% (v/v) MRSA and incubated at 37°C for 12 h.[13] The formation of clear halo on activity gel was observed and compared with protein gel.

Results

The isolated strain WASM9-25M was identified as *Bacillus* sp. according to its 16S rRNA gene sequence. The partially purified bacteriocin (PPB) from strain WASM9-25M showed antagonistic activity against MRSA. Temperature stability experiment revealed that PPB was completely stable at high temperature up to 100°C for 30 min (Table 2). The complete inactivation was observed after treatment with trypsin. There is only 10% of activity left after treatment with other proteolytic enzymes (α-chymotrypsin and proteinase K). These enzymes are a key criterion for bacteriocin characterization. With regard to pH sensitivity, antibacterial activity of PPB was maintained at high level within the pH range of 3.0-9.0 (Table

2). PCR was used to analyze for genes involved in the biosynthesis of antimicrobial peptides reported to be produced by *Bacillus* sp. PCR and nucleotide sequencing analysis of *Bacillus* sp. strain WASM9-25M showed that the strain harbored the gene encoding iturin A, lichenysin synthetase C and bacillomycin D (Figure 1). There are two PCR products (500 and 1,200 bp) obtained from LicCF and LicCR primer. This might be due to the primer which is non-specific. However, the sequence of 1,200 bp PCR product showed high similarity (95% identity) to lichenysin synthetase C. The molecular weight of bacteriocin from *Bacillus* sp. strain WASM9-25M was determined by Tris-Tricine SDS-PAGE analysis. As shown in Figure 2, a single protein band with clear halo revealed a bacteriocin activity. The band had an apparent molecular mass around 3.14 kDa.

Table 2. Effects of proteolytic enzymes, temperature and pH on PPB of strain WASM9-25M

Treatments and conditions	Residual activity (%)
None (control)	100
Enzyme treatment	
Trypsin	0
α–chymotrypsin	10
Proteinase K	10
Temperature	
40°C, 30 min	100
40°C, 60 min	100
60°C, 30 min	100
60°C, 60 min	100
80°C, 30 min	100
80°C, 60 min	100
100°C, 30 min	100
100°C, 60 min	50
121°C, 15 min	0
pH	
3.0	100
4.0	100
5.0	100
6.0	100
7.0	100
8.0	100
9.0	100

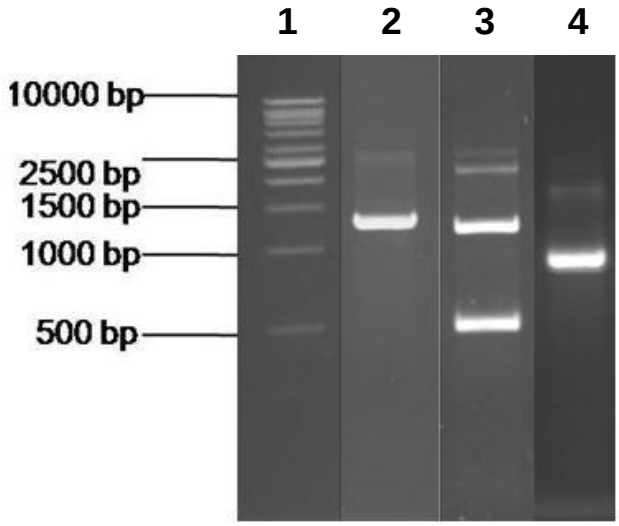

Figure 1. 1% Agarose gel electrophoresis showing PCR products of strain WASM9-25M. Lane 1: 100 bp DNA marker (Vivantis, Malaysia), Lane 2, 3 and 4: PCR products obtained from primers specific to iturin A, lichenysin synthetase C and bacillomycin D gene, respectively.

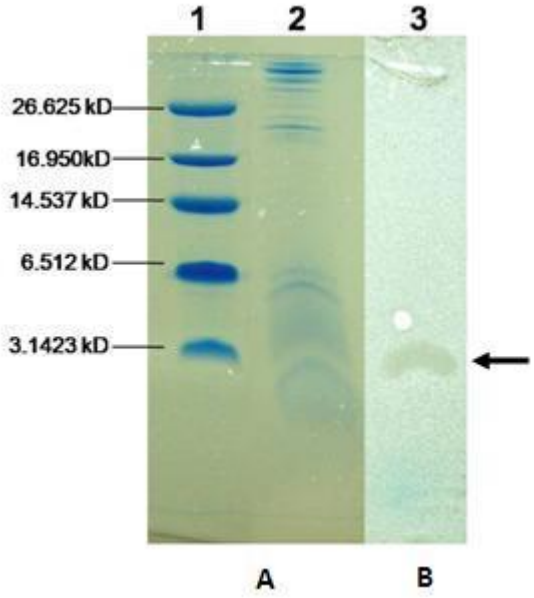

Figure 2. Tris-Tricine SDS-PAGE analysis of PPB prepared from *Bacillus* sp. strain WASM9-25M.Coomassie brilliant blue stained gel (A) and activity gel (B) shows the clear zone (arrow) after overlaid with MRSA. Lane 1: Peptide molecular weight marker; lane 2: PPB; and lane 3: PPB (overlaid with MRSA).

Discussion

The infections with methicillin resistant *Staphylococcus aureus* or MRSA have been increasing and become a serious problem worldwide. Novel anti-MRSA agents are urgently needed. The use of bacteriocin as an alternative agent to overcome the problem is promising. The isolated *Bacillus* sp. strain WASM9-25M showing anti-MRSA was isolated from environment.[8] It is well documented that bacteriocin or bacteriocin-like substances are commonly produced by different *Bacillus* species. Most of them can inhibit only gram-positive bacteria and less effective against gram-negative strains. The partially purified bacteriocin (PPB) prepared from strain WASM9-25M was active against MRSA. The sensitivity of PPB to proteinase K, trypsin and α-chymotrypsin suggests that the antimicrobial substance produced by isolated strain WASM9-25M is peptide or bacteriocin. Therefore, the isolated strain WASM9-25M bacterium can be regarded as bacteriocin-producing bacteria. The bacteriocin from strain WASM9-25M was heat stable as evidenced by its ability to reserve the activity at 100°C for 30 min. Moreover, it was stable within a wide range of pH (3-9). The heat stable property was also observed in other bacteriocins, *i.e.*, entomocin 9 and AMS T6-5, produced by *B. thuringienesis* HD9[7] and *B. licheniformis* T6-5.[14] The heat and wide pH stability is a characteristic of the class II small bacteriocins. It was shown by PCR and nucleotide sequencing that strain WASM9-25M harbors genes for producing iturin A, lichenysin synthetase C and bacillomycin D. Iturin A, lichenysin C and bacillomycin D are bacterial cyclic lipopeptides with molecular mass of 1.058, 1.000 and 0.989 kDa, respectively.[15] The molecular weight of bacteriocin from strain WASM9-25M as determined by Tris-Tricine SDS-PAGE analysis was 3.14 kDa. In conclusion, *Bacillus* sp. strain WASM9-25M produced a bacteriocin with anti-MRSA activity. This strain is non-pathogenic and derived from the nature. The biochemical properties such as thermal stability and wide range pH stability are remarkable. The bacteriocin produced by this microorganism might be used as an alternative source for controlling MRSA in the future.

Acknowledgments

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References

1. Chen CJ, Huang YC. New epidemiology of *Staphylococcus aureus* infection in Asia. Clin Microbiol Infect. 2014 Jul;20(7):605-23.
2. Chaiwarith R, Pacharasupal P, Sirisanthana T. Epidemiology, clinical characteristics and treatment outcomes of healthcare-associated methicillin-resistant *Staphylococcus aureus* bloodstream infections at Chiang Mai University Hospital: a retrospective study. Southeast Asian J Trop Med Public Health. 2014 Jul;45(4):897-905.

3. Krishnan PU, Miles K, Sketty N. Detection of methicillin and mupirocin resistance in *Staphylococcus aureus* isolates using conventional and molecular methods: a descriptive study from a burns unit with high prevalence of MRSA. J Clin Pathol. 2002 Oct;55(10):745-48.

4. Klaenhammer TR. Bacteriocins of lactic acid bacteria. Biochemie. 1988 Mar; 70(3):337-49.

5. Lee NK, Jin HE, Jun HK, Paik HD. Antimicrobial effect of bacteriocin KU24 produced by *Lactococcus lactis* KU24 against methicillin-resistant *Staphylococcus aureus*. J Food Sci. 2013 Mar;78(3):M465-9.

6. Pattnaik P, Grover S, Batish VK. Effect of environmental factors on production of lichenin, a chromosomally encoded bacteriocin-like compound produced by *Bacillus licheniformis* 26L-10/3RA. Microbiol Res. 2005;160(2):213-8.

7. Cherif A, Chehimi S, Limem F, Hansen, BM, Hendriksen NB, Daffonchio D, Boudabous A. Detection and characterization of the novel bacteriocin entomocin 9 and safety evaluation of its producer, *Bacillus thuringiensis* ssp. *entomocidus* HD9. J Appl Microbiol. 2003;95(5):990-1000.

8. Aunpad R, Na-Bangchang K, Pipatsatitpong D. Bacteriocins with anti-MRSA activity produced by water and soil isolated bacteria. Ann Microbiol. 2007 Mar;57(1):9-14.

9. Rosaldo AS, Seldin L. Production of a potentially novel anti-microbial substance by *Bacillus polymyxa*. World J Microbiol Biotechnol 1993 Sep;9(5): 521-8.

10. Eden PA, Schmidt TM, Blakemore RP, Pace NR. Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. Int J Syst Bacteriol. 1991 Apr;41(2):324-5.

11. Tagg JR, McGiven AR. Assay system for bacteriocins. Appl. Microbiol. 1971 May;21(5):943.

12. Schagger H, von Jagow G. Tricine-sodium dodecyl sulphate-polyacrilamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. Anal Biochem. 1987 Nov 1;166(2):368-79.

13. Gao Y, Jia S, Gao Q, Tan Z. A novel bacteriocin with a broad inhibitory spectrum produce by *Lactobacillus sake* C2, isolated from traditional Chinese fermented cabbage. Food Cont. 2010 Jan;21(1):76-81.

14. Korenblum E, von der Weid I, Santos ALS, Rosado AS, Sebastian GV, Coutinho CMLM, Magalhaes FCM, de Paiva MM, Seldin L. Production of antimicrobial substances by *Bacillus subtilis* LFE-1, *B. firmus* H2O-1 and *B. licheniformis* T6-5 isolated from an oil reservoir in Brazil. J Appl Microbiol. 2005;98(3):667-75.

15. Benitez LB, Velho RV, Medina LFC, Brandelli A. Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM 5006. J Microbiol. 2010 Dec;48(6):791-7.

16. Ramarathnam R, Bo S, Chen Y, Fernando WGD, Xuewen G, de Kievit T. Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Can J Microbiol. 2007 Jul;53(7):901-11.

17. Grover M, Nain L, Singh SB, Saxena AK. Molecular and biochemical approaches for characterization of antifungal trait of a potent biocontrol agent *Bacillus subtilis* RP24. Curr Microbiol. 2010 Feb;60(2):99-106.

18. Nieminen T, Rintaluoma N, Andersson M, Taimisto AM, Ali-Vehmas T, Seppälä A, Priha O, Salkinoja-Salonen M. Toxinogenic *Bacillus pumilus* and *Bacillus licheniformis* from mastitic milk. Vet Microbiol. 2007 Oct 6;124(3-4):329-39.

19. Hsieh FC, Li MC, Lin TC, Kao SS. Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. Curr Microbiol. 2004 Sep;49(3):186-91.

20. Klein C, Kaletta C, Schnell N, Entian KD. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl Environ Microbiol. 1992 Jan; 58(1):132-42.

21. Stein T, Düsterhus S, Stroh A, Entian KD. Subtilosin production by two *Bacillus subtilis* subspecies and variance of the sbo-alb cluster. Appl Environ Microbiol. 2004 Apr;70(4): 2349-53.