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G + C, DNA/DNA HYBRIDIZATION AND CELL WALL COMPOSITION OF EXTREMOPHILIC BACILLI

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ABSTRACT

Ninetten extremophilic Bacillus strains previously isolated from different interesting niches were separated into three taxonomic groups according to their G + C, and cell wall compostion. Group I comprised the obligate alkalophilic strains ,G+C content 36.2 to 38.6 mol % , peptidoglycan types : L-Or.. , L-lys, & M-Dpm , glucuronic acid present (10strains) Group II comprised the obligate and facultative haloalkalophilic strains , G + C content 41.5 to 43.1 mol % peptidoglycan type : m-Dpm, Teichoic acids present (mainly glycerol phosphate) (8 strains) , Group . III comprised one haloalkalophiclic thermophilic strain , G + C content 49.5 mol % ptidoglycan type m-Dpm , presence of glucuronic acid . DNA homology with other type strains were also presented .

INTRODUCTION

Since the first isolation of alkalophilic Bacillus by Vedder (1934) few reports were known concerning their taxonomy . In 1982 Gordon and Hyde[12] placed similar strains in the B. firmus - B.lentus group . In 1990 Fritze et al. , (1990) investigated the G+ C ratios and DNA / DNA reassociation of 78 alkalophilic Bacillus strains can be divided into separate G + C content clusters that have been ahown to correlate with certain physiological properties under neutral and alkaline conditions .

We have isolated 19 alkalophilic and / or haloalkalophilic Bacillus strains (Ghanem *et al* . , .1990 a , b & c & 1993) from some interested niches such as salt marches , 5000 years old ancient pharohs and wall

paintings of some ancient tombs .

Growth and physiological characteristics of these bacteria were previously studied (8,9,10,11). The present study is investigating the usefullness of G + C content, DNA homology levels and peptidoglycan type as valuable taxonomic markers for the taxonomy of extremophilic *Bacillus* strains .

MATERIALS AND METHODS

BACTERIAL STRAINS . Twenty four bacterial strains were used in this investigation . Nineteen strain were isolated from different localities in Egypt and previously tentatively identified (Ghanem et al., .1990 a, b & c & 1993). Others were purchased from the American Type culture collection (ATCC), Rockville, Maryland . A list of all strains is presented in Table (1).

MEDIA AND GROWTH CONDITIONS . The following media were used : Medium 1 , for the growth of atrains H1,H3, H4 & H5 (g/1) : glucose, 10.0; peptone, 5.0; yeast extract, 3.0; K2HPO4,1.0; MgSO4. 7H2O, 0.2; agar-agar, 13.0 and supplemented with 3% NaCl; pH was adjusted to 10.5 using 10% Na2CO3 . Medium 2 , for the growth of wadi El Natroun and lake Qaroun isolates : Medium 1 supplemented with 15% NaCl. Medium 3, for the growth of antiquities isolates . Type strains were grown on medium 1 at pH 7.5.

CELL WALL ANALYSIS : Cell walls of strains under investigation were prepared as described by Aono and Horikoshi

(1983) . Cell wall preparations were suspended in 5% TCA, incubated overnight at 60 C , centrifuged at 7000 g for 30 min . Extraction was repeated twice or more .

Amino acids, uronic acids and phosphorus were determined as previously described (Aono and Horikoshi, 1983).Amino sugars were assayed after hydrolysis with 4 N HCl by the Elson - Morgan reaction (Ashwell, 1957). Glycerol was assayed in the 1 M HCl hydrolysate according to wieland (1974).

DNA BASE COMPOSITION. Logarithmic phase cultures were harvested by centrifugation and DNA was extracted according to Marmur (1961). The average G + C values were determined by thermal denaturation, De Ley and Van Muylem (1963) and were calculated by using the equation of Marmur and Doty (1962) as modified by De Ley (1970).

DNA-DNA HOMOLOGY. For quantitative DNA homology, whole cell DNA was nick translated with (methyl-^{3}H) dCTP(80 ci/mmol; BRL) to specific activity of 1-2 X 10^{6} cpm/ug of DNA. The DNA concentration was set so that the fragment size was about 2000 base pairs (bp) and the probe was then sonicated to 500 bp. for hybridization experiments, the S1 nuclease method (crosa et al., 1973) was followed with slight modification (Grimont et al., 1978 and 1980). The degree of polynucleotide sequence homology was calculated by determining the ratio the average counts in the nuclease-treated and untreated samples. results were then normalized to the homologous

Table: 1 List of bacterial s ains used in the present study and their phenotypic characteristics.

Strain	Isolation source	Characteristics*			
	Sample t a	Sample from	enzymic activites	emprature relation	colony color
H1	Soil	desert	cellulolytic	ne ophilic	cream white
H3	Soil	desert	cellulolytic	mesophilic	yellow
H4	Soil	agricultural	cellulolytic	mesophilic	orange
H5	S	agricultural	cellulolytic	amilic	yellow
WN12	Wadi natrum	salt march	ND	thermoduric	cream white
WN31	Wadi Natrum	salt march	ND	thermoduric	cream white
LQ3	La Qa oun	salt lake	Lipolytic	e ne	cream white
LQ4	un	salt lake	Chitinolytic	esophilic	cream white
N25	omb	wall decorations	Amylolytic	mesophilic	cream white
N28	Nefertari tomb	wall decorations	Amylolytic	ophilic	cream white
T3	Tut n' en tomb	wall decorations	Amylolytic	ophilic	cream white
T6	Tu ukh Amen tomb	wall decorations	Amylolytic	thermophilic	cream white
T12	b	wall decorations	Chitinolytic	ophilic	cream white
T18	Tut Ankh Amen tomb	soil	Lipolytic	mesopt ic	cream white
LS67	y	mummified skin	Proteolytic	ophilic	cream white
LA83	Royal Ku mummy	mummified skin	Proteolytic	esophilic	cream white
M83	Royal King's mummy	mummified hair	Proteolytic	mesophilic	cream white
MO172	Royal K g's mummy	mummified nail	Proteolytic	ophilic	orange red
R20	Royal King's mummy	under coffin	Proteolytic	mesophilic	brownish
BACILLUS SUBTILIS	ATC 051		ND	mesophilic	brownish
B ALKALOPHILUS	ATC 27647		ND	mesophilic	cream white
B ALKALOPHILUS SUBSP HALODURANCE	ATCC 27557		ND	mesophilic	cream white
B LENTUS	ATCC 10840		ND	mesophilic	cream white
B FIRMUS	ATCC 14575		ND	mesophilic	cream white

* All strains listed except the type strains were isolated and characterized by Ghanem et al., 1990 a & b, 1991 & 1993

reaction, after the percentage of S1-resistant material in the control tubewas subtracted.

RESULTS

The nineteen extremophilic Bacillus strains which were included in previous studies (Ghanem et al., .1990 a, b & c & 1993) as well as five type strains were investigated in this study .Cell wall structure of 22 strains (Table, 2)showed that L-ornithine was the characteristic peptidoglycan type (position 3) of strains H3, H4, LS67, & MOT72; L-lysine for strains H1, H5, & LQ4, and m-Dpm for the rest of strains .Teichoic acids were detected as glycerol phosphate in the cell walls of strains T3, N28, T18, M83, R20, LQ3, WN12, no other polyols were detected. uronic acids were detected in the Cell walls of the other strains.

DNA BASE COMPOSITION.of the 19 strains ranged from 36.2 to 49.5 mol% G + C (Table, 3). Accordingly the 19 strains were divided into three DNA groups as follows: Group I : 10 strains with G = C content of 36.2 to 38.6 mol%, Groupe II : 8 strains with G + C content of 41.5 mol% and Group III: one strain with a very high G + C content of 49.5 mol%. interestingly the DNA group I was shown to comprise all the obligate alkalophilic strains which showed marked cellulolytic, chitinolytic and amylolytic activities. DNA group II comprised the facultative haloalkalophilic thermophilic strains that showed marked

Table 2: Composition of the cell walls of the extremophilic strains under investigation and those of type strains

DNA groups	strain	Chemical composition													
		Asp	Glu	Gly	m-Dpm	L-Orn	L-Lys	Ala	GlcN	Mur	Uronic acid	P	Glyc	Glc	Gal
I	H1	ND	D	D	ND	ND	D	D	D	D	D	D	ND	D	D
I	H3	ND	D	D	ND	D	ND	D	D	D	D	D	ND	D	D
I	H4	ND	D	D	ND	D	ND	D	D	D	D	D	ND	D	D
I	H5	ND	D	D	ND	ND	D	D	D	D	D	D	ND	D	D
I	LS67	ND	D	D	ND	D	ND	D	D	D	D	D	ND	D	D
I	MOT72	ND	D	D	ND	D	ND	D	D	D	D	D	ND	D	D
I	LA83	D	D	D	D	ND	ND	D	D	D	D	D	ND	D	ND
I	T12	D	D	D	D	ND	ND	D	D	D	D	D	ND	D	ND
I	LQ4	ND	D	D	ND	ND	D	D	D	D	D	D	ND	D	ND
I	N25	D	D	D	D	ND	ND	D	D	D	D	D	ND	D	ND
II	T3	ND	D	D	D	ND	ND	D	D	D	ND	D	D	D	ND
II	T18	ND	D	D	D	ND	ND	D	D	D	ND	D	D	D	ND
II	N28	D	D	D	D	ND	ND	D	D	D	ND	D	D	D	D
II	M83	D	D	D	D	ND	ND	D	D	D	ND	D	D	D	ND
II	R20	D	D	D	D	ND	ND	D	D	D	ND	D	D	D	ND
II	LQ3	ND	D	D	D	ND	ND	D	D	D	ND	D	D	D	ND
II	WN12	ND	D	D	D	ND	ND	D	D	D	ND	D	D	D	ND
II	WN31	D	D	D	D	ND	ND	D	D	D	ND	D	D	D	D
III	T6	ND	D	D	D	ND	ND	D	D	D	D	D	ND	D	D
Type strain	Bacillus subtilis	ND	D	ND	D	ND	ND	D	D	D	D	D	D	D	ND
Type strain	B. alcalophilus	ND	D	D	D	ND	ND	D	D	D	D	D	D	D	ND
Type strain	B. alcalophilus subsp halodurans	D	D	D	D	ND	ND	D	D	D	D	D	ND	D	D

ND = NOT DETECTED
D = DETECTED
ABBREVIATIONS: Asp, asparaginr; Glu, glutamic acid; Gly, glycine; m-Dpm, mesodiaminopimilic acid; L-orn, ornithin; L-Lys, lysine; Ala, alanine; GlcN, glucoesamine; Mur, muramic acid; P, phosphorus; Glyc, glycerol; Glc, glucose; Gal, galactose

amyloytic, lipolytic and proteolytic activities, and group III comprised the facutative haloalkalophilic thermoduric strain that showed marked amylolytic activities.

DNA homology results represented in Table (4) showed high correlation of some strains of group I to *B. alkalophilus ATCC 27647. Bacillus* strains LA83, MOT72, H4. and T12 showed high correlation percentage < 70%, strains N25 and H3 > 60% and strains H1, H5, LS67, AND LQ4 < 43%. other type strains were considered unrelated to any of the strains of group I where all correlations were < 40%.strains T18, N28, R20, WN12, WN31 AND group IIshowed higher correlations to B. alcalophilus subsp halodurans ATCC 27557 as 81, 73, 72, 69, 67 and 52% respectively. Two strains of the same group (M83, LQ3) showed low homology (<49%) to *B. alkalophilus* subsp halodurans. strain T6 the unique strain that possessed higher G+

Table 3. G + C content of the extremophilic *Bacillus* strains under investigation. (G + C of type, strains were also determined during this investigation

mol% G + C												
Group I			Group II			Group III			Type strains			
strain	Tm	SD	strain	Tm	SD	strain	Tm	SD	strain	Tm	SD	From literature Tm
H1	37.7	0.4	T3	42.6	0.6	T6	49.5	0.5	ATCC 27647	37.5	0.7	37.0 a
H3	36.4	0.3	T18	42.5	0.5				ATCC 10840	36.7	0.3	36.3 a
H4	36.2	0.3	N28	34.1	0.3				ATCC 27557	42.1	0.4	42.5 b
H5	38.6	0.2	M83	24.9	0.3				ATCC 6051	42.6	0.5	42.9 a
LS67	38.5	0.2	R20	41.5	0.3				ATCC 14575	41.2	0.5	41.4 a
MOT72	38.1	0.3	LQ3	41.5	0.4							
LA83	36.9	0.4	WN12	42.5	0.3							
T12	37.5	0.2	WN31	41.9	0.3							
LQ4	36.8	0.2										
N25	37.8	0.2										

a = Fahmy et al., (1985)
b = Fritze et al., (1990)

Table 4: DNA/DNA homology levels among three groups

strain	Group I										Group II								Group III
	H1	H3	H4	H5	LS67	MOT7	LA83	T12	LQ4	N25	T3	T18	N28	M83	R20	LQ3	WN12	WN31	T6
ATCC 27647	43	6	72	27	18	80	87	70	13	67	18	23	17	16	18	23	19	28	16
ATCC 10840	33	28	39	40	25	17	16	18	17	19	23	15	8	5	14	15	12	7	7
ATCC 27557	18	27	13	15	12	10	9	18	5	29	52	81	73	39	72	20	69	67	15
ATCC 6051	10	5	34	23	15	26	14	22	19	8	30	23	13	27	14	20	22	12	9
ATCC 14575	17	15	19	21	25	19	15	20	34	28	18	26	13	25	45	22	11	13	8
H1	100	43	68	53	30	22	67	39	58	66	12	5	23	18	30	33	25	22	10
H3		100	39	26	30	65	44	39	12	8	27	25	43	18	35	23	17	10	16
H4			100	43	54	29	53	24	17	34	35	34	34	23	29	6	43	26	12
H5				100	31	22	43	65	12	31	42	23	35	32	14	27	46	34	34
LS67					100	83	67	56	67	54	34	22	25	54	51	38	24	15	45
MOT72						100	23	54	18	46	23	35	10	18	16	12	13	10	10
LA83							100	80	63	75	13	23	41	37	18	12	10	9	7
T12								100	90	75	5	14	8	21	13	42	35	26	30
LQ4									100	50	21	34	26	23	20	27	28	18	10
N25										100	8	21	17	10	27	23	25	17	19
T3											100	13	22	19	20	38	60	49	45
T18												100	73	62	60	56	71	64	23
N28													100	74	65	60	58	71	37
M83														100	57	59	62	73	48
R20															100	64	59	68	40
LQ3																100	34	66	37
WN12																	100	80	13
WN31																		100	10
T6																			100

Results sre the averages of five experiments with three independent preparations. The average hybridization between probe and unlabelled DNA from the same strain was in the range of 70 + 4% to 76 + 2%. Results were normalized to 100%.

C content (49.5 mol%) showed no correlation to any type strain as well as to any other strain of groups I & II.correlation among members of each group showed either higher correlations (< 70 to 90%), moderate correlation (> 50 to 69%) or low correlation (<50%). correlation between group is too low except those of strain LS67 (group I) which showed moderate homoiogy (54 and 51%) to group II strains M83 and R20 respectively .

DISCUSSION

Taxonomy of alkalophilic Bacillus strains still needs further investgation. Since the isolation of B.alcalophilus (Vedder 1934) no profound approach was achieved until the studies of Gordon and hyde (1982) and more recently Fritze et al., (1990). Gordon and Hyde placed the alkalophilic Bacillus strains fairly loosely in the B. firmus - B. lentus complex . However, Fritze et al., (1990) concluded that the complex of alkalophilic Bacillus strains could be divided into separate G + C content clusters. Nineteen *Bacillus* strains previously isolated and characterized(Ghanem et al., .1990 a, b & c & 1993) were divided according to their growth physiology into three groups. Group I included the obligate alkalophilic strains. They showed optimal growth at pH 10.5 with lower pH of 9.5 - 9.8 and higher limits of 11 - 11.5. Group II included the facultative haloalkalophilic strains that showed minimal growth at pH 7.5, optimal growth at pH 10.0 and minimal growth at 12.0 and resist NaCl concentration of 15%. Group III comprised one strain which grew in a pH 9.0, resisted high

temperatures (70 C) and high NaC1 concentration 12.5%. Based on DNA base composition those isolated were separated into three G + C cluster I was characterized by extreme alkalophilicity an G + C content of 36.2 mol%. Cell wall structure showed some specific variations in the diaminoacid type (position 3) which resulted in further dividing to 3 subgroups one containing L-Orn., the 2nd containing L-Lys. and the 3rd containing m-Dpm. on the other hand other peptidoglycan constituents showed structural stability, except for the presence of Asp. Interestingly DNA homology among this group appeared to be invalid criteria for taxonomy where only two strains that showed >80% homology while others were less homologous. Some strains showed high homology to the type strain B. alcalophilus ATCC 27647 which suggested their identification as B. alcalophilus new strains.

Group II included the facultative haloalkalophilic strains that showed G + C content between 41.5 to 43.1 mol%.

Cell wall composition showed structural homology among members of group II where thy share a common structure, tiechoic acids (glycerol phosphate). On the basis of DNA homology some strains were closely related to B. alcalophilus subsp halodurans ATCC 27557 while others were far related. Again DNA homology among members of group II could not be used as a taxonomic criterion.

Group III comprised a unique strain which had high DNA content (49.5 mol%), cell wall structure similar to group I with m-Dpm as the diaminoacid. This strain was DNA-heterologous to all tested strains.

results revealed that B. alcalophilus comprised many species according to DNA reassociation data (table, 4) this was confirmed if we considered the proposal of de ley (1978) that members of one species should not differ by more than about +1 mol% in G + C ratio. The same conclusion was true concerning B. alcalophilus subsp halodurans which we propose a new species (B.haloalcalophilus) and members of group II. Bacillus strain T6 (group III) had high DNA content (49.5 mol%), heterologous to all type strains as well as other investigated strains. Therefore we recommend it to be a new species among the extremophilic bacilli and may propose a new name B. Pharonies as it was isolated from Tut Ankh Amen tomb (one of the Egyptian Pharohs).

Dividing extremophilic Bacillus strains according to certain physiological properties such as alkalophilicity, haloalkalophilicity (mesophilic) and thermostability in addition to certain cell wall components such as teichoic acids and teichuronic acids and G + C content clusters might be a valuable and necessary tool in the near future.

However, this needs collaborative efforts of the interested bacteriologists.

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عزل وتعريف بعض أنواع السلالات على أساس نسبة G + C وكذلك تركيب الجدار الخلوى

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تم عزل ١٩ صنفا وسلالة من الجنس العضوى (باسيللس) والذى ينمو فى بيئات متباينة ، وقد تم تقسيم السلالات إلى ٣ مجموعات بناءا على نسبة G+C وكذلك تركيب الجدار الخلوى .

واتضح من الدراسة أن المجموعة الأولى لا تعيش إلا فى بيئة قلوية (اجبارية القلوية) بينما المجموعة الثانية من الممكن أن تتعايش إما فى بيئة قلوية أو غير قلوية (اختيارية القلوية) ، أما المجموعة الثالثة فهى تتعايش فى بيئة ملحية قلوية .