Methods for Confirming the Gram Reaction of Gram-variable *Bacillus* Species Isolated from Tobacco*

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SUMMARY

Bacillus is a predominant genus of bacteria isolated from tobacco. The Gram stain is the most commonly used and most important of all diagnostic staining techniques in microbiology. In order to help confirm the Gram positivity of *Bacillus* isolates from tobacco, three methods using the chemical differences of the cell wall and membrane of Gram-positive and Gram-negative bacteria were investigated: the KOH (potassium hydroxide), the LANA (L-alanine-4-nitroanilide), and the vancomycin susceptibility tests. When colonies of Gram-negative bacteria are treated with 3% KOH solution, a slimy suspension is produced, probably due to destruction of the cell wall and liberation of deoxyribonucleic acid (DNA). Gram-positive cell walls resist KOH treatment. The LANA test reveals the presence of a cell wall aminopeptidase that hydrolyzes the L-alanine-4-nitroanilide in Gram-negative bacteria. This enzyme is absent in Gram-positive bacteria. Vancomycin is a glycopeptide antibiotic inhibiting the cell wall peptidoglycan synthesis of Gram-positive microorganisms. Absence of lysis with KOH, absence of hydrolysis of LANA, and susceptibility to vancomycin were used with the Gram reaction to confirm the Gram positivity of various *Bacillus* species isolated from tobacco. *B. laevolacticus* excepted, all *Bacillus* species tested showed negative reactions to KOH and LANA tests, and all species were susceptible to vancomycin (5 and 30 µg). [Beitr. Tabakforsch. Int. 19 (2000) 97–101]

ZUSAMMENFASSUNG

Bacillus ist eine vorwiegende Gattung von Bakterien, die aus Tabak isoliert werden. Die Gram-Färbung ist die meistverwendete und wichtigste diagnostische Färbetechnik in der Mikrobiologie. Zur Bestätigung der Gram-Positivität von *Bacillus*-Isolaten aus Tabak wurden drei Methoden verwendet, die chemische Unterschiede der Zellwände und Membranen von Gram-positiven und -negativen Bakterien ausnutzen: der KOH- (Kaliumhydroxid), der LANA- (L-Alanine-4-nitroanilid) und der Vancomycinempfindlichkeits-Test. Wenn Kolonien Gram-negativer Bakterien mit 3%iger KOH-Lösung behandelt werden, entsteht eine schleimige Suspension, wahrscheinlich durch die Zerstörung der Zellwand und Freisetzung von Desoxyribonukleinsäure (DNA). Gram-positive Zellwände widerstehen der KOH-Behandlung. Der LANA-Test weist eine Zellwand-Aminopeptidase nach, die L-Alanine-4-nitroanilid in Gram-negativen Bakterien hydrolysiert. Dieses Enzym fehlt in Gram-positiven Bakterien. Vancomycin ist ein Glykopeptid-Antibiotikum, das die Peptidoglycan-Synthese in der Zellwand Gram-positiver Mikroorganismen hemmt. Das Fehlen der Lyse mit KOH, das Ausbleiben der LANA-Hydrolyse und die Empfindlichkeit gegen Vancomycin wurden zusammen mit der Gram-Färbung dazu benutzt, die Gram-Positivität verschiedener von Tabak isolierter *Bacillus*-Arten zu bestätigen. Außer *B. laevolacticus* zeigten alle untersuchten *Bacillus*-Arten negative Reaktionen mit KOH- und LANA-Tests und alle Arten waren Vancomycin-empfindlich (5 und 30 µg). [Beitr. Tabakforsch. Int. 19 (2000) 97–101]

RESUME

Bacillus est un genre prédominant parmi les bactéries isolées du tabac. La coloration Gram est la coloration la plus utilisée et la plus importante pour sa valeur diagnostique en microbiologie. Afin d'aider à confirmer la positivité de la réaction Gram de souches de *Bacillus* isolées du tabac, trois méthodes utilisant les différences chimiques de la paroi cellulaire et de la membrane des bactéries Gram-positives et Gram-négatives ont été évaluées: ce sont les tests KOH (hydroxyde de potassium), LANA (L-alanine-4-nitroanilide) et la susceptibilité à la vancomycine. Lorsque des bactéries Gram-négatives sont traitées avec une solution de KOH à 3%, une suspension visqueuse est produite. Elle est probablement due à la destruction de la paroi cellulaire et à la libération d'ADN. La paroi des bactéries Gram-positives résiste au traitement au KOH. Le test de LANA révèle la présence d'une aminopeptidase pariétale qui hydrolyse la L-alanine-4-nitroanilide. Cette enzyme est présente chez les bactéries Gram-négatives, mais absente chez les bactéries Gram-positives. La vancomycine est un antibiotique glycopeptidique inhibant la synthèse du peptidoglycan pariétal des bactéries Gram-positives. L'absence de lyse par le KOH, l'absence d'hydrolyse du LANA, et la susceptibilité à la vancomycine ont été utilisées conjointement avec la réaction Gram, afin de confirmer la Gram-positivité de diverses espèces de *Bacillus* isolées du tabac. Sauf pour *B. laevolacticus*, toutes les espèces de *Bacillus* testées ont révélé une réponse négative aux tests KOH et LANA. Toutes les espèces testées ont été sensibles à 5 et 30 µg de vancomycine. [Beitr. Tabakforsch. Int. 19 (2000) 97–101]

INTRODUCTION

The Gram reaction is a differentiation stain of taxonomic value in the identification of unknown bacterial strains. Bacteria are recognized as Gram-positive (blue-black) if

they retain the complex of crystal violet and iodine in the face of attempted decolorization with alcohol, or as Gram-negative (pink or red) if decolorization occurs as shown by bacteria accepting the safranin used as counterstain. *Bacillus* strains may lose their Gram positivity within the first 20 h of incubation (1). Because preliminary information given by the Gram reaction provides clues to the type of microorganisms present and dictates the further tests required to characterize them, a confirmation of the Gram reaction is essential. Rapid tests shown to correlate with the Gram reaction include the dissolution of the cell wall and cytoplasmic membrane by 3% KOH (2) and the presence of a cell wall aminopeptidase (3). Failure of KOH to dissolve the cell wall and absence of an aminopeptidase would be indexes for Gram positivity. Vancomycin susceptibility has been used in determining the Gram reaction of non-enterobacterial rods (4) and all the *Bacillus* strains gave positive vancomycin tests. The first objective of this study was to examine the applicability of the KOH, LANA and vancomycin susceptibility tests to confirm the Gram positivity of *Bacillus* species associated with tobacco. The second objective was to examine the effect of various incubation times on the Gram reaction *versus* the KOH, the LANA, and the vancomycin susceptibility tests.

MATERIAL AND METHODS

Bacterial strains

Fifty-five *Bacillus* strains and two Gram-negative strains listed in Table 1, were grown on Tryptic Soy Agar (TSA) (Difco Laboratories, Detroit, MI, USA) for 24 h at 30 °C. They were then Gram stained and tested with the KOH and LANA reagents. Sixteen *Bacillus* strains listed in Table 2, were grown on TSA medium for 10, 14, 18 or 24 h at 30 °C and tested as described above.

Table 1.
Gram reaction, KOH, LANA and vancomycin susceptibility tests of Gram-positive and Gram-negative rods grown on TSA for 24 h at 30 °C

Bacterial species (no. of strains)	Gram ¹	KOH ²	LANA ³	Vancomycin ⁴
<i>Bacillus amyloliquefaciens</i> (7)	+v	–	–	+
<i>Bacillus laevolacticus</i> (2)	+v	+	–	+
<i>Bacillus laterosporus</i> (2)	+v	–	–	+
<i>Bacillus licheniformis</i> (8)	+v	–	–	+
<i>Bacillus megaterium</i> (5)	+v	–	–	+
<i>Bacillus pumilus</i> (19)	+v	–	–	+
<i>Bacillus subtilis</i> (12)	+v	–	–	+
<i>Sphingomonas paucimobilis</i> (1)	–	+	+	–
<i>Stenotrophomonas maltophilia</i> (1)	–	+	+	–

¹ +: Gram-positive reaction, –: Gram-negative reaction, +v: variable Gram

² +: Lysis and string formation, –: absence of lysis and absence of string formation +/-: light string formation

³ +: Presence of endopeptidase and yellow colour, –: absence of endopeptidase and no colour

⁴ +: Susceptible at 5 and 30 mg, –: resistant

Table 2.
Influence of incubation time on Gram reaction, KOH, LANA, and vancomycin susceptibility tests on *Bacillus* species¹ grown on TSA for 10, 14, 18 and 24 h at 30 °C

<i>Bacillus</i> species (Strain designation)	Incubation time (h)	GRAM	KOH	LANA	Vancomycin
<i>Bacillus amyloliquefaciens</i> (ATCC 23350)	10	+	NA ²	- ³	+ ⁴
	14	+v	NA		
	18	+v	-		
	24	+v	-		
<i>Bacillus amyloliquefaciens</i> (ITL B051)	10	+	-	-	+
	14	+	-		
	18	+	-		
	24	+	-		
<i>Bacillus laevolacticus</i> (ATCC 23492)	10	+	-	-	+
	14	+v	-		
	18	+v	-		
	24	+v	-		
<i>Bacillus laevolacticus</i> (ITL B024)	10	+v	+	-	+
	14	-	+		
	18	-	+		
	24	+	+		
<i>Bacillus laterosporus</i> (ATCC 64)	10	+v	NA	-	+
	14	+v	NA		
	18	+v	+/-		
	24	-	-		
<i>Bacillus laterosporus</i> (ITL B043)	10	+	-	-	+
	14	-	-		
	18	+	-		
	24	+	-		
<i>Bacillus licheniformis</i> (ATCC 14580)	10	+	-	-	+
	14	+v	-		
	18	+v	-		
	24	-	-		
<i>Bacillus licheniformis</i> (ITL B014)	10	+	-	-	+
	14	+v	-		
	18	+v	-		
	24	+	-		
<i>Bacillus megaterium</i> (ATCC 14581)	10	+	-	-	+
	14	+	-		
	18	+v	-		
	24	+v	-		
<i>Bacillus megaterium</i> (ITL B004)	10	+	-	-	+
	14	+	+/-		
	18	+	-		
	24	+v	-		
<i>Bacillus pumilus</i> (ATCC 7061)	10	+	-	-	+
	14	+v	-		
	18	+	-		
	24	+v	-		
<i>Bacillus pumilus</i> (ITL B028)	10	+	-	-	+
	14	+	-		
	18	+v	-		
	24	+	-		

Table 2 (contd).

<i>Bacillus</i> species (Strain designation)	Incubation time (h)	GRAM	KOH	LANA	Vancomycin
<i>Bacillus subtilis</i> (ATCC 6051)	10	+	–	–	+
	14	+	–	–	+
	18	+v	–	–	+
	24	+	–	–	+
<i>Bacillus subtilis</i> (ITL B029)	10	+v	–	–	+
	14	+v	–	–	+
	18	+v	–	–	+
	24	+v	–	–	+

¹ See legend of Table 1.

² NA: Not applicable because of insufficient growth.

³ A negative reaction was observed for all the incubation times.

⁴ A positive reaction was observed for all the incubation times.

Gram staining

The Gram reaction was performed according to the Bacto 4-step staining procedure (Difco). The Bactrol Gram slide (Difco) was used for evaluating and controlling the quality of Gram stain reagents and technique.

KOH test

One to five colonies were picked from the surface of TSA medium with an inoculation loop and stirred in one drop of 3% KOH placed on a glass slide, for 5 to 10 sec at room temperature. Then the loop was lifted up slowly from the mixture. As seen in Gram-negative bacteria, the reaction was positive when, on raising the loop, a thread of slime followed it 0.5 cm or more.

LANA test

Whatman no. 1 (Whatman International Ltd, Maidstone, England) filter paper discs (diameter: 0.5 cm) impregnated with 10 µL of a 4% L-alanine-4-nitroanilidehydrochloride were laid upon isolated colonies grown on TSA. A LANA positive reaction, as seen in Gram-negative bacteria, was indicated by the development of a yellow color reaction within 15 min at room temperature.

Vancomycin susceptibility

The antibiotic susceptibilities of the strains were assessed on Mueller-Hinton Agar (Difco) by using filter paper disks impregnated with 5 and 30 µg of vancomycin. The test was based on the Kirby-Bauer technique (5). A positive test (susceptibility) was indicated by the formation of a clear zone (absence of growth) around the disks.

RESULTS

The Gram reaction of the *Bacillus* species tested changed from Gram-positive to Gram-negative during incubation, some before 18 h. Only one false positive KOH result (i.e. string formation) was observed for *Bacillus laevolacticus* (Tables 1 and 2). As previously reported (1), results of the KOH and LANA tests were not influenced by the age of the culture (Table 2). All *Bacillus* species tested were susceptible to vancomycin. The two Gram-negative controls responded as expected.

DISCUSSION

The Gram stain may yield false results in cultures of gram-positive rods such as the *Bacillus* species. The KOH and LANA tests are rapid, simple and useful adjuncts to the Gram reaction performed on tobacco isolates. We confirmed that both tests correlate well with the expected Gram positivity of *Bacillus* species. Out of 55 *Bacillus* strains tested, only *B. laevolacticus* (ITL strain ITL B024) gave a positive KOH test. This was also reported for one strain each of *B. cereus* (6), and *B. macerans* (1). Along with the vancomycin susceptibility test, these tests help to confirm the Gram positivity of *Bacillus* species isolated from tobacco. We recommend performing the Gram reaction on *Bacillus* cultures grown for 10–14 h at 30 °C in cooled incubators under temperature-programmed operation. In the range of incubation times tested, i.e. 10 to 24 h, the KOH, LANA and Vancomycin tests are not influenced by the age of the culture, as previously reported (7, 8). The demonstration of the presence of spores can then be used in older cultures to confirm the compatibility of the above tests with *Bacillus* species.

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