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Cellular carbohydrate patterns of the avian *Pasteurella haemolytica* like *"Actinobacillus salpingitidis"* complex

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نموذج السكريات خلايا الدجاج المركبة PHAS

في هذا البحث تم دراسة خلايا المركب PHAS للدجاج، و ذلك بطريقة الكروماتوغراف الغازي للسكريات المشتقة بالأندننتريل براستيلا و ميتلوكسيم. و مكن هذا التحليل من التعرف على 13 نوعا كميائيا تؤكد تغاير الصنف الوراثي عن طريقة تهجين لحامض دي أوكسيريبونيكلييك و حامض دي أوكسيريبونيكلييك.

الكلمات المفتاحية: أكتينوبسلاس سلبجيتيديس – بستوريلا هيمولتيك – الكروماتوغراف الغازي

Profils des glucides cellulaires du complexe pseudo *Pasteurella haemolytica* aviaire/"*Actinobacillus salpingitidis*"

Des souches représentant les différents phénotypes du complexe pseudo *Pasteurella haemolytica* aviaire/ "*Actinobacillus salpingitidis*" (PPHAAS) ont été étudiées via l'analyse de leurs profils en glucides, dérivés en aldonitriles péracétylés et en méthyloximes, par chromatographie capillaire gazeuse et par spectrométrie de masse. L'analyse a permis de reconnaître 13 chémotypes confirmant ainsi l'hétérogénéité observée par la caractérisation phénotypique conventionnelle, et par l'hybridation de l'acide déoxyribonucléique-acide déoxyribonucléique.

Mots clés: *Actinobacillus Salpingitidis* - *Pasteurella haemolytica* Aviaire - Profil glucidique - Chromatographie en phase gaseuze

Cellular carbohydrate patterns of the avian *Pasteurella haemolytic* like *"Actinobacillus salpingitidis"* complex

Strains representing the phenotypic different types of the avian *Pasteurella haemolytica* like *"Actinobacillus salpingitidis"* (APHLAS) complex were analyzed for their cellular carbohydrate contents by capillary gas-chromatography-mass spectrometry of the peracetylated aldononitrile and O-methyloxime derivatives. Analysis of the carbohydrate patterns allowed the recognition of 13 chemotypes confirming the heterogeneity observed by conventional phenotypic characteriz ation with biochemical reactions and by deoxyribonucleic acid-deoxyribonucleic acid hybridization.

Key words: Avian *Pasteurella-haemolytica*-like Actinobacillus salpingitidis - Carbohydrate profile - Gas chromatography

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INTRODUCTION

Strains of the so-called avian *Pasteurella haemolytica*-like/"*Actinobacillus salpingitidis*" (APHLAS) complex (Bisgaard, 1977 ; Mráz *et al.*, 1976) are frequently isolated from the respiratory tract of healthy birds, but are also associated with pathological lesions like chronic respiratory disease, salpingoperitonitis (Bisgaard, 1977 ; Bisgaard & Dam, 1981 ; Kohlert (1968) ; Mushin *et al.*, 1980), enteritis, hepatitis and drop in egg production (Hacking & Pettit ,1974; Matthes *et al.*, 1969 ; Shaw *et al.*, 1990) . The phenotypic and genetic investigations of Piechulla *et al.* (1985) have shown that the APHLAS isolates constitute a genus-like heterogenous group different from *P. haemolytica* strains isolated from ruminants and pigs. Avian isolates have been allocated in the vicinity of the genus *Actinobacillus*. Strains classified with the APHLAS complex do not belong to the genus *Actinobacillus sensu stricto* and probably represent more than one species according to the same authors, and the process of species delineation within the APHLAS group is still progressing.

The analysis of the carbohydrates of Gram-negative bacteria by gas chromatography (GC) and GC-mass spectrometry (MS) has proved to be a useful tool in the taxonomic evaluation of a range of fastidious organisms (Böhning *et al.*, 1989 ; Engelhard & Mutters, 1991 ; Mouahid *et al.*, 1990; Mouahid *et al.*, 1991) .

Based upon differences in production of acid from L(+) arabinose, D(+) xylose, meso-inositol, D(-) sorbitol, maltose, trehalose and dextrin 24 different biovars have been demonstrated within the APHLAS complex (Bisgaard, unpublished results).

This report describes the characteristic cellular carbohydrates from whole-cell hydrolysates of biovars of APHLAS, by capillary gas-chromatography-mass spectrometry .

MATERIALS AND METHODS

1. Bacteria

The bacterial strains investigated are listed in Table 1. The microorganisms were grown on chocolate agar (5% v/v sheep blood heated for 20 min to 80°C in Tryptic Soy agar (Difco, Detroit, USA)) and incubated at 36°C in a moist atmosphere containing 5 to 10 % carbon dioxide (Anaerocult C, Merck, Darmstadt, FRG).

2. Phenotypic characterization

The identity of the strains was confirmed by inoculation of biochemical test media as described by Mannheim *et al.* (1989). Additional tests included API ZYM (Cell suspension in 0.9 % NaCl containing about 5 mg bacterial dry weight, harvested from chocolate agar culture grown at 36°C for 24 hrs) and API 20 NE (API System S. A., La Balme les Grottes, Montalieu- Vercieu, France).

3. Cytochemical characterization

Cells grown on chocolate agar (24 hrs, 36 °C) were removed from the plate with distilled water and washed by centrifugation. The derivatization technique used was a modification of the method described by Böhning *et al.* (1989). After hydrolysis and hexane extraction, hydroxylamine hydrochloride (16 mg/ml; Sigma, Deisenhofen, FRG), 4-dimethylamino-pyridine (53 mg/ml, Sigma) and o-methoxylamine hydrochloride (33 mg/ml, Aldrich, Steinheim, FRG) were dissolved in pyridine:methanol (3:1 (vol./vol.)) and 0.4 ml of mixed reagents were added to the sugars for simultaneous derivatization into peracetylated methyloximes (PAMO) and peracetylated aldononitriles (PAAN), in a single derivatization tube. Furthermore, derivatives were washed once with 2ml 1NHCL and extracted by adding 3ml H_2O.

Carbohydrate derivatives were analyzed by a Hewlett Packard model 5890A gas chromatograph equipped with an automatic sampler HP 7673A. A HP-1 (cross linked methyl silicone gum) capillary column (25 m x 0.2 mm x 0.1 µm) was used. The heating program ran from 90°C to 250°C with a heating rate of 8°C/min and was held at 250°C for 5 min. Helium served as carrier gas.

1 µl each of the samples was injected splitlessly with a sampling time of 30s. N-octadecane served as an internal standard with a retention time of 14.18 min.

The carbohydrates were identified using a model HP 5971A mass selective detector (MSD) and analyzed with a HP G1030A MS analytical station by comparison with mass spectra of standard reference sugars.

Table 1. Bacterial strains investigated

Serial n°	Strain	biovar *	Collection n°	Source	Year and place of isolation
1	M. Bisggard 10.672/6	1	MCCM 00924	Chicken, salpinx	Langaa, DK, 1979
2	H. Gerlach 1.307/89	1	MCCM 00925	Budgerigar, liver and heart	Munich, FRG, 1989
3	H. Gerlach 1.359/89	1	MCCM 00926	Muscovry duck	Munich, FRG, 1989
4	M. Bidgaard 36.961/sv 4	3	MCCM 00934	Chiken, pharynx	Langaa, DK, 1988
5	M. Bisgaard 12.158/5	3	MCCM 00976	Chiken; salpinx	Langaa, DK, 1980
6	H Gerlach 2. 396/79=F465	4	HIM 648-3	Chiken	Munich, FRG, 1979
7	M. Bisgaard 10.672/9	4	MCCM 00521	Chiken, salpinx	Langaa, DK, 1979
8	H. Gerlach 5.821/88	4	MCCM 00937	Ara melitensis	Munich, FRG, 1988
9	O Mrãz 558/71	5	CCM 5975	Hen, upper respiratory	CS FR, 1971
10	H. Gerlach 2.740/89	5	MCCM 00979	Pigeon, liver and heart	Munich, FRG, 1989
11	O Mrãz 558/71	8	CCM	Hen, liver	CSFR, 1971
12	M. Bisgaard 25.841/13	8	MCCM 00935	Chiken, spleen	Langaa, DK, 1985
13	M. Mrãz 558/71	9	CCM	Hen, oviduct	CSFR, 1971
14	H. Gerlach 2.737/89	11	MCCM	Turkey	Munich, FRG, 1989
15	M. Bisgaard 36.961/Sv 5	11	MCCM 01047	Chicken, pharynx	Langaa, DK, 1988
16	H. Gerlach 2. 962/88	12	MCCM 00978	Budgerigar, liver & heart	Munich, FRG, 1988
17	M.Bisgaard 14.542/2	12	MCCM 01048	Chicken, liver	Langaa, DK, 1981
18	M Bisgaard 19.945/2.2	12	MCCM 01049	Goose, cloac	Langaa, DK, 1983
19	M. Bisgaard 20116 (G16)	12	MCCM 01050	Pheasant, liver	Langaa, DK, 1983
20	K.H. Hinz IPDH 697-78	15	MCCM 00983	Chicken	Hannover, FRG,1978
21	H.Gerlach 3348/80 F219	17	HIM 685-4	Goose, air sleeve	Munich, FRG, 1980
22	H. Gerlach 3076	17	MCCM 01051	Duck	Munich, FRG
23	M. Bisgaard 10.816/12	18	MCCM 01052	Chicken, salpinx	Langaa, DK, 1980
24	M.Bisgaard 13251/1	18	MCCM 00938	Goose, trachea	Langaa, DK, 1981
25	H. Gerlach 442/89	18	MCCM 01053	Budgerigar, liver & heart	Munich, FRG, 1989
26	M. Bisgaard 13190/1	18	MCCM 01054	Duck, trachea	Langaa, DK, 1981
27	M. Bisgaard 20216/3 (G25)	19	MCCM 00933	Goose, pharynx	Langaa, DK, 1983
28	O. Mraz 26b/65	20	CCM 5995	Hen	CSFR, 1965
29	H. Gerlach 236/81	21	HIM 698-7/8	Parakeet, liver, heart	Munich, FRG, 1981
30	W. Mannheim Sittich 1	21	HIM 637-5	Parakeet, heart	Marburg, FRG, 1978
31	H. Gerlach 4224	22	MCCM 01080		Munich, FRG
32	H. Gerlach 139/89	23	MCCM 01055	Chicken, liver and heart	Munich, FRG, 1989
33	M. Bisgaard 220/89	24	MCCM 01056		

* : biovar according to Bisgaard
CCM : Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia.
HIM : Bakterien-Stammsammlung, Hygiene-Institut, Marburg, F. R. Germany.
MCCM : Medical Culture Collection Marburg, F.R. Germany.

RESULTS

• Phenotypic characterization

The phenotypic features used for the differentiation of the strains investigated are given in Table 2. Differences in acid production from L-arabinose, D-xylose, m-inositol, D-sorbitol, maltose, trehalose and dextrine confirmed the recognition of 17 biovars.

The API ZYM tests of the strains investigated (Table 3) exhibited strong reactions for alcaline phosphatase, leucine arylamidase, acid phosphatase and naphtol-AS-BI- phosphohydrolase (Reactions no. 2, 6, 11 & 12 respectively). Further positive reactions were recorded for esterase (C4), esterase lipase (C8), ß-galactosidase and α-glucosidase (reactions no. 3, 4, 14 & 16). The API 20 NE system assigned the numerical profile 1020004 to all of the strains, corresponding to positive reactions for nitrate reduction, ß-galactosidase (ONPG) and oxidase.

• Carbohydrate patterns

Gas chromatography of simultaneous derivatized PAMO and PAAN of cellular carbohydrates revealed chromatogramms mainly clear of extraneous peaks.Furthermore derivatizations of duplicate cultures yielded similar results.

Table 2. Some conventional phenotypic features differentiating the strains investigated

Serial n°	Biovar	Arabinose	Xylose	M-inositol	Sorbitol	Maltose	Trehalose	Dextrin
1, 2, 3	1	-	+/(+)	+/(+)	+	+	+/(+)	+/(+)
4, 5	3	-	+	+	+	+	-	+
6, 7, 8	4	-	+	+/(+)	+	-	+/(+)	-/(+)
9, 10	5	-	+	-	-	+	+	(+)
11, 12	8	+	+	-	-	+	+	+
13	9	-	+	+/(+)	-	+	-	+
14, 15	11	-	+	+	+	-	-	-/(+)
16, 17, 18, 19	12	-	+	+/(+)	-	-	+/(+)	-/(+)
20	15	-	-	+	+	-	-	-
21, 22	17	-	+	+/(+)	-	-	-	-
23, 24, 25, 26	18	-	+	-	-	-	+/(+)	-/(+)
27	19	-	+	-	-	-	-	-
28	20	-	-	-	-	-	+/(+)	-
29, 30	21	-	-	+	-	-	-	-
31	22	-	+	-	+	-	-	-
32	23	-	-	-	-	+	+	+
33	24	-	+/(+)	+/(+)	-	+	+	+/(+)

+: Positive reaction after 24-48 h, -: Negative reaction after 21 days, (+): Delayed reaction.
Strain serial no. 30 required NAD for growth and produced acid from salicin and from esculine. Strains 29 and 30 were ß-galactosidase-negative galactosidase-negative and did not produce acid from D-mannitol and from lactose. All other strains produced acid from D-glucose, D-galactose, D-mannose, D-fructose, lactose, raffinose and D-mannitol, and were ß-galactosidase positive. They were negative for starch, dulcitol, salicin and esculine. All strains were negative for arginine dihydrolase, lysine and ornithine decarboxylase, urease, gelatinase and indole formation.

Table 3. API-ZYM reactions of the strains investigated

Serial n°	API-ZYM reactions No							
	2	3	4	6	11	12	14	16
1	5	2	1	3	5	2	3	4
2	5	2	2	3	5	1	0	3
3	5	1	2	3	5	3	1	4
4	5	3	2	4	5	4	0	1
5	5	2	3	4	5	3	3	3
6	4	2	3	3	5	4	3	1
7	5	2	3	3	5	3	0	5
8	5	1	2	3	5	3	1	4
9	5	3	4	3	5	4	1	4
10	5	2	3	3	5	4	2	5
11	5	1	2	2	5	3	1	4
12	5	1	2	3	5	4	3	4
13	5	1	2	3	5	1	3	3
14	5	1	2	3	5	5	2	0
15	5	2	2	3	5	5	3	1
16	5	2	2	4	5	5	3	5
17	5	2	3	4	5	5	4	4
18	5	1	1	3	5	4	4	5
19	5	3	1	3	5	1	2	1
20	5	1	2	1	5	1	2	2
21	5	2	3	3	5	5	4	0
22	5	2	3	3	5	4	4	0
23	4	1	2	3	5	4	4	4
24	5	1	2	4	5	4	4	4
25	5	2	3	4	3	1	3	3
26	4	1	2	5	5	0	5	4
27	5	4	3	1	5	3	4	0
28	5	2	3	4	4	3	3	4
29	4	2	1	3	5	4	3	1
30	2	0	0	0	5	0	0	0
31	5	1	2	4	5	5	3	1
32	5	2	2	4	5	2	2	4
33	5	2	2	4	5	4	4	4

*: Reaction no. 2= alcaline phosphatase, 3= esterase (C4), 4= esterase-lipase (C8), 6= leucine arylamidase, 11= acid phosphatase, 12= phosphohydrolase, 14= ß-galactosidase, 16= a-glucosidase. The numbers 1-5 indicate the increasing amounts of reaction product as detected by the color intensity. All strains were negative in reactions no. 5, 7, 8, 9, 10, 13, 15, 17-20.

The carbohydrate retention time values are given in Table 4. Aldononitrile acetate derivatives yielded a single peak for each sugar recognized whereas methyloxime acetates yielded two peaks for most of the sugars detected. All strains investigated exhibited ribose, glucose, galactose and glucosamine. The general distribution of other carbohydrates allowed the recognition of a large group of strains (chemotype X) exhibiting, in addition to former sugars, galactosamine and meso-erythrite. Additional chemotypes were discriminated by different distribution patterns for talose (I and III), adonitol (II), 1,6-anhydro-ß-D-glucose (VII), fructose (II, IV, V and XI), mannose (VI and XII), 2-keto-3deoxyoctonate (KDO) groups (II, VII, IX and XIII), and glucoheptose (I, VI, VII and XI).

Table 4. Carbohydrate retention time values (RT, min) of the peracetylated O-methyloximes (PAMO) and the peracetylated aldononitriles (PAAN), and corresponding carbohydrates

RT	Carbohydrates	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
12,05	meso-erythrite	0	0	0	0	0	0	0	0	0	1	0	0	0
12,26	ribose (A)	1	1	1	1	1	1	1	1	1	1	1	1	1
13,21	1,6-anhydro-ß-D-glucose (A)	0	0	0	0	0	0	1	0	0	0	0	0	0
13,45	ribose	1	1	1	1	1	1	1	1	1	1	1	1	1
13,77	ribose	1	1	1	1	1	1	1	1	1	1	1	1	1
14,38	adonitol	0	1	0	0	0	0	0	0	0	0	0	0	0
15,36	1,6-anhydro-D-glucose	0	0	0	0	0	0	1	0	0	0	0	0	0
15,47	talose	1	0	1	0	0	0	0	0	0	0	0	0	0
15,5	mannose	0	0	0	0	0	0	1	0	0	0	0	1	0
15,67	glucose	1	1	1	1	1	1	1	1	1	1	1	1	1
15,98	galatose	1	1	1	1	1	1	1	1	1	1	1	1	1
16,32	talose	1	0	1	0	0	0	0	0	0	0	0	0	0
16,44	galatose	1	1	1	1	1	1	1	1	1	1	1	1	1
16,51	glucose	1	1	1	1	1	1	1	1	1	1	1	1	1
16,75	glucose	1	1	1	1	1	1	1	1	1	1	1	1	1
16,82	mannose	0	0	0	0	0	0	1	0	0	0	0	1	0
16,99	galatose	1	1	1	1	1	1	1	1	1	1	1	1	1
17,08	fructose	0	1	0	1	1	0	0	0	0	0	1	0	0
17,28	glucosamine	1	1	1	1	1	1	1	1	1	1	1	1	1
17,48	KDO	0	1	0	0	0	0	1	0	1	0	0	0	1
18,13	galactosamine	1	1	1	1	0	1	1	1	1	1	0	1	0
18,28	glucoheptose	1	0	0	0	0	1	1	0	0	0	1	0	0
18,64	glucosamine	1	1	1	1	1	1	1	1	1	1	1	1	1
18,68	galactosamine	1	1	1	1	0	1	1	1	1	1	0	1	0
19,5	glucoheptose	1	0	0	0	0	1	1	0	0	0	1	0	0

DISCUSSION

The type strain of *P. haemolytica* was investigated by Brondz & Olsen (1985) who detected four major sugars (glucose, galactose, D-glycero-D-mannoheptose and L-glycero-D-mannoheptose) and four major fatty acids. The low genetic relatedness of the *P. haemolytica* type strain with the avian *P. haemolytica*-like isolates (Piechulla *et al.*, 1985) and the use of the whole cell methanolysis and trifluoro-acetic-anhydride derivatization could explain the lack of detection of ribose and other minor sugars contained by the avian isolates.

In terms of biochemical features, the APHLAS group contains several distinct biovars. Genetic investigations by DNA:DNA hybridization also revealed distinct sub-groups (Piechulla *et al.*, 1985). The biochemical or DNA-DNA hybridization-related heterogenity within this group was also reflected in their sugar patterns.

Moreover, strains that are genetically highly related (Piechulla *et al.*, 1985) were found to belong to the same carbohydrate chemotype. Two strains,

serial number 9 and 11, belonged to the same chemotype although not related on species level. Overall a good correlation was observed between the genetically established groups and the differentiation on the basis of cellular carbohydrates.

To investigate whether differences in cellular carbohydrates might reflect genetical groups, it is necessary to include additional isolates, such investigations might be useful in establishing species within the APHLAS group as a basis for clinical and epidemiological evaluation of these bacteria. In the mean-time, the thirteen chemotypes described here may serve as epidemilogical markers.

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