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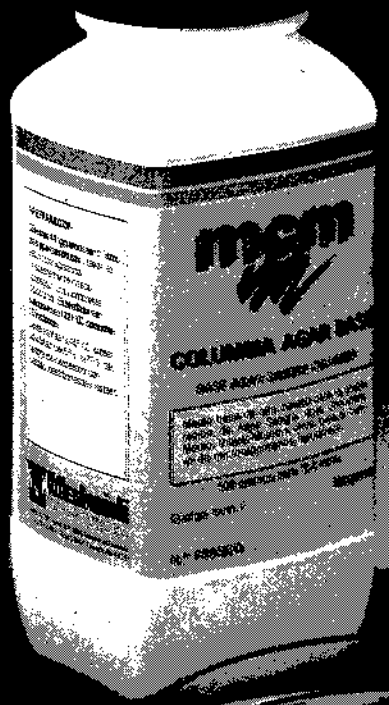

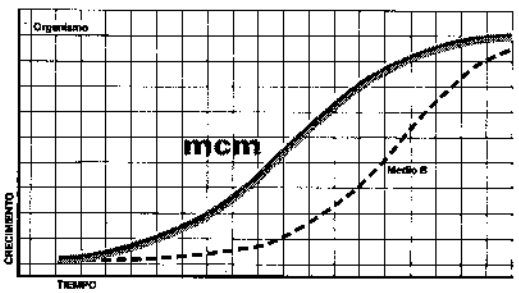

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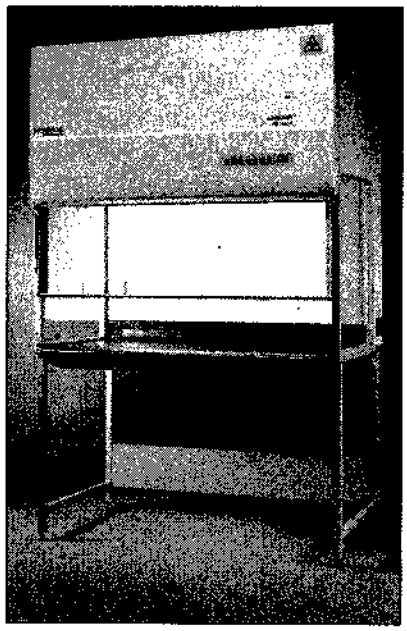

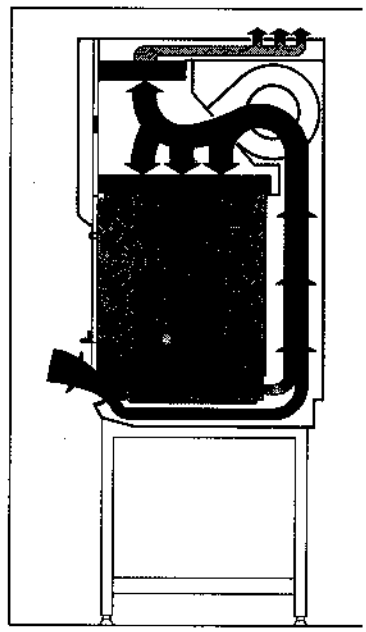

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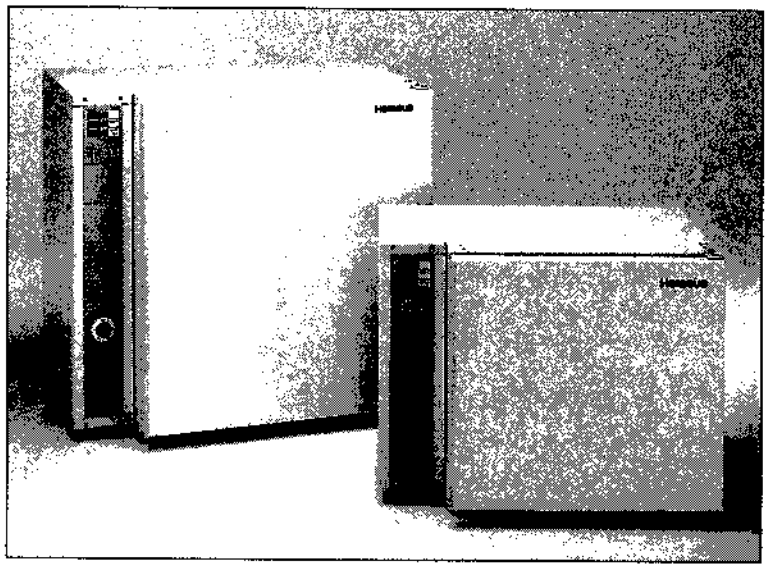

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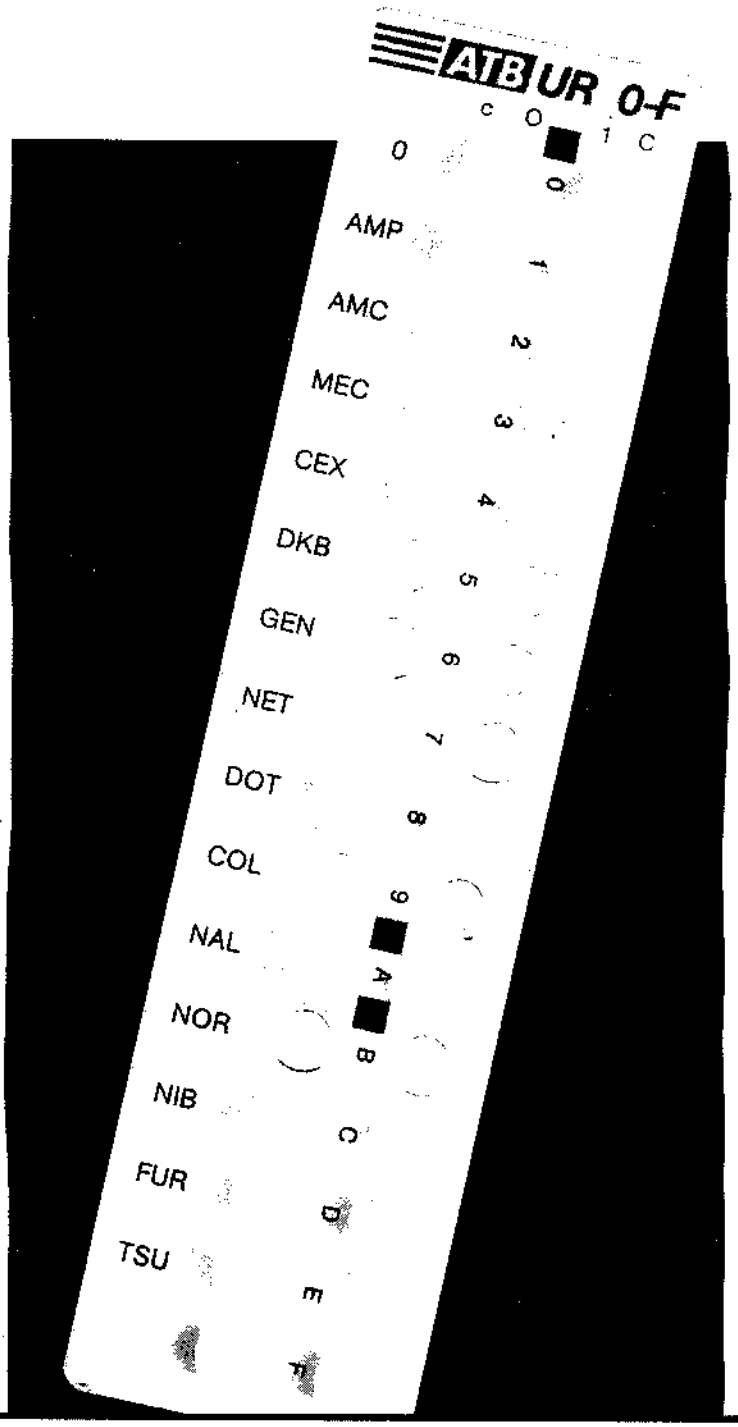

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MICROBIOLOGIA SEM 7 (1991), 57-72 **MINIREVIEW**

Enterotoxins, colonization factors and serotypes of enterotoxigenic *Escherichia coli* from humans and animals

Jorge Blanco*, Miguel Blanco, José I. Garabal and Enrique A. González

Departamento de Microbioloxía e Parasitoloxía, Facultade de Veterinaria, Universidade de Santiago, Campus de Lugo (España).

(Receivced April 10/June 19, 1991.)

Summary

Enterotoxigenic *Escherichia coli* (ETEC) strains may synthesize both thermolabile (LT-I and LT-II) and thermostable (STa and STb) enterotoxins. Whereas thermolabile enterotoxins are high molecular weigth proteins (85,000 d-90,000 d) composed by a single enzimatic A subunit combined with five B subunits which enable toxin for the receptor recognition, thermostable enterotoxins are small peptide chains with molecular weigth between 1,900 d and 5,000 d. In addition to the synthesis of enterotoxins, the ability of ETEC strains to cause diarrhoea is also conditioned by the possession of colonization factors which enable bacteria adhere-to and colonize the luminal surface of small bowel. Colonization factors in ETEC strains were located in rigid fimbriae and flexible fibrils constituted by protein subunits ranging in size from 14,500 d to 31,000 d and usually responsible for mannose-resistant haemagglutination with determined erythrocyte species. Both enterotoxins and colonization factors are controlled by plasmids. There exist plasmids which may code separatedly enteroxins and colonization factors, and besides there also exist recombinat plasmids coding together these two virulence factors. Human ETEC strains may synthesize LT-I and/or STa enterotoxins, they may possess the colonization factors named CFA/I, CFA/II, CFA/III or CFA/IV, and they belong mainly to serogroups O6, O8, O15, O20, O25, O27, O63, O77, O78, O114, O115, O126, O128, O139, O148, O153, O159 and O167. ETEC strains from porcine origin synthesize LT-I, STa and/or STb, they possess the colonization factors K88, P987, K99 or F41, and they usually belong to serogroups O8, O9, O20, O45, O64, O101, O115, O138, O141, O147, O149 and O157. Bovine and ovine ETEC strains are usually STa producers harbouring on the bacterial surface K99 or F41 colonization factors and they belong to serogroups O8, O9 and O101. Nevertheless, some particular bovine ETEC strains synthesizing LT-II have been described. Thus, a high specificity level between ETEC strains causing diarrhoea in humans and domestic animals can be observed. This is mainly due to the specific recognition between bacterial colonization factors and the epithelial receptors during host-parasite interaction.

Key words: E. coli, *enterotoxins, adhesins, diarrhoea, colibacillosis.*

Resumen

Los *Escherichia coli* enterotoxigénicos (ETEC) pueden sintetizar enterotoxinas termolábiles (LT-I y LT-II) y/o termoestables (STa y STb). Las termolábiles son proteínas de elevado peso mole-

(*) Corresponding author.

cular (85.000 d a 90.000 d) constituidas por una subunidad A y cinco B, mientras que las termoestables son pequeños polipéptidos de 1.900 d a 5.000 d. Pero para que un ETEC cause diarrea además de sintetizar enterotoxinas debe poseer un factor de colonización que le permita adherirse y colonizar el epitelio del intestino delgado. Los factores de colonización pueden ser fimbrias rígidas o fibrillas flexibles que están constituidas por subunidades proteicas de 14.500 d a 31.000 d y presentan a menudo actividad hemaglutinante en presencia de manosa. Tanto las enteroxinas como los factores de colonización suelen estar controlados por plásmidos que pueden codificar individualmente o conjuntamente para ambos tipos de factores de virulencia. Los ETEC humanos son LT-I$^+$ y/o STa$^+$, presentan los factores de colonización CFA/I, CFA/II, CFA/III o CFA/IV y suelen pertenecer a los serogrupos O6, O8, O15, O20, O25, O27, O63, O77, O78, O114, O115, O126, O128, O139, O148, O153, O159 y O167. Los de origen porcino sintetizan LT-I, STa y/o STb, presentan los antígenos adhesivos K88, P987, K99 o F41 y usualmente se engloban en los serogrupos O8, O9, O20, O45, O64, O101, O115, O138, O141, O147, O149 y O157. Los ETEC bovinos y ovinos suelen producir solamente la enterotoxina STa, expresar en su superficie K99 (a menudo también F41), y casi siempre pertenecen a los serogrupos O8, O9 y O101. No obstante, algunos *E. coli* bovinos también producen la variedad de enterotoxina termolábil LT-II. Por lo tanto, vemos que existe una especifidad de huésped entre los ETEC que causan diarrea en seres humanos y animales domésticos, lo cual es debido fundamentalmente a los distintos receptores celulares presentes en los enterocitos.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) constitute one of the more important groups among *E. coli* that cause diarrhoea (5, 48). ETEC were originally described by Smith and Halls (110), Gorbach *et al.* (52) and Sack *et al.* (101), and they are nowadays considered as the major cause of infantile diarrhoea in children living in developing countries (2, 18), as well as the most frequently responsible ethiological agent for travelers' diarrhoea (79). ETEC are rarelly isolated from sporadic cases of diarrhoea in developed countries (8, 11) but occasionally they are responsible for outbreaks of diarrhoea in children's hospitals and nurseries (11, 22, 30). Colibacillosis due to ETEC strains are frequent in domestic animals, affecting mainly to newborn and postweaning animals. Thus, they are responsible for an important economic damage in intensive explotations of porcine, bovine and ovine (59, 62). Nevertheless, a high host specificity in ETEC strains has been demonstrated because enterotoxigenic strains causing human enteric infections are considerably different to those responsible for animal colibacillosis. The pathogenicity of ETEC causing diarrhoea is due to the combination of two virulence factors: i) the ability to synthesize enterotoxins and ii) the expression of specific adhesins which enable bacteria to colinize the intestinal epithelium (48, 59, 62).

Enterotoxins

At present two groups of *E. coli* enterotoxins habe been identified. The first group is constituted by thermolabile enterotoxins (LT-I and LT-II) the activity of which has been lost after heating at 60° C for 30 min. The second group is formed by thermostable enterotoxins (STa and STb) whose enterotoxic activity remains stable after heating at 100° C for 15 min. Both, thermostable and thermolabile enterotoxins are synthesized and released by the ETEC strains into the small intestine lumen and their activity is to increase the secretion of fluid without damaging the epithelial cells (102). The most relevant characteristics of *E. coli* enterotoxins are summarized in Table 1. Table 2 shows the activity of *E. coli* enterotoxins in different enterotoxigenicity assays.

TABLE 1
CHARACTERISTICS OF ENTEROTOXINS PRODUCED BY *E. coli*

Characteristic	Heat-labile toxins		Heat-stable toxins	
	LT-I	LT-II	STa (ST-I)	STb (ST-II)
Nature	Protein	Protein	Protein	Protein
Molecular weight (d)	85,000-90,000	87,000	1,900-1,979	5,000
Subunits	1A:5B	1A:5B	No	No
Molecular weight of subunits (d)	A 25,000 B 11,500	A 28,000 B 11,800		
Receptors	GM1 Ganglioside		Not identified	
Activation of	Adenylate cyclase		Guanylate cyclase	Not known
Effect	cAMP[a] increase		cGMP[b] increase	Not known
Physiologic activity	Prolonged hypersecretion after a lag period		Rapid hypersecretion of short duration	
Immunogenicity	Good	Good	Poorly	Poorly
Controlled by	Plasmids	Chromosome	Plasmids	Plasmids

[a] Cyclic adenosine monophosphate.
[b] Cyclic guanosine monophosphate.

TABLE 2
ACTION OF LT, STa AND STb ENTEROTOXINS IN THE CONVENTIONAL ASSAYS FOR ENTEROTOXIGENICITY

	In vivo assays				*In vitro* assays
	Intestinal loops of				Cell lines
	Pigs of				
Enterotoxin	1-2 w[a]	7-9 w	Rabbits	IMT[b]	Y-1, CHO or Vero
LT[c]	+	+	+	–	+
STa	+	–	+	+	–
STb	+	+	+	–	–

[a] Weeks of age.
[b] Infant mouse test.
[c] LT-I and LT-II have a similar biological activity.

Thermolabile enterotoxin (LT) is a protein with mol. wt. 85,000-90,000 antigenically, structurally and functionally related with cholera toxin (CT) synthesized by *Vibrio cholerae* (99). Like CT, LT is formed by one A subunit and by five B subunits. The initial step for their activity is the recognition and attachment of B subunits to the receptors of GM1 gangliosides on the epithelial surface (86). The translocation of the A subunit inside epithelial cells and the dissociation in A_1 and A_2 subunits are followed by the ADP-ribosilation caused by A1 on adenilatecyclase which results in an increased level of intracellular cAMP (cyclic adenosine monophosphate) (41). The necessary gene-

tic information for synthesis of LT is located in conjugative plasmids with Tox A and Tox B genes organized in a well characterized operon (109).

Thermolabile enterotoxins synthesized by *E. coli* of human (LTh) and porcine (LTp) origins have practically the same molecular structure. However, some particular antigenic and genetic differences have been observed. Studies about the amino acid sequences of A and B subunits of LTh, LTp and CT suggest that *E. coli* would be transformed into enterotoxigenic by adquiring a foreign gene, that LT and CT diverged in the remote past and that LTh and LTp diverged very recently (123). Different monoclonal antibodies against CT, LTh and LTp have been obtained and their reactivity was observed with the homologous toxin, with all three toxins, with LTh and LTp or with LTh and CT (36).

The detection of LT enterotoxin may be carried out by the following methods: ileal loop test (24), Y-1 (25), CHO (54) and Vero (12, 46) cells; immunological methods such as ELISA-AcLT (125), staphylococcal coagglutination (100) and passive latex agglutination (103); DNA hybridization (40, 84) and haemagglutination of erythrocytes coated with GM1 gangliosides (39).

Pickett *et al.* (95) have detected a new thermolabile enterotoxin (LT-II) with the same biological activity than LT (also named LT-I) and CT although immunologically unrelated with them. LT-II is produced in low titres and is mostly cell-associated. Genes for LT-II appear to be located in the bacterial chromosome. These LT-II genes do not establish hybridization with LT (LT-I) genes under conditions of low stringency (95). LT-II, like LT (LT-I), activates the adenylate cyclase, causes rounding of Y-1 cells, increases permeability in rabbit skin and fluid accumulation in the rabbit ileal loop test, but in opposition to LT-I can cause the death to mice when it is injected intraperitoneally (61). Holmes *et al.* (61) after purification of LT-II have established that this toxin is a protein with a mol. wt. of 87,000 d composed by A and B subunits of mol. wt. 28,000 d and 11,800 d respectively. Recently, two LT-II toxin (LT-IIa and LT-IIb) variants have been described by Guth *et al.* (58).

Seriwatana *et al.* (106) after testing 168 human LT^+ *E. coli* and 47 bovine LT^+ in the Y-1 cells assay, have observed that only 1.8% of ETEC from human origin were $LT\text{-}II^+$, whereas 72.3% of bovine ETEC strains were $LT\text{-}II^+$. Thus, practically all the ETEC strains producing LT from human origin and probably of porcine origin are $LT\text{-}I^+$, whereas the majority of bovine *E. coli* strains producing LT synthesize the LT-II variant.

On the other hand, *E. coli* thermostable enterotoxins are low mol. wt. proteins presenting an immunological reduced activity. Two thermostable enterotoxins STa and STb (also named ST-I and ST-II) were recognized. Whereas the STa enterotoxin is methanol soluble and causes deshidratation to lactant mice, the STb enterotoxin is methanol insoluble and does not affect to lactant mice but causes fluid accumulation in the intestinal loops of postweaned pigs (Table 2) (102).

The actuation of STa enterotoxin is based upon guanylate cyclase activation increasing the intracellular level of cGMP (cyclic guanosine monophosphate). The final result is a rapid secretion of fluid occurring within a shorter period of time than that produced by LT (55). STa is synthesized as a precursor polipeptide chain of 72 amino acids that after peptidic cleavage during maduration is released as a small peptide formed by 18 amino acids (85). Heat stability and resistance to enzimatic hydrolisis showed by STa are due to the presence of six cysteine residues which establish disulphide linkages (20). Genes specifying STa are located in plasmids which may also contain genes for LT, for colonization factors and/or for resistance to antibiotics (26, 29, 43).

The amino acid sequence in STa enterotoxins synthesized by human, porcine and bovine *E. coli* strains has already been determined and their primary structure is very similar to all of them, existing differences only in the position of some amino acids at the N-terminal part (20). STa genes in *E. coli* from human and animal origin have also been cloned and DNA hybridization probes have been developed for STa detection. Application of these DNA probes has discovered the existance of two varieties of the STa toxin: STa1 (STap or STIA) and STa2 (STah or STIB) (84, 85). Seriwatana *et al.* (105) after applying DNA probes to 337 STa^+ strains of human origin have observed that:

72 % hybridized with the gene of STa2, 25 % with the gene of STa1 and 3% with the genes of both STa1 and STa2. All ETEC STa$^+$ strains from animal origin synthetize the STa1 variety. Hybridization of genes for the two varieties of STa enterotoxin may by carried out under loose conditions, not under demanding conditions, showing that only a partial homology exists between both STa genes. Although STa enterotoxins are poorly antigenic in natural conditions, specific antisera for both varieties have been obtained and these sera were able to show that STa toxins produced by strains from human and animal origins are antigenically related (37).

The classical conventional assay for detecting STa is the infant mouse test (IMT), which is employed in the majority of studies (7). Nevertheless, different *in vitro* assays for detecting STa have been developed, including radioimmunoassay (37), ELISA (104) and DNA hybridization (40, 84).

STb enterotoxin is synthesized by some porcine ETEC strains and rarely by human strains (27). The STb action mechanism is not known but it appears not to act via cAMP or cGMP. When STb was tested in the piglet ileal loop assay, it was possible to deduce that STb caused active bicarbonate secretion (121). STb was synthesized as a polipeptide chain of 71 amino acids containing a peptidic signal of 23 amino acids, and after the cleavage it was released as a mature protein of 48 amino acids and with a mol. wt. of 5,000 d (73). STb plasmidic genes were cloned and besides it was shown that there was not homology between them and those coding STa1 and STa2 (27).

Detection of STb was initially carried out in the piglet ileal loop text (44), but at the present moment the method usually employed is a gene probe (27) and an ELISA has recently been developed (60).

Colonizations factors

Enterotoxigenic *E. coli* possess adhesive mechanisms which enable bacteria to adhere and colonize the small bowel mucosal surface, supporting the washing action of fluids in the lumen and the intestinal peristaltism (38, 48). Adhesive properties in ETEC are associated with the expression of proteinaceous filament appendages located on the bacterial surface. Those filamentar appendages may constitute rigid structures (fimbriae) or flexible filaments (fibrils), both made from protein subunits of mol. wt. between 14,500 d and 31,000 d. Genetic information for these adhesins or colonization factors is usually located in plasmids which normally contain genes for enterotoxins synthesis. Furthermore, the majority of colonization factors exhibit mannose resistant haemagglutination (MRHA) with specific erythrocyte types, and they may be expressed by bacteria growing at 37° C, not at 18° C. There is a close association between expression of specific colonization factors and bacteria belonging to determined O:K:H serotypes. General characteristics of adhesins detected in ETEC strains from human and animal origin are recovered in Tables 3 and 4 respectively. Toxic phenotypes of human and animal *E. coli* strains expressing the different colonization factors are summarized in Tables 5 and 6.

Adhesins in ETEC strains from human origin were found in both fimbriae and fibrils, and they were labelled colonization factor antigens (CFAs) (21, 31, 32, 38, 45, 53, 63, 118, 119). The first colonization factor antigen was named CFA/I reported and discovered by Evans *et al.* (32) in the strain H10407 of serotype O78:H11. CFA/I was defined as a fimbria with 6-7 nm in diameter which confers to bacteria an increased surface hydrophobicity level due to the high proportion of hydrophobic amino acids (37 %) (33, 34, 50, 120). The presence of CFA/I gives bacteria the ability to haemagglutinate human, bovine and chicken erythocytes in the presence of D-mannose (6, 33, 34, 120). CFA/I is controlled by plasmids which usually contain genes for STa enterotoxin and occasionally for synthesis of LT and STa enterotoxins (28, 32, 87).

Evans *et al.* (31) reported the existence of another colonization factor unrelated with CFA/I, named CFA/II. CFA/II showed a different haemagglutination pattern to the reported by CFA/I,

TABLE 3
CHARACTERISTICS OF COLONIZATION FACTOR ANTIGENS IN HUMAN ETEC AND SEROGROUPS MORE FREQUENTLY DETECTED AMONG STRAINS WITH COLONIZATION FACTORS

Colonization factor antigen[a]	Coli surface antigen	Mol. wt. of subunits (d)	Morphology	Diameter (nm)	MRHA[b]	Serogroups
CFA/I (F2)	None	15,058	Fimbrial	6-7	+	O4, O15, O20, O25, O62, O63, O78, O90, O110, O114, O126, O128, O153
CFA/II (F3)	CS1	16,300	Fimbrial	6-7	+	O6, O8, O9, O78, O80,
	CS2	15,300	Fimbrial	6-7	+	O85, O115, O128, O139,
	CS3	14,700	Fibrillar	2-3	+	O154, O168
CFA/III	None	18,000	Fimbrial	6-7	–	O25
CFA/IV (E8775)	CS4	17,000	Fimbrial	6-7	+	O6, O25, O27, O92
	CS5	21,000	Fimbrial	6-7	+	O115, O148, O153
	CS6	14,500-16,000	Fibrillar	2-3	–	O159, O167, O169
PCF O159:H4	None	19,000	Fimbrial	6-8	–	O159
PCF O148:H28	?	?	Fibrillar	3	–	O148

[a] Additional denominations of colonization factors are indicated in parenthesis.
[b] Mannose-resistant haemagglutination.

TABLE 4
CHARACTERISTICS OF COLONIZATION FACTOR ANTIGENS IN ANIMAL ETEC AND SEROGROUPS MORE FREQUENTLY DETECTED AMONG STRAINS WITH COLONIZATION FACTORS

Colonization factor antigen[a]	Origin of strains	Mol. wt. of subunits (d)	Morphology	Diameter (nm)	MRHA[b]	Serogroups
K88 (F4)	Porcine	23,500-26,000	Fibrillar	2.1	+	O8, O9, O20, O45, O138, O147 O149, O157
P987 (F6)	Porcine	20,000	Fimbrial	7.0	–	O8, O9, O20, O64, O141, O149
PCF O141	Porcine	17,000	Fimbrial	< 5	–	O141
CS1541[c]	Porcine	18,000-19,000	Fibrillar	3-5	–	O8
F42	Porcine	31,000	Fimbrial	5-7?	+	?
F41	Porcine and Bovine	29,500	Fibrillar	3.2	+	O9, O20, O64, O101
K99 (F5)	Porcine and Bovine	18,500	Fibrillar	4.8	+	O8, O9, O20, O64, O101, O149
Att25 (FY ó F 17)	Bovine	20,000	Fibrillar	3-4	–	O101, O141
CS31A (31a)[d]	Bovine	29,000	Fibrillar	2	–	O8

[a] Additional denominations of colonization factors are indicated in parenthesis. Att25 and CS31A, unlike other adhesins detected in ETEC, are expressed by non enterotoxigenic strains. Whereas non enterotoxigenic Att25' strains belong to serogroups O8, O9, O15, O78 and O101, non enterotoxigenic CS31A' strains belong the serogroups O2, O17, O20, O78, O86, O117 and O153.
[b] Mannose-resistant haemagglutination.
[c] CS1541 antigen presents a serological cross-reaction with P987.
[d] CS31A is structurally and immunologically related with K88.

showing activity only with calf and chicken erythrocytes in the presence of D-mannose (6, 33, 34, 120). Reports published by Cravioto *et al.* (19) and Smyth (112) have demonstrated that composition of CFA/II was more complex than the CFA/I fimbria. These authors discovered that strains possessing CFA/II may express three coli surface antigens (CS1, CS2 and CS3), the synthesis of which is controlled by a single plasmid, usually coding LT and STa as well (28, 108). Thus, depending of strain these coli surface antigens may be expressed in different combinations (112) and the CFA/II producing strains may express CS1 and CS3, CS2 and CS3 or CS3 only. CS3 is invariably present in all the CFA/II producing strains and their morphology seems a flexible fribil with 2-3 nm in diameter, whereas CS1 and CS2 are rigid fimbriae with 6-7 nm in diameter (19, 108, 112).

Honda *et al.* (63, 64) working with several ETEC from human origin belonging to O25 serogroup have reported the presence of another colonization factor named CFA/III. CFA/III enables bacteria to colonize the intestinal epithelium of rabbits and lactant mice, and was it shown to be a highly hydrophobic fimbria with 6-7 nm in diameter showing a complete lack of ability to agglutinate erythrocytes in the presence of D-mannose. Whereas surface hydrophobicity due to the expression of CFA/III on bacterial surface was thermostable, CFA/I and CFA/II was thermolabil. *E coli* strains expressing CFA/III normally produce LT enterotoxin only.

Thomas *et al.* (119) have reported the presence of a new colonization factor named CFA/IV in the prototype strain E8775. CFA/IV, like CFA/II is composed by three different coli surface antigens (CS4, CS5 and CS6). CS4 and CS5 are rigid fimbriae with 6-7 nm in diameter whereas CS6, like the CS3 antigen in CFA/II, is a flexible fibril with 2-3 nm in diameter. CFA/IV is responsible for mannose-resistant haemagglutination of human and bovine erythrocytes (119). Genetic information for CFA/IV synthesis is located in plasmids and there were detected plasmids coding together CS5-CS6-STa, CS6-STa, CS6-LT and CS4-CS6 (78).

Two putative new colonization factors have been recently reported, and none of which showed haemagglutinating activity in the presence of D-mannose. The first was described by Tacket *et al.* (118) in O159:H4 ETEC strains and is a fimbria coded in the same plasmid that codes LT and STa as well. The second one has shown a morphology like curly fibrils with 3 nm in diameter and was detected by Knutton *et al.* (70) in ETEC strains producing LT and STa belonging to serotype O148:H28.

The first intestinal colonization antigen discovered in *E. coli* strains from animal origin was K88. This was originally described by Orskov *et al.* (94) in *E. coli* strains isolated from piglets with diarrhoea and it was a protein in nature with the appearance of a fibril with 2 nm in diameter (115). After diverse studies three K88 antigenic variants were described: K88ab, K88ac and K88ad (57). Synthesis of K88 is controlled by a transmissible plasmid (115). K88 antigen confers to bacteria the ability to haemagglutinate guinea pig erythrocytes in the presence of D-mannose (115).

The practical totality of ETEC STa^+ strains which are responsible for bovine and ovine colibacillosis express on their surface the colonization factor antigen K99 (92), initially named «Kco» by Smith and Linggood (111). K99 antigen is a proteinaceous fribil with 4.8 nm in diamater that haemagglutinate human, guinea pig and sheep erythrocytes in the presence of D-mannose (23, 65, 92, 111). Moon *et al.* (80) in 1977 have proved that K99 antigen, previously detected only in bovine and ovine ETEC (81, 88), was also found in some particular porcine ETEC lacking K88. This research group has observed that K99 was only present in the porcine ETEC belonging to class II non-typical porcine enteropathogenic strains (producing only STa) and being included within the serogroups O9, O64, and O101, from which O9 and O101 are considered classical bovine enteropathogens.

A new fimbrial antigen named P987 was discovered in 1977 by Isaacson *et al.* (67) and Nagy *et al.* (90). P987 was detected in some porcine ETEC strains lacking K88 and K99. P987 fimbria has 7 nm in diameter and does not confer haemagglutinating activity to the bacteria (67, 68). The P987 genetic control remains unknown but it was suggested that P987 genes could be located in the bac-

TABLE 5
ENTEROTOXIC PHENOTYPES OF HUMAN ETEC. RELATION WITH THE EXPRESSION OF COLONIZATION FACTOR ANTIGENS

Colonization factor antigen	Enterotoxic phenotypes		
	LT	LT STa	STa
CFA/I	–	+	+*
CFA/II	–	+	–
CFA/III	+	–	–
CFA/IV	–	+	+
PCF O159:H4	–	+	–
PCF O148:H28	–	+	–
None	+*	+	+

* = Phenotypes more frequently detected.
– = Not detected or with very low frequency only.

TABLE 6
ENTEROTOXIC PHENOTYPES OF ANIMAL ETEC. RELATION WITH THE EXPRESSION OF COLONIZATION FACTOR ANTIGENS

Colonization factor antigen	Origin[a]	Enterotoxic phenotypes					
		LT	LTSTa	LSTb*	STa*	STb*	STaSTb*
K88	Porcine	+	+	+*	–	–	–
P987	Porcine	–	–	–	+*	+	+
PCF O141	Porcine	–	–	–	–	–	+
CS1541	Porcine	–	–	+	–	–	–
F42	Porcine	–	–	–	+	–	–
F41	Porcine and Bovine	–	–	–	+	–	–
K99	Porcine and Bovine	–	–	–	+*	+	+
Att25	Bovine	+	–	–	+*	–	–
CS31A	Bovine	–	–	–	+	–	–
None	Porcine	+	+	+	+*	–*	+*

[a] = The majority of bovine ETEC produce STa only and express K99 and/or F41 antigens.
* = Phenotypes more frequently detected.
– = Not detected or with a low frequency only.

terial chromosome (38). P987 antigen, like K99, allows to bacteria the ability to colonize the small bowel distal part, whereas those bacteria possessing K88 colonize the proximal and distal parts of the intestine.

Morris *et al.* (83) found another colonization antigen named F41 which was expressed by the bovine enteropathogenic *E. coli* strain B41 and which can also express K99 antigen. F41 is commonly synthesized by $K99^+$ ETEC strains from porcine, bovine and ovine origins belonging to O9, O64 and O101 serogroups (35, 77, 122). Despite this, porcine ETEC strains expressing exclusively F41 and belonging mainly to O64 and O101 serogroups have been isolated (35, 114). F41 is a fibril with 3.2 nm in diameter that confers haemagglutinating activity of human, guinea pig and sheep erythrocytes in D-mannose presence. The location of genes coding F41 remains unknown.

During the last years other five additional putative colonization antigens were detected in

ETEC strains from animal origin; three of them (PCF O141, CS1541 and F42) were discovered in porcine *E. coli,* and the other two (Att25 and CS31A) in bovine strains. However, the role of these putative colonization antigens in intestinal colonization, such as PCF O159:H4 and PCF O148:H28 from human *E. coli,* must be established in future studies.

The PCFO141 adhesive fimbria was recently discovered by Kennan and Monckton (69) in porcine O141:K85ab ETEC strains STa^+STb^+ isolated from post-weaning pigs with diarrhoea in Australia. PCF O141 was found to be a fimbria with a diameter size lower than 5 nm and lacking of haemagglutinating activity in D-mannose presence. It is formed by protein subunits with a mol. wt. of 17,000 d and like the majority of colonization antigens was not expressed by bacteria growing at 18° C. CS1541 was detected by Broes *et al.* (13, 14) in porcine ETEC producing LT and STb isolated in Canada and belonging to serotype O8:KX105. CS1541 was demostrated to be immunologically related with P987. Like P987, CS1541 antigen was not expressed at high levels in *in vitro* conditions. Thus, it was necessary to perform the detection of this new antigen by immunofluorescence methods in the piglet intestines. CS1541 antigen is a fibril with 3-5 nm in diameter which resembles the appearance of K99 and that it is formed by protein subunits of mol. wt. 18,000-19,000 d showing a high hydrophobicity level (13). The role of CS1541 as colonization antigen needs to be clarified because the strains possessing CS1541 were able to develop diarrhoea in colostrum deprived piglets even though intestinal colonization seems to be moderate and those strains lack ability of attaching to isolated piglet enterocytes (14). In contrast with the remaining colonization antigens discovered in ETEC strains, CS1541 may be expressed by bacteria growing at both 37° C and 18° C incubation temperatures and does not show haemagglutinating activity. On the other hand F42 antigen was found by Yano *et al.* (124) in porcine *E. coli* producing STa isolated in Brazil. F42 is an haemagglutinating fimbria with mannose resistant activity which may be expressed by bacteria growing at 37° C but not at 18° C incubation temperatures, and it is formed by protein subunits with mol. wt. 31,000 d (74). Strains STa^+ and $F42^+$ produce the typical symptoms of colibacillar diarrhoea when they are administered to colostrum deprived piglets (74).

Some particular bovine ETEC besides expressing K99 antigen may produce another adhesin named Att25 in Belgium (96) and FY in France (16) which was recently classified as F17. Results obtained by Morris *et al.* (82) and Lintermans *et al.* (75) after examining both the Att25 antigen discovered by Pohl *et al.* (96) and the FY antigen discovered by Contrepois *et al.* (16), suggest that they are really the same adhesin type antigen. Att25 is a fibrillar adhesin with 3-4 nm in diameter constituted by protein subunits with mol. wt. 20,000 d (75, 76). It has not been found a clear association between the possession of Att25 and the production of enterotoxins, although a close association has been observed between strains $Att25^+$ and calves with both diarrhoea or septicaemia (97, 98). Two categories of $Att25^+$ strains were established: (i) enterotoxigenic strains producing STa and K99, and (ii) non enterotoxigenic strains lacking of K99 and possessing only Att25 (16, 97). These two categories of Att25 producing strains showed haemagglutination activity with bovine erythrocytes and were adhesive to calf enterocytes in the presence of D-mannose (75, 107). Adhesion to intestinal villi in strains synthesizing both Att25 and K99 was only inhibited when enterocytes were previously incubated with these two purified fimbriae but not when the preincubation was performed with only one fimbriae type (75). Furthermore it was feasible to determine that the colostra from cows vaccinated with bacteria producing K99 and F41 and from cows vaccinated with bacteria producing only Att25 do not confer protection to lactant calves, when a ETEC $K99^+F41^+Att25^+$ strain was orally administered. Nevertheless the mixture of these two colostra previously indicated was able to protect against experimental oral infection with a bacteria producing K99, F41 and Att25 (17). All the results mentioned above suggest that Att25 adhesin, like F41, may contribute to enhance colonization properties in bovine ETEC synthesizing K99. The role of non enterotoxigenic strains producing Att25 in enteric infections has not been clarified yet, likewise some particular results indicate that they may cause systemic colibacillosis, like $CS31A^+$ strains (16, 97).

TABLE 7
HUMAN, PORCINE AND BOVINE ETEC SEROTYPES

Enterotoxigenic *E. coli* serotypes		
Human	Porcine	Bovine
O6:K15:H- o H16	O8	O8:K25:K99
O6:K-:H51	O8:K87:K88	O8:K85:K99
O8:K25:H9	O8:K99	O9:K30:K99
O8:K40:H9	O9:K103:P987	O9:K35:K99
O15:H-	O9:K99	O9:K(A)?:K99
O15:K-:H11	O20:K101:P987	O20:K?:K99
O20:H- o H21	O20:K101:K99	O101:K28:K99
O25:H-	O45:K88	O101:K30:K99
O25:K7:H42	O64:P987	O101:K(A)?:K99
O27:H7 o H20	O64:K99	O101:K-:K99
O63:H-	O64:F41	
O77:H18	O101:K99	
O77:K13:H45	O101:K99, F41	
O78:H-	O115	
O78:K-:H11 o H12	O138:K81	
O114:H- o H49	O138:K81:K88	
O115:H40 o H51	O141:K85	
O126	O141:K85:P987	
O128: H7, H12 o H21	O147:K89:K88	
O139:H28	O149:K89:K88, P987	
O148:K-:H8	O149:K91	
O148:H28	O149:K91:K88	
O153:K-:H45	O149:K99	
O159:H4, H20, H21 o H34	O157	
O167:H5	O157:K88	

Girardeu *et al.* (42) have carried out the molecular characterization of the CS31A adhesin described by Contrepois *et al.* (15) in bovine *E. coli* causing diarrhoea and septicaemia. This adhesin showed a fibrilliar morphology constituted by protein subunits with a mol. wt. 29.000 d and it was structurally and immunologically related with K88 (42). Similarly to the majority of colonization factors, CS31A seems to be controlled by a plasmid, in this occasion with a mol. wt. 105 Md (15). Although strains producing CS31A and strains producing Att25 showed mannose-resistant haemagglutinating activity (MRHA) with diverse erythrocyte species, results obtained with purified fimbriae (75) and with transconjugant strains (42) suggest that the MRHA is due to other unknown fimbriae type expressed by these strains. The surface antigen CS31A was initially detected in strains isolated from claves with diarrhoea, some of which were enterotoxigenic (STa^+) and belonged to serotype O8:K85ab:K99. However, it was recently found that CS31A is also common among non enterotoxigenic *E. coli* belonging to serogroups O8, O20, O78, O86, O117 and O153, isolated from hypogammaglobulinemic newborn calves with septicaemia. Contrepois *et al.* (15) showed that oral infection of calves not receiving colostrum with *E. coli* 31a (reference strain for CS31A) was generally followed by septicaemia and death in less than 48 h. Post-mortem examination revealed pneumonia and oedema on the kidneys and gall bladder. Oral infection of calves receiving colos-

trum had no effect, but intravenous inoculation produced arthritis within 15 days. Moreover, Shimizu *et al.* (107) induced lethal diarrhoea in neonatal calves by challenge with the combination of a K99$^+$STa$^+$ *E. coli* strain and either a CS31A$^+$ strain or a CS31A$^+$ strain plus an Att25$^+$ *E. coli* under the experimental conditions in which lethal diarrhoea was not induced by challenge with a K99$^+$STa$^+$ *E. coli* strain alone. CS31A$^+$ strain and Att25$^+$ strain did not induce any observable symptoms in calves that had received colostrum from unvaccinated cows. Strain producing K99 and STa induced diarrhoea but not lethal disorders in calves (107). We think that it would be interesting to investigate the production of CNF2 necrotizing factor in those septicaemic *E. coli* synthesizing CS31A, because we have recently detected the production of CNF2 in faecal bovine *E. coli* strains belonging to serogroups O8, O78, O86, O117 and O153 (unpublished results). The group directed by De Rycke together with our own research group (24) have demonstrated that necrotizing *E. coli* (NTEC) may synthesize two cytotoxic necrotizing factors named CNF1 and CNF2. CNF1 is synthesized by *E. coli* strains causing urinary infections and bacteremia in humans (3, 4), whereas CNF2 is synthesized by faecal bovine *E. coli* isolated from diarrhoeic and healthy calves (12, 49, unpublished results). It would by feasible that strains producing CNF2 isolated from calves may possess the same critical role in extraintestinal infections of bovine than strains producing CNF1 in extraintestinal infections of humans.

Serotypes

An important characteristic in ETEC strains is that the majority of them belong to a reduced number of O:K:H serotypes that are rarely found among non-enterotoxigenic *E. coli.* Some of these serotypes were found in isolates from well defined geographical areas whereas other serotypes may be isolated from diverse regions of the world. Table 7 summarizes the most frecuently found serotypes among ETEC strains of human, porcine and bovine origins. Orskov and Orskov (93), during the last decades, have serotyped a high amount of human ETEC strains isolated in countries around the world and they have concluded that the majority of them (approximately 72%) belonged to only seven serogroups (O6, O8, O15, O25, O78, O115 and O128) and that the most frequently O:K:H combinations (serotypes) detected were O6:K15:H16 or H-, O8:K40:H9, O15:K-:H11, O25:K7:H42 and O78:K-:H11, H12 or H-. In Spain the serotype most frequently detected among human ETEC strains was O153:K-:H45 (producing STa). Strains belonging to this serotype were the cause of various infantile diarrhoea outbreaks suffered in Valencia, Madrid, Talavera de la Reina and La Coruña (11, 30). Other serotypes commonly detected in human ETEC strains isolated in Spain were O6:K15:H16 (producing LT and STa) and O27:K-:H7 (producing STa). O153:K-:H45 ETEC strains isolated in Spain synthesized CFA/I and those of serotype O6:K15:H16 synthesized CFA/II, whereas O27:K-:H7 strains isolated in our country developed after growing on Minca-Is medium an adhesin that haemagglutinated bovine and sheep erythrocytes in the presence of D-mannose and besides it would represent a putatively new colonization factor (10). Genes coding STa and CFA/I in O153:K-:H45 strains were located in non-conjugative plasmids of approximately 82 and 87 Md in size (51). Studies carried out with human ETEC strains isolated in differents countries show that approximateley 50% synthesized CFA/I or CFA/II, 15% expressed CFA/III and 5% possessed CFA/IV (6, 38, 48).

The majority of ETEC strains isolated from newborn piglets and postweaning pigs with diarrhoea was found to belong to the serotype O149:K91:K88 and to synthesize LT and STb or LT, STa, and STb, being usually haemolytic (56, 71, 89, 91, 113, 114, 122). ETEC strains K88$^+$ belonging to serogroups O8 and specially O157 are also relatively frequent (56, 71, 113, 122). Nevertheless, the systematic administration of vaccines containing K88 antigen during the last decade has contributed to the selection of enterotoxigenic strains possessing K99 or P987 colonization antigens and the re-

sult was the increase of the incidence of ETEC strains with these colonization antigens mainly in piglets younger than a week, in Europe (114), in USA (122) and Japan (91). Thus, in addition to the classical porcine ETEC with K88 antigen the following serotypes of enterotoxigenic *E. coli* have also been isolated with high frequencies: O8:K99 (122); O9:K99, F41 or P987 (35); O20:K99, F41 or P987 (35, 122); O64:K99, F41 or P987 (35, 114); O101:K99, F41 (114, 122); O141:P987 (114, 122) and O149:K99 (91). Other particularly frequent strains detected were those named $4F^{-}$ strains (lacking of K88, P987, K99 and F41) belonging to serogroups O8 and O157 (91). Among porcine ETEC with K88 antigen, the majority synthesize LT enterotoxin in combination with STa and/or STb, whereas among strains with P987, K99 or F41 the majority of them synthesize only STa and some STb also (Table 6) (35, 89, 114, 122). Porcine ETEC O138:K81 synthesizing STa, such as enteropathogenic O139:K82 and O141:K85ab strains, may also cause the oedema disease of postweaning pigs. ETEC strains of these three later serotypes synthesize a variety of verotoxin (VT) named *oedema disease principle* (EDP) (46, 89). With respect to bovine ETEC strains, the majority of them synthesize only STa enterotoxin, they express K99 antigen and belong to serogroups O8, O9 and O101 (1, 66, 72, 77). Bovine strains of serogroups O9 and O101 express K99 and F41 conjointly, whereas O8 strains usually express K99 antigen only (77). We have carried out studies on ETEC incidence in piglets and calves with diarrhoea in Sapin, and we have isolated ETEC strains from approximately 30% of piglets with diarrhoea (47, unpublished results), and only from 1% of calves with diarrhoea (12, unpublished results). Among porcine ETEC strains isolated in Spain, those belonging to serotypes O149:K91:K88 producing LT and STa, and of serotype O141:K85ab:P987 producing STa were predominant (9, 47, 116). Nevertheless, Suárez *et al.* (117) isolated ETEC strains of serogroup O153 $K88^{+}$ or $P987^{+}$ from diarrhoeic piglets in Castilla-León. Concerning bovine ETEC strains we have isolated them only from two calves with diarrhoea and in both cases these strains were of serotypes O9:K(A)35 and O141; they expressed K99 and F41 conjointly and synthesized STa (12, unpublished results). On the other hand, we have isolated at a high frequency (20%) strains possessing Att25 from both diarrhoeic and healthy calves. We have isolated a total amount of 64 Att25 producing strains and none of them synthesized STa enterotoxin, only one synthesized LT and three synthesized CNF2. The most frequently determined serogroups among Att25 producing strains isolated in Spain were O8, O9, O1, O17, O23 and O153. Like Att25 producing strains isolated in other countries, practically all of Spanish isolates haemagglutinated bovine erythrocytes in the presence of D-mannose. Spanish Att25 producing strains are just now under research because in addition to Att25 antigen they synthesize other adhesin types with MRHA activity provisionally named B59a and B16c (unpublished results).

In summary, as it might be concluded along this review, though enterotoxigenic *E. coli* causing diarrhoea in humans and animals produce similar types of enterotoxins, they express differents classes of colonization factor antigens and they belong to separate serotypes.

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Increase of hemolysin expression in *Escherichia coli* by cloned DNA sequences of *Serratia marcescens*

Pilar Díaz and Antonio Juárez*

Departament de Microbiologia. Facultat de Biologia. Universitat de Barcelona. Avda. Diagonal 645, 08028-Barcelona. Spain.
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Summary

A genomic library of Sau 3AI-generated *Serratia marcescens* DNA fragments was constructed in *Escherichia coli* 5K harbouring plasmid pANN202-312. Two clones capable of increasing the external hemolytic activity of the parental strain were isolated and characterized. The recombinant plasmids purified from these clones (designated pPSF29 and pPSH18) contained approximately 30 and 20 Kb, respectively, of *Serratia* DNA. Introduction of either plasmid in *Escherichia coli* caused a marked increase in hemolysin production and a concomitant decrease in cell viability.

Key words: secretion, hemolysin, S. marcescens, E. coli.

Resumen

Se describe la construcción de una genoteca en *Escherichia coli* 5K portadora del plásmido pANN202-312, a partir de los fragmentos de DNA de *Serratia marcescens* generados por digestión parcial con *Sau* 3AI. A partir de esta genoteca se aislaron y caracterizaron dos clones capaces de incrementar la actividad hemolítica externa de la cepa original. Los plásmidos recombinantes purificados a partir de estos clones (designados pPSF29 y pPSH18) contenían aproximadamente 30 y 20 Kb, respectivamente, de DNA procedente de *Serratia marcescens*. Los fragmentos clonados ocasionaban un incremento significativo en la producción de hemolisina en *E. coli* y causaban además un descenso paralelo en la viabilidad de las células portadoras de cualquiera de ellos.

Introduction

Escherichia coli is the bacterial host most frequently used for studying foreign gene expression. One of the drawbacks of this microorganism concerns its limited ability for extracellular protein secretion (24). The only well documented example of a protein secreted by *E. coli* is α-hemolysin (12). In contrast, *Serratia marcescens* is an enteric bacterium which produces various extracellular proteins, including nucleases, proteases and lipases (5, 6, 16). While some of them are also secreted

(*) Corresponding author.

when cloned and expressed in *E. coli* (2, 7), others remain in the periplasmic space of this microorganism (21). Thus, *Serratia marcescens* seems to be endowed with a general extracellular secretory system, not present in *Escherichia coli*. Different workers have studied expression and secretion in *E. coli* of *S. marcescens* exoenzymes (2, 7) but the process of extracellular secretion is still poorly understood in *S. marcescens* as well as in other gram negative bacteria (22, 24).

As an alternative approach, we tried to analyze if any *S. marcescens* function could be cloned in *E. coli* and facilitate protein secretion by this microorganism. As a model, we used plasmid pANN202-312 which contains all four *E. coli* hemolysin genes, but lacks a sequence (*hlyR*) necessary in cis for the correct secretion of this protein (27). Cells harbouring this plasmid express hemolysin but do not release it efficiently to the external medium. It has been shown that in the absence of *hlyR*, premature termination of the transcription occurs between *hlyA* and *hlyB*. This fact would result in a poor expression of the hemolysin specific secretion genes, and cells lacking *hlyR* would not export properly hemolysin into the external medium (14). We report here the construction of a *S. marcescens* library in *E. coli* 5K harbouring plasmid pANN202-312 and the analysis of clones initially isolated because of their highly hemolytic phenotype.

Materials and Methods

Bacterial strains and vectors

Bacterial strains, cosmids and plasmids used are listed in Table 1. LB medium (19) or blood-agar (5% blood in LB-agar) were routinely used for the maintenance and growth of the strains and clones. When required ampicillin, chloramphenicol, tetracycline and kanamycin were prepared and used for supplementing the culture media at the concentrations described by Maniatis *et al.* (18).

DNA isolation and cloning techniques

Plasmid and chromosomal DNA isolation, transformation, restriction and ligation were performed as previously described (18). DNA restriction analysis was carried out on agarose gels as described by Maniatis *et al.* (18).

TABLE 1
BACTERIAL STRAINS AND VECTORS

Strain/vector	Relevant Genotype	Reference
E. coli 5K	F^-, r_k^-, m_k^-, *rpsL, thr, thi, leu, lac Z*	(13)
E. coli SMR10	(λ *cos2*, Δ*B*, *red3*, *gam* am210, cIts857, *Xis*1, *nin*5, Sam 7)/λ	(28)
E. coli HB101	F^-, *hsd S20* (r^-_B m^-_B), *supE 44*, *ara-14*, *galK-2*, *lacY1*, *proA2*, *rpsL20*, *xyl-5*, *mtl-1*, λ^-, *recA13*	(18)
S. marcescens	wild strain 2170 c	(10)
pANN202-312	*hlyC*$^+$, *hlyA*$^+$, *hlyB*$^+$, *hlyD*$^+$, Cm^r	(13)
pANN202-312R	*hlyR*$^+$, *hlyC*$^+$, *hlyA*$^+$, *hlyB*$^+$, *hlyD*$^+$, Cm^r	(17)
pBR322	Amp^r, Tet^r	(3)
pHC79	Amp^r, Tet^r, derivative of pBR322 carrying the *cos* sequence of phage λ	(11)

Bacterial strains and plasmids used in this work.

Cloning of chromosomal DNA fragments from *Serratia marcescens* into *Escherichia coli* 5K was carried out by ligation of the *Sau* 3AI-generated *S. marcescens* DNA fragments, upon partial digestion (fragments of about 30 Kb), to cosmid pHC79 and packaging using bacteriophage as a vector. Packaging extracts were prepared according to the method described in (25). New *E. coli* clones harbouring cosmid pHC79 were selected on the basis of their Cmr, Apr phenotype, as the *Serratia marcescens* DNA fragments had been cloned into the Bam HI site within the Tcr determinant of the vector. Screening for superhemolytic recombinant colonies was done by plating on blood-agar.

Hemolysin purification and antiserum production

Hemolysin was purified from 400 ml cultures of strain *E. coli* 5K (pANN202-312R), which contains the complete hemolysin determinant from plasmid pHly152 cloned in plasmid pACYC184 and overproduces hemolysin (27). Late exponential cultures of this strain were pelleted and the cell-free external hemolysin was precipitated with 10% TCA, separated on 8 % SDA-PAGE and transferred to nitrocellulose filters (26). The fragment of the filter containing the 110 KDa hemolysin band was cut longwise and melted in the presence of DMSO. After exhaustive dialysis to remove the DMSO, hemolysin was acetone-precipitated and suspended in water. NZ rabbits (male, 1-2 kg) were immunized with the purified hemolysin and periodically bled until sacrified. The titer of anti-hemolysin antiserum was determined by ELISA techniques, using purified hemolysin as antigen.

Cell fractionation and hemolysin assay

Cell fractionation was performed as described previously (23). Periplasmic and cytoplasmic protein content was determined on the basis of the Bradford procedure (4). Protein analysis was carried out on 8 % to 13 % polyacrylamide-SDS gels (15). The hemolytic activity of the culture supernatants was measured as previously described (20). Supernatant, periplasmic and cytoplasmic hemolysin content of the recombinant clones was tested by immunoblot assay and determined by scanning the developed filters on a Shimadzu CS 9000 densitometer. Inner and outer membrane proteins from the recombinant clones were isolated and separated according to the method described in (8), slightly modified.

Results and Discussion

Gene bank analysis and clone selection

As *E. coli* 5K(pANN202-312) shows very narrow hemolytic haloes on blood-agar plates, a genomic library containing chromosomal *Serratia marcescens* DNA cloned into *Escherichia coli* 5K(pANN202-312), was screened for ampicillin and chloramphenicol resistant (Ap^r, Cm^r) colonies producing large haloes of hemolysis on LB-blood-agar plates. Twelve colonies with such a phenotype were detected among 5000 clones screened. The average size of the hemolytic haloes of these clones was approximately 4.5 mm, the average diameter of the colonies being 1.3 mm. In contrast, similar size colonies of strains 5K(pANN202-312) showed haloes of about 3 mm diameter.

Two clones showing a large halo of hemolysis were selected, purified, and further characterized. Isolation of plasmid DNA from these clones and restriction analysis showed that both strains harboured cosmid pHC79 with an insert of DNA from *Serratia marcescens*. The recombinant plasmids, designated pPSF29 and pPSH18, contained different inserts of approximately 29 Kb and 18 Kb, respectively, as determined by restriction analysis (data not shown).

Hemolysin production of strain 5K(pANN202-312) harbouring plasmids pPSF29 and pPSH18

To quantify the overall amount of hemolysin produced by strain *E. coli* 5K(pANN202-312) harbouring either pPSF29 or pPSH18, we first measured the external hemolytic activity. Table 2 shows the hemolytic activity found in the external medium for all strains assayed at the early and late log phase. Clones harbouring either pPSF29 or pPSH18 showed small differences in external hemolysin production after 4 hours of growth, compared to strain 5K(pANN202-312). However, a maximum level in external hemolytic activity was detected for cells harbouring plasmid pPSH18 after 6 hours of growth, whereas no further increase could be detected for those harbouring plasmid pPSF29. The results shown in Table 2 indicate that there is an increase in external hemolytic activity for clones harbouring either pPSF29 or pPSH18, althoug much lower for the former.

To further characterize the hemolysin production of strains harbouring plasmids pPSF29 or pPSH18, external hemolytic activity was measured throughout the growth curve. The results obtained (Fig. 1) showed significant differences in external hemolysin production for cultures harbouring either recombinant plasmid with respect to 5K(pANN202-312). As shown in Figure 1, the biggest differences in external hemolytic activity could be detected after 2 and 6 hours of growth for clones pPSF29 and pPSH18, respectively. Nevertheless, for the strain harbouring plasmid pPSH18, the highest hemolytic activity could be correlated to a significant decrease in the growth kinetics, suggesting that the increase in the production of hemolysin altered cell viability.

In order to find out whether the differences in external hemolysin production for clones carrying a *S. marcescens* DNA fragment were due to a higher synthesis level or to a more efficient secretion of the synthesyzed toxin, the concentration of hemolysin at each cell compartment was de-

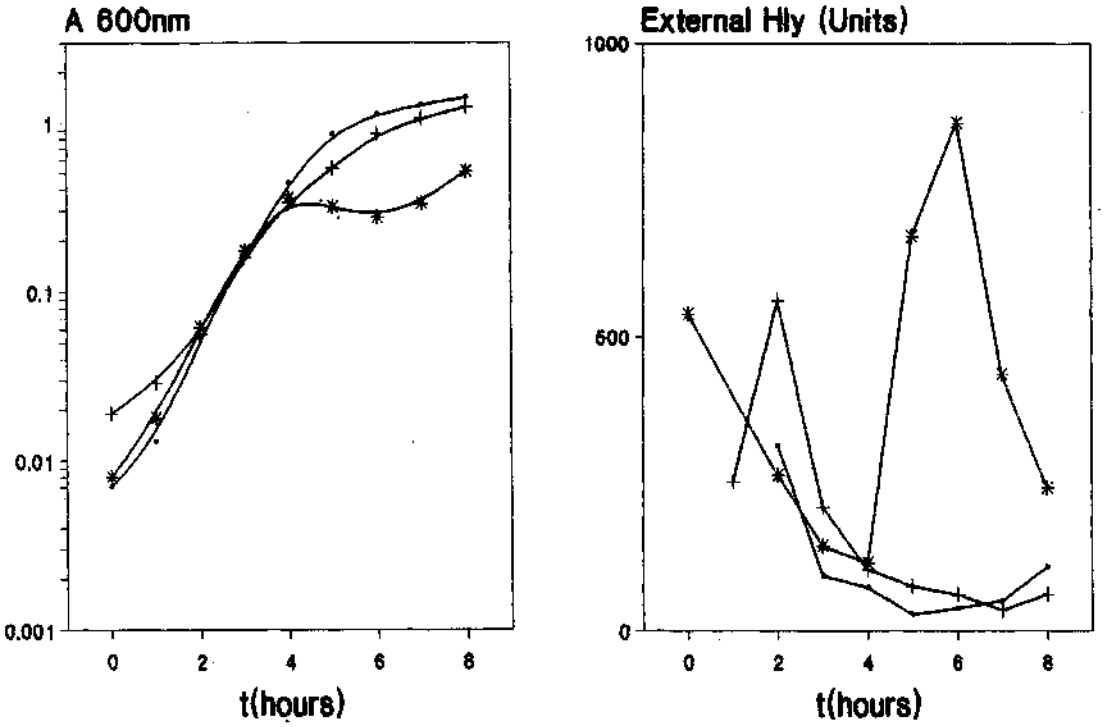

Fig. 1. Pannel A shows the growth kinetics, measured as the absorbance at 600 nm, of strain 5K (pANN202-312) (•) and strains 5K (pANN202-312) harbouring plasmids pPSF29 (+) and pPSH18 (★). Pannel B corresponds to the external hemolysin production along the growth curve for the three strains assayed.

termined. Upon cell fractionation, equal amounts of protein were analyzed on 8% SDS-polyacrylamide gels, and their content in hemolysin quantitated by the desintometer tracing of nitrocellulose filters after western blot assay. The results obtained (Table 3) showed that the amount of external hemolysin was higher for the clones harbouring plasmids pPSF29 or pPSH18 than that found for strain 5K(pANN 202-312). These results correlate well with those obtained when analyzing the hemolytic activity in the external medium (see Table 2). In addition, we observed that the total amount of internal hemolysin (Table 3) was higher in clones harbouring either pPSF29 or pPSH18 than in strain 5K(pANN202-312). Cells harbouring plasmid pPSH18 showed a higher overall production, as well as a higher external hemolysin production, with respect to those harbouring plasmid pPSF29, where hemolysin seemed to accumulate internally rather than being released into the external medium. These results are in good agreement with the fact that both plasmids contained different inserts of foreign DNA, and may therefore cause different alterations in the hemolysin expression mechanisms.

Analysis of membrane and periplasmic proteins from strains harbouring plasmids pPSF29 and pPSH18

To determine whether the presence of the cloned *S. marcescens* DNA could account for changes in the cell envelopes, outer an inner membrane proteins were isolated and analyzed by SDS-polyacrylamide gel electrophoresis. Comparison of the electrophoretic protein patterns showed no differences in the composition of outer membrane proteins for strains harbouring plasmids pPSF29 or pPSH18 and strain 5K(pANN202-312). Significative differences in protein composition were neither observed among them when periplasmic or cytoplasmic proteins were analyzed (data not shown).

'Instability of the S. marcescens *sequences which modify hemolysin expression in* E. coli

Upon repeated re-streaking of colonies harbouring plasmids pPSF29 or pPSH18, they began to segregate colonies producing small (3.0 mm) hemolysis haloes. The segregation continued when colonies producing large (4.5 mm) hemolysis haloes were picked up and further purified. Finally, 100% of the clones had lost the highly hemolytic phenotype.

To avoid the fact that undesired recombinational events could account for this phenomenon, plasmids pPSF29 and pPSH18 were transformed into *E. coli* strain HB101 harbouring plasmid pANN202-312. However, segregation of colonies forming small haloes was also observed in strain HB101. In this case, the two phenotypes found after each culture were similar to those of 5-K(pANN202-312) clones harbouring either pPSF29 or pPSH18, or those of the cosmid free 5-K(pANN202-312) strain. All highly hemolytic colonies had a 29 or 18 Kb insert in cosmid pHC79, whereas all poorly hemolytic colonies contained pHC79 DNA without any detectable insert, as determined from restriction analysis. This phenomenon was also apparent when subcloning was intended.

For subcloning, plasmid pPSF29 was digested with different restriction enzymes (*Eco* RI, *Sal* I, and *Pst* I), cloned into plasmid pBR322 and expressed in strain 5K(pANN202-312). As a result, four highly hemolytic clones were obtained from this plasmid digested with *Sal* I. No subclones could be obtained from plasmid pPSH18. Characterization of the subclones showed that they contained a fragment of DNA from *Serratia marcescens* of about 3 Kb. Cells harbouring these subclones showed a similar pattern of hemolysin expression than cells harbouring plasmid pPSF29. As mentioned above, upon suculturing such subclones, they segregated colonies producing small hemolysis haloes which contained plasmid DNA lacking the 3 Kb insert.

TABLE 2
EXTERNAL HEMOLYSIN ACTIVITY (ARBITRARY UNITS)

Strain	$A_{600} = 0,3$	$A_{600} = 0,9$
5K (pANN202-312)	40	56
5K (pANN202-312, pPSF29)	50	46
5K (pANN202-312, pPSH18)	54	236

External hemolysin production by strains 5K (pANN202-312), 5K (pANN202-312, pPSF29) and 5K (pANN202-312, pPSH18), determined at two different stages of the growth curve. Units of external hemolysin were calculated as described in Materials and Methods, comparing the capacity of the supernatants to release hemoglobin from fresh erythrocytes. The values expressed correspond to the average of 5-7 measures performed.

TABLE 3
RELATIVE HEMOLYSIN PRODUCTION

Strain	External	Internal
5K (pANN202-312)	1.00	1.00
5K (pANN202-312, pPSF29)	4.62	3.05
5K (pANN202-312, pPSH18)	16.00	2.60

Hemolysin production by strains 5K (pANN202-312) harbouring plasmids pPSF29 and pPSH18, expressed as arbitrary units of absorbance. Quantitation of the amount of hemolysin contained in each cell compartment was determined by the densitometer tracing of immunoblot filters. The amount of hemolysin found at each cell compartment of cosmid free strain 5K (pANN202-312) was taken as the reference level.

Effect of the cloned sequences on the viability of E. coli *cells*

We could detect a significant effect on the viability of the *E. coli* clones and subclones carrying a fragment of *S. marcescens* DNA. When subculturing on blood-agar plates clones harbouring plasmids pPSF29 or pPSH18 as well as the subclones containing the 3.0 Kb *Sal* I fragment from cosmid pPSF29 cloned into pBR322, the highly hemolytic colonies showed an important decrease in their viability. When these colonies were picked up and re-streaked, they produced either poorly hemolytic colonies or highly hemolytic microcolonies which did not continue to grow. This result is in good agreement with the effect observed in Figure 1(A) for cells harbouring plasmid pPSH18, showing an important decrease in viability upon reaching the highest production of external hemolysin. In some cases, viability could only be ensured by subculturing on plates lacking chloramphenicol, allowing then plasmid-free clones to grow. These results suggest that the increase in hemolysin expression caused by the cloned DNA sequences alters cell viability.

The results obtained indicate that whereas hemolysin expression can be significatively increased in *E. coli* by *S. marcescens* DNA sequences, such an increase causes a progressive loss in cell viability. When selecting for the presence of both the hemolytic plasmid and the cosmid, many survivors showed to harbour deletions in the cosmid DNA, affecting the inserts initially characterized.

Although many genes of strains belonging to the Enterobacteria can be cloned and stably maintained in *E. coli* (1, 3, 7, 9, 28), some functions, which can alter or modify the expression of certain proteins such as hemolysin, even if they belong to a related species such as *Serratia marcescens*, can not be stabilized for more than a few generations in *E. coli*, even in a recA background.

From the results obtained, it seems feasible to find some *Serratia marcescens* functions able to modulate gene expression in *Escherichia coli*. However, increased expression or secretion of cloned foreign proteins may require alternative strategies to cope with viability problems.

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Distribution of oxidizing bacterial activities and characterization of bioleaching-related microorganisms in a uranium mineral heap

María Isabel de Silóniz, Paloma Lorenzo and Julián Perera*

Departamento de Bioquímica y Biología Molecular, I. Facultades de Ciencias Biológicas y Químicas. Universidad Complutense de Madrid. 28040 Madrid. Spain.

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Summary

The occurrence and activity of bioleaching-related microorganisms are highest at 0.25 m under the surface of a uranium mineral heap, and decrease at points deeper than 1 m inside the heap.

Thiobacillus ferrooxidans, Th. acidophilus, Th. thiooxidans and *Leptospirillum ferrooxidans* have been found and isolated from different sites inside the heap. Other mesophilic iron –or sulphur– oxidizing bacteria and some moderate thermophile have also been isolated from deep (1 m and 4 m) sites and characterized. Several fungi and some yeasts are present in this bioleaching habitat.

Key words: bioleaching, oxidizing bacterial activities, acidophiles, Thiobacilli.

Resumen

La presencia y actividad de microorganismos relacionados con la biolixiviación son máximas a 0,25 m bajo la superficie de una era de mineral de uranio, y disminuyen en puntos situados a profundidades superiores a 1 m en el interior del montón.

Thiobacillus ferrooxidans, Th. acidophilus, Th. thiooxidans y *Leptospirillum ferrooxidans* han sido encontrados y aislados a partir de diferentes puntos del interior del montón. Se han aislado y caracterizado igualmente otras bacterias mesófilas oxidantes de hierro o de azufre y algún termófilo moderado, a partir de sitios profundos (1 m y 4 m). Algunos hongos y levaduras se encuentran también presentes en este hábitat de biolixiviación.

(*) Corresponding author.

Introduction

Bioleaching involves the attack of a mineral ore based on the direct or indirect action of different microorganisms (4). In this process, a big number of metals, in form of their corresponding insoluble minerals, are oxidized and transformed to soluble forms as a result of the bacterial activity (11). Bioleaching could be a good alternative to the classic hydrometallurgical processes (6, 7). However, the fundamentals of the process and the ways to get better or faster yields has begun to be known only recently (5). At the moment, the use of bioleaching is restricted to the processing of very poor ores or marginal minerals like those of copper, uranium or gold (6, 18). The biological basis of the leaching process is just partially known. The microorganism populations occurring in the bioleaching habitats are only partially characterized and the implications of the different species in the whole process is known just for some of them (3, 8, 9, 12, 16). The iron –and sulphur– oxidizing activities are mostly carried out by species of the *Thiobacillus* genus (*ferrooxidans* and *thiooxidans*) and *Leptospirillum ferrooxidans* (16). These activities are thought to be in the centre of the metal-solubilizing reactions and the estimation of their levels is most important to foresee the leaching results (16). On the other side, the occurrence of several other microbial species in bioleaching habitats has been also reported (12, 15, 17), although their participation in the whole population maintenance is very poorly understood and needs a great deal of further studies.

In this paper, some new data on the distribution of oxidizing bacterial activities along a uranium mineral heap are reported. Furthermore, several bacterial isolates from different depths into the heap, including some probably new strains, have been identified and partially characterized.

Materials and methods

Site description and mining and leaching procedures

This study has been developed with uranium mineral from the Mina Fe, at Saelices el Chico, Ciudad Rodrigo (Salamanca, Spain), which is being worked by the Empresa Nacional del Uranio, S. A. (ENUSA) according to an extractive method based on a static leaching procedure.

The primary mineral is pitchblende, together with pyrite or carbonates. The open air working is made by explosion. The mineral is crushed to sizes smaller than 100 mm, and used to build leaching heaps where it is washed with an acidic solution.

The washing liquids, with the solubilized uranium, go to an organic solvent extraction step prior to a saline solution re-extraction. The final precipitation with air-dissolved ammonia leads to ammonic diuranate which, after drying yields a uranium concentrate with a 85 % of U_3O_8.

Sample collection and treatment

Samples were collected from an active, average heap («Marginal 11, Módulo 6»), considered as rather representative of the leaching process at Ciudad Rodrigo mine.

Solid samples were aseptically taken from different depths inside the heap (0.25, 1 and 4 m) and put into 100 ml sterile bottles containing 50 ml of MS solution (0.2 g/l $(NH_4)_2SO_4$, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l KCl, 0.1 g/l $K_2HPO_4 \cdot 3H_2O$) pH 2.0 (samples 2, 3 and 4, respectively).

Once into the bottles, samples were kept for 8 hours with occasional shaking to help microorganisms in solid samples going into solution.

To know the level of contamination owing to sample manipulation, a blank sample was also co-

llected by opening another sterile MS solution containing bottle, at different depths inside the heap (sample 0).

Liquid samples were collected from irrigation water (sample 1) and effluent solution (sample 5) into sterile bottles and in aseptic conditions.

Sample measurements

When taking solid samples, temperature at each level inside the heap was recorded and pH was estimated by suspending a fixed amount of mineral in destilated water (1:1) and determining the pH of the resulting suspension.

The amounts of solid samples were calculated from the weights of the bottles before and after their collection.

Simultaneously to the liquid sample collection, temperature and pH of both washing and effluent liquids were recorded. Oxygen content in them was also measured.

Quantification of microbial population

1. Iron-oxidizing bacteria

To count iron-oxidizing microorganisms, the most probable number (MPN) method (18) was followed. To ease the handling of such a number of samples, the procedure was carried out in 24-well multi-dishes (Cell Wells, Corning). For every sample, ten 10-fold dilutions were made on MS medium, pH 2.0. 150 µl of each were used to inoculate triplicate, 1.5 ml of ferrous sulfate solution (13.9 g/l $FeSO_4 \cdot 7H_2O$ in MS solution, pH 2.0) in three labelled wells.

After inoculation, the multi-dishes were sealed with parafilm paper to avoid evaporation. Incubation was performed at 30° C for 30 days. Growth of iron-oxidizing bacteria was indicated by the formation of ferric iron with its characteristic color.

To enumerate moderately thermophylic and thermophylic microorganisms the same process was repeated twice, and incubations were carried out at 45° C and 65° C.

In every case, a negative (without inoculation) control was used. For the assays at 30° C, 150 µl of a pure *Thiobacillus ferrooxidans* ATCC 23270 culture were used as positive control.

2. Sulphur-oxidizing bacteria

The same procedure as above was followed to enumerate sulphur-oxidizing microorganisms in collected samples, except that inoculations were made on medium with sulphur (5 g/l sulphur in MS solution, pH 3.0). Bacterial growth was followed by the decrease of the insoluble sulphur and the increase of turbidity.

3. Yeasts and fungi

The quantification of yeasts and fungi was made on the basis of the number of colonies grown on malt-agar medium. 150 µl of 10-times concentrated samples, 150 µl of the samples and 150 µl of 10-fold dilutions made from the samples, were plated on malt-agar medium and incubated 72 hours at 30° C.

Isolation and identification of microbial strains

Microorganisms isolation was usually made from enriched liquid cultures on media specific for those microbial groups considered more interesting.

Medium with ferrous iron or with sulphur (in MS solution, see above) were used for the enrichment of iron or sulphur oxidizing, mesophilic liquid cultures, respectively.

Cultures of moderate thermophilic bacteria were enriched at 45° C in liquid media with ferrous and yeast extract (13.9 g/l $FeSO_4 \cdot 7H_2O$, 0.2 g/l yeast extract, in MS solution), or with pyrite (10 g/l pyrite in MS solution). Media for the enrichment of thermophilic cultures were the same as above, but incubation temperature was raised to 65° C.

Pure strains were prepared from isolated colonies grown after several passes on solid medium.

Iron- or sulphur-oxidizing, mesophilic microorganisms were isolated on plates of agarose in medium with ferrous (5 g/l agarose, 13.9 g/l $FeSO_4 \cdot 7H_2O$ in MS solution) or thiosulfate (5 g/l agarose, 5 g/l $Na_2S_2O_3$ in MS solution), respectively. Enriched liquid cultures on ferrous or sulphur medium were used.

Moderate thermophiles were isolated from enriched cultures in ferrous/yeast extract medium by using agarose/ferrous/yeast extract plates at 45° C.

Isolation of yeast and fungi was performed from the same solid medium used for their quantification.

On the basis of their morphology, isolated microorganisms were firstly classified as yeast, fungi and bacteria. From this moment on specific classification rules for each group were followed (1, 2, 13, 21).

Results and discussion

Samples data

The source and some physical data of the different samples are shown in Table 1. Temperature

TABLE 1
MINERAL SAMPLES DATA AND QUANTIFICATION OF OCCURRING MICROORGANISMS

Sample source	Temp °C	pH	$[O_2]$ ppm	Fe-oxidizing			S-oxidizing			Fungi	Yeast
				germs	grow	at	germs	grown	at		
				30° C	45° C	70° C	30° C	45° C	70° C		
Watering	26.8	2.4	9.5	60 cel/ml	—	—	$>10^5$ cel/ml	—	—	—	—
0.25 m deep	22.1	2.8		2×10^5 cel/g	10^4 cel/g	—	4×10^5 cel/g	10^2 cel/g	—	700 cel/g	—
1 m deep	24,1	2.65		10^3 cel/g	—	—	2×10^2 cel/g	—	—	10 cel/g	—
4 m deep	26.6	2.65		10^3 cel/g	15 cel/g	—	10^2 cel/g	—	—	—	—
Effluent	22.0	2.6	8.9	60×10^2 cel/ml	—	—	6×10^2 cel/ml	—	—	7 cel/ml	7 cel/ml

in solid samples increases with the depth inside the heap, which could be related with a higher both chemical and biological activity in these levels.

Microbial oxidant activity

The results of some quantification assays using multi-dishes are shown in Figure 1. Positive and negative cultures are clearly distinguished. The multidish method developed and reported here has proved to be very useful for this kind of work, allowing to handle more than one thousand assays simultaneously.

The concentrations (number of viable cells or colony forming units, CFU, per g of wet weight) of iron– and sulphur-oxidizing bacteria, yeasts and fungi in the different solid samples collected from the heap are shown in Table 1. The microbial occurrence and activity are highest at 25 cm under the heap surface and decrease with the depth. This situation is in accordance with previous results (18) and can be correlated with the oxigen availability and the concentration of toxic products (metal ions, metabolic degradation products, etc.) at these levels. This distribution stands not only for mesophilic but also for moderately thermophilic organisms. However, no extreme thermophylic (65° C) activity was detected at any depth in this study.

The occurrence and levels of oxidant bacteria, yeasts and fungi in liquid samples are also depicted in Table 1. The difference of iron-oxidizing activity in both liquids can be explained on the basis of a bacterial population established inside the heap, whose washing out leads to the detection of activity in the effluent.

Microbial strains isolation

After several passes through the medium of choice, isolated colonies could be obtained from different enriched cultures.

These colonies were identified and characterized on the basis of their colony and microscopic morphology and also of different physiological and biochemical tests. The results of these studies are outlined in Table 2.

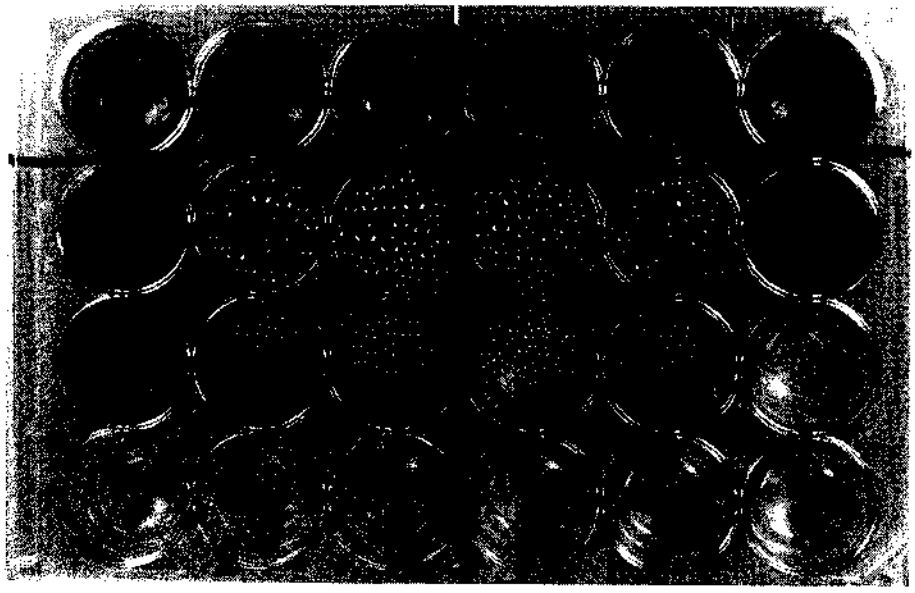

Fig. 1. Results of some quantification assays of oxidant microbial activities using multi-dishes (see text).

TABLE 2
CHARACTERISTIC OF ISOLATED MICROORGANISMS

Strain	*Thiobacillus ferrooxidans* 1.18	*Leptospirillum ferrooxidans* A3	T3.2	YN3G	YN4	TM4
Morphology.	Ends-rounded, small, single or paired bacilli. 0.84 × 2.4 μm.	Pleomorphics, vibrioid or spiral form; different sizes.	Long, occasionally Y-shaped bacilli. 1.2 × 0.5 μm.	Long, normally paired bacilli. 0.8 × 3.6 μm.	Normally paired bacilli. 1.2 × 2.8 μm.	Bacilli.
Reproduction.	Binary division with the help of septum.	Binary division.	Binary, occasionally multiple division. Equal or unequal, with the help of septum.	Binary division.	Equal or unequal, binary division.	Binary division.
Colonies in solid medium (agarose).	In Fe (II) medium. Spherical, orange, coated with ferric hydroxid. < 1 mm.	In Fe (II) medium. Spherical, orange, centered ferric deposit. > 1 mm.	In thiosulphate medium. Lobulate edges, rounded. White milky colour, stronger in the centre. > 1 mm.	In Fe (II) medium. Rounded, white, centered orange ferric deposit. > 1 mm.	In Fe (II) medium. Rounded, white, centered orange ferric deposit. > 1 mm.	In Fe (II) medium. Rounded, white, centered orange ferric deposit. > 1mm.
Growth in liquid medium.	In Fe (II), medium becomes amber due to Fe oxidation. In pyrite, medium turns turbid and yellow-green.	In Fe (II), medium becomes amber due to Fe oxidation. In pyrite, medium turns turbid and yellow-green.	In sulphur, medium becomes very turbid. Sulphur changes appearance and turns dusty.	In Fe (II), medium becomes amber due to Fe oxidation.	In Fe (II), medium becomes amber due to Fe oxidation.	In Fe (II), medium becomes amber due to Fe oxidation.
Type of nutrition.	Autotroph.	Autotroph.	Facultative autotroph.	Chemolithoheterotroph.	Chemolithoheterotroph.	Chemolithoheterotroph.
Relation to oxigen.	Aerobe.	Aerobe.	Aerobe.	Aerobe.	Aerobe.	Aerobe.
Source of energy.	Fe (II), S, thiosulfate, tetrathionate, pyrite.	Fe (II), pyrite.	Fe (II) plus yeast extract, S, thiosulfate, pyrite.	Fe (II) plus yeast extract.	Fe (II) plus yeast extract.	Fe (II) plus yeast extract.
Source of C.	CO_2	CO_2	CO_2	—	—	—
Growth temp.	30-35° C	30-35 ° C	30-35° C	35-45° C	35-45° C	40-45° C
Growth pH.	2-2.5	2-2.5	3-4	2-2.5	2-2.5	2-2.5
G+C content of DNA.	58 % molar.	37.5-48% molar.	58% molar.	45% molar.	48% molar.	Non determined.
Source for isolation.	Watering.	Watering.	Uranium mineral, 1 m deep.	Uranium mineral, 1 m deep.	Uranium mineral, 4 m deep.	Uranium mineral, 4 m deep.

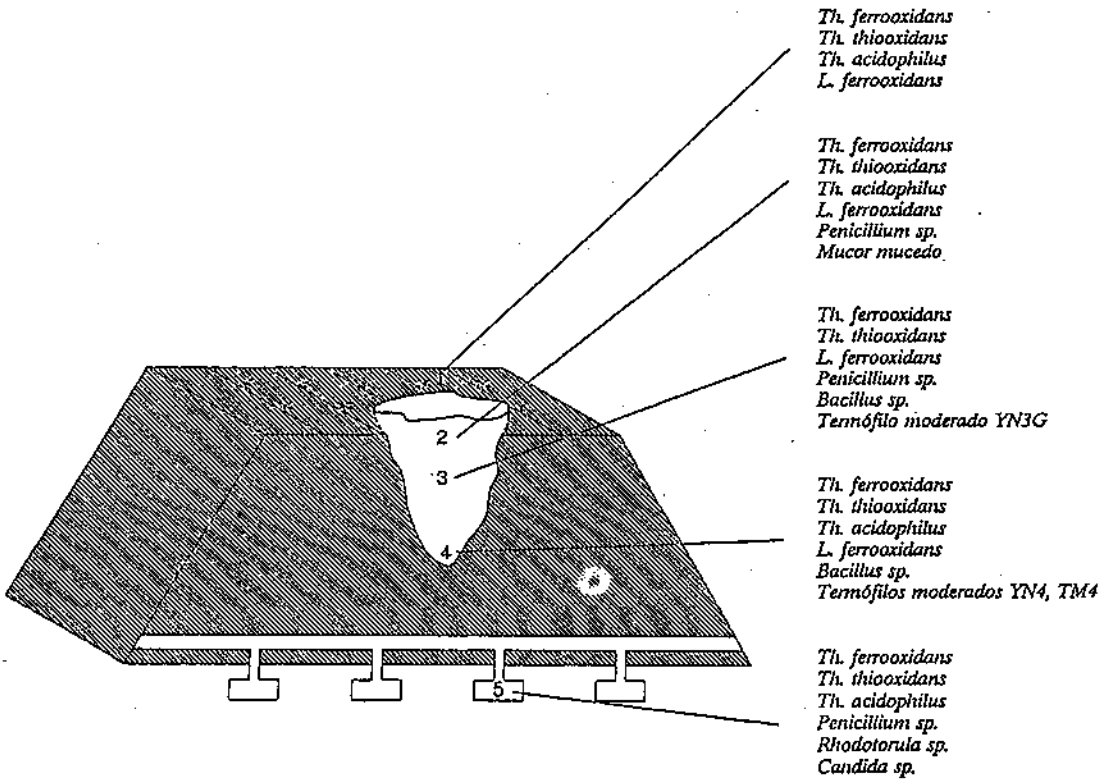

Fig. 2. Microorganisms detected and isolated from the different sites studied in the mineral heap. Site 1: watering liquid; site 2: solid sample at 0.25 m deep; site 3: solid sample at 1 m deep; site 4: solid sample at 4 m deep; site 5: effluent liquid (see text).

Among the colonies isolated on ferrous medium, *Thiobacillus ferrooxidans* was detected in every sample, very often together with *Th. acidophilus*. From deeper solid samples *Leptospirillum ferrooxidans* was also isolated. Respect to the sulphur-oxidizing organisms, *Thiobacillus thiooxidans* could be isolated from three samples, and other strains (T3.2 and T4.2) from the other two, these showing some significant differences with *Th. thiooxidans*.

On ferrous/yeast extract plates *Th. acidophilus* was very easily isolated. Some other strains were also obtained: YN3G and YN4. In searching specifically for moderate thermophilic organisms, only from the deepest solid sample colonies could be isolated, corresponding to a new moderate thermophilic strain (TM4).

The morphologically different colonies grown on malt-agar plates were isolated and identified. Among fungal species, some of the genera *Penicillium* and *Mucor* were detected. Some yeasts of the genera *Rhodotorula* and *Candida* were also identified in the effluent liquid.

In Figure 2, a scheme showing the distribution of different microorganisms isolated from the mineral heap is depicted.

Further characterization studies on these bacterial isolates are in progress.

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Proteins specified by swine transmissible gastroenteritis virus: identification of non-structural proteins by two-dimensional electrophoresis

María D. Laviada, Maribel de Diego and José M. Escribano*

Instituto Nacional de Investigaciones Agrarias, Departamento de Sanidad Animal. Embajadores, 68. 28012 Madrid, Spain.
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Summary

Four virus-induced non-structural proteins with apparent molecular weights of 11-14 kilodaltons (kDa) were identified by two-dimensional electrophoresis in cells infected by TGEV. Differences in the number of non-structural proteins were observed among virulent and attenuated TGEV strains as well as with two antigenically related feline and canine coronaviruses.

Key words: TGEV, non-structural proteins, two-dimensional electrophoresis.

Resumen

Se han identificado mediante electroforesis bidimensional cuatro proteínas no estructurales inducidas por el VGPT en células infectadas, con pesos moleculares comprendidos entre 11 y 14 kilodaltons. Diferencias en el número de estas proteínas no estructurales fueron observadas entre cepas virulentas y atenuadas del VGPT, así como entre los coronavirus felino y canino antigénicamente muy relacionados.

Introduction

Transmissible gastroenteritis (TGE) virus is a coronavirus that causes a highly contagious enteric disease in swine, especially severe in newborn pigs (14).

The TGEV genome is composed of a single stranded RNA with positive polarity of about 30 Kb and with a MW of 6.8×10^6 (3). At least 8 intracellular mRNAs are synthesized during a TGEV productive infection (12, 17), forming a 3'-coterminal nested structure. In addition to the previously identified genes encoding the three structural proteins, S, M, N (8) and mRNA 1, whose translation products are one or two polymerases, four regions, A, B, C and D, have been identified (17). These correspond to the non-overlapping portions of the four remaining mRNA species and may code for, so far unidentified, non-structural polypeptides.

(*) Corresponding author.

Antisera raised against synthetic oligopeptides derived from open reading frame (ORF) D reacted with a polypeptide of 14K MW in TGEV-infected cells (7). The other mRNA species 3, 4 and 5 had no product assigned to them from either infected cells or virions (4, 17). By comparison of intracellular RNAs of an avirulent and the parent virulent strain of TGEV, differences in RNAs 3 and 4 were found, suggesting that those genes may contribute to TGEV pathogenesis (18).

Six virus-specific RNA species have been detected in cells infected with feline infectious peritonitis virus (FIPV). A comparison between TGEV and FIPV genome maps showed a lower number of subgenomic mRNA species in the feline virus (corresponding to genome regions between S and M genes), suggesting that transcription units have been lost or gained during coronavirus divergence (5). No information about the size and number of RNA species in cells infected by canine coronavirus (CCV) is available at present.

Apparently, mRNA 8 is the unique non-structural region translationally active in TGEV-infected cells, producing a polypeptide of 14-17K that induces antibodies in infected pigs (7, 15). Since not all the products of TGEV-specific RNA species have been detected in infected cells, we have studied virus induced minor polypeptides in a productive infection. Protein induction by TGEV in infected cells was compared to that of highly antigenically related coronaviruses CCV and FIPV (13), analyzing similarities in the number and MW of the polypeptides characterized.

Materials and methods

Cells, viruses and sera

Four TGE viruses were grown in swine testicular (ST) cells. The TGEV attenuated strains PUR-54, BRE-79 and SHI-56 were kindly provided by Dr. Enjuanes (Centro de Biología Molecular, Madrid) and, the highly virulent strain MAD-88 corresponds to a virus isolation in Spain previously described (10).

The feline infectious peritonitis virus (FIPV) strain 1146 and canine coronavirus (CCV) strain 353716 were kindly provided by Dr. Carmichael (Baker Institute, Ithaca, N.Y.) and grown in feline lung cells and canine tumor (A-72) cells respectively.

A sow twice-infected by 5×10^8 tissue culture infectious doses 50 of MAD-88 strain each, was bled at 7 days after farrowing and the serum, presenting an ELISA titer higher than 1:10,000 was used in radioimmunoprecipitation.

Isotopic labeling of infected cells, polyacrylamide gel electrophoresis and radioimmunoprecipitation

The ST, A-72 and feline lung cells inoculated at a multiplicity of infection of 10 were pulse labeled for 2 h with 500 μCi/ml of ^{35}S-methionine (800 Ci/mmol; Amersham) in Eagle minimal essential medim lacking methionine.

All procedures for one-dimensional electrophoresis were as previously described (6) in acrylamide-N, N'diallyltartardiamide (DATD) gels. The labeled infected-cell extracts (10^5 cpm) were immunoprecipitated as described (1, 11) using *Staphylococcus aureus* for the adsorption of the antigen-antibody complexes.

Two-dimensional gel electrophoresis

The buffers, solutions and procedures employed for two-dimensional electrophoresis were essentially the same as those described by Bravo (2) with minor modifications.

Isoelectric focusing (IEF) gels (0.2 × 15 cm) were prepared using a combination of ampholines (LKB) to give a pH range between 7 and 4.5 in 4% acrylamide. The gels were prerun at 200, 300 and 400 V for 15, 30 and 60 min respectively. The gels were loaded with about 5×10^5 cpm of trichloroacetic acid-precipitable material and then the gels were run for 20 h at 400 V. Focused gels were gently shaken in 5 ml of equilibration buffer for 10 min and stored at –70° C until used in second dimension electrophoresis.

Nonequilibrium pH gradient electrophoresis (NEPHGE) gels (0.2 × 15 cm) were prepared using a combination of ampholines to give a pH range between 7 and 9.5 in the same concentration of acrylamide than IEF gels. The same precipitable counts used in the acid gels were applied and the gels were run at 400 V for 4.5 h without prerun. After the run, the gels were equilibrated as before and kept at –70° C until be used.

All the second dimension separations were performed on 17% acrylamide-DATD gels. After second dimension run at 15 mA overnight, the gels were dried and exposed to X-ray films.

Results

Proteins specified by TGE virus

^{35}S-methionine pulse labeling swine testicular (ST) cells infected by the Purdue strain of TGEV followed by SDS-PAGE analysis was used to resolve and to identify the virus-induced polypeptides. The labeling infection was carried out at different hours post infection (0-16 hpi) and at a multiplicity of infection of 10. The major structural proteins, S, N and M with relative MW of 220K, 48K and 29K respectively, were detected at 4 hpi (Fig. 1). Two additional polypeptides with relative MW of about 11K and 14K were also detected at 6 and 4 hpi respectively and were designated NS1 and NS2 (Fig. 1).

Infected cell extracts obtained at 8 hpi were immunoprecipitated using a serum from a twice-infected sow. The antibodies were able to immunoprecipitate the three structural proteins S, N and M and the non-structural protein NS1 but not NS2 (Fig. 1).

Since several of the subgenomic mRNA species must encode putative similar MW polypeptides, we used two-dimensional gel electrophoresis to separate proteins with similar MW. After this analysis, both NS1 and NS2 were differentiated clearly into two spots each, with different isoelectric points (Fig. 2). The NS1 band characterized in one-dimensional PAGE showed two polypeptides with isoelectric points at pH 7.8 and 7.2. NS2 also comprised two polypeptides with isoelectric points at pH 7.1 and 6.6 (Fig. 2). When we used a gel with a pH gradient between 6 and 8, NS1 and NS2 were also resolved in the same gel as two spots in both cases (data not shown) indicating that both NS2 polypeptides were not the same protein agregated in one of the NEPHGE or IEF gels. Protein N is highly basic with a net charge of +23 at neutral pH (9) and was not observable using the pH gradient employed. Only when the NEPHGE gels were run less than 30 min, the N protein was visualized (data not shown).

In order to compare protein induction in infected cells at the same multiplicity of infection by other TGEV strains, we analyzed infected cells by the attenuated strains BRE-79 and SHI-56 and by the virulent strain MAD-88 at 8 hpi. Two differences were observable between strains. First, strains PUR-54 and SHI-56 switch off host cells protein synthesis more effectively than the others. Second, none of the NS2 forms were detected in the only virulent strain, MAD-88 (Fig. 3). In addition, M protein in the strain SHI-56 showed two glycosylation forms.

Pulse-chase experiments using different intervals between labeling and the protein analysis (2 and 4 hours), did not eliminate any of the protein spots in NS1 and NS2 (data not shown). Also, Tunycamicin (0.5 μg/ml), that inhibited glycosylation of S and M proteins, did not affect any of the NS1 and NS2 forms (data not shown).

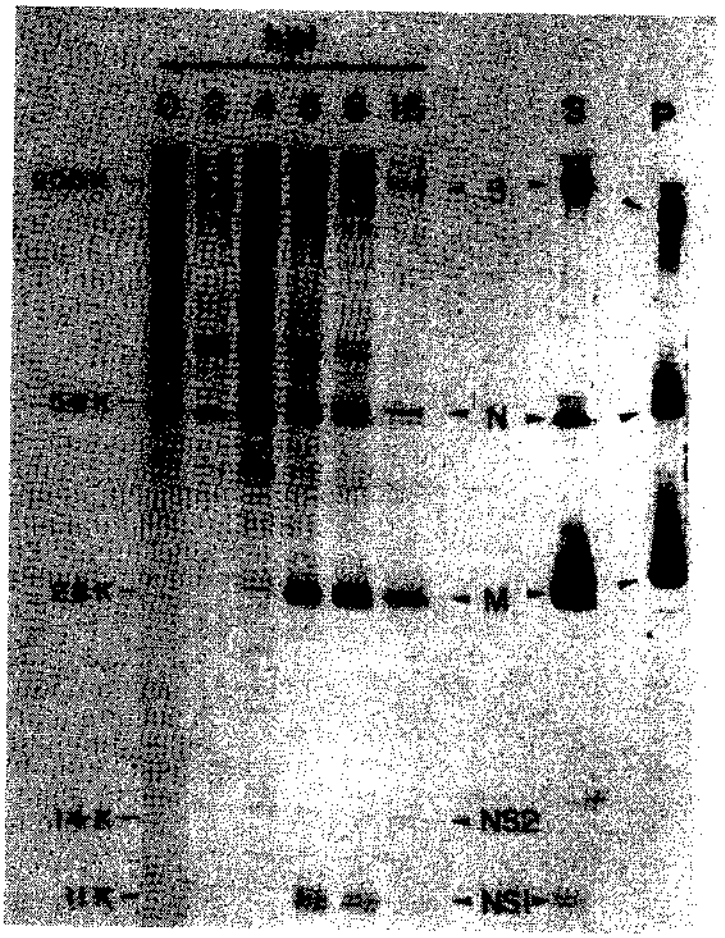

Fig. 1. Kinetic of synthesis of TGEV-induced proteins in infected ST cells labeled at different hours post infection (0-16 hpi). NS1 and NS2 correspond to TGEV-induced proteins with MW 11 K and 14 K respectively, not present in purified virus (P). NS1 was immunoprecipitated from a cell extract (8 hpi) by serum (S) from an experimentally infected sow.

Proteins specified by canine and feline coronaviruses

By analysis of protein induction in cells infected by FIPV and CCV, we showed three differences with cells infected by TGEV. First, N proteins had different apparent MW. TGEV N protein had higher MW (about 2 kDa) than the ones induced by FIPV and CCV (data not shown).

Second, M protein when glycosylated showed similar MW in the three viruses, however, when they were synthesized in the presence of tunycamicin the transmembrane protein of TGEV had a higher MW (about 2 kDa) than those induced by FIPV and CCV (data not shown). On the other hand, the number of different glycosylated forms of M protein found in infected cells by the three viruses were different. FIPV-infected cells showed forms varying from the unglycosylated (the most abundant form) to at least four other partially glycosylated forms that differ in the MW and isoelectric points (Fig. 4). CCV-infected cells showed only two glycosylated forms of M protein that differ essentially in the isoelectric point (Fig. 4). With the exception of the SHI-56 strain, in TGEV infected cells only the completely glycosylated form of M protein was detected.

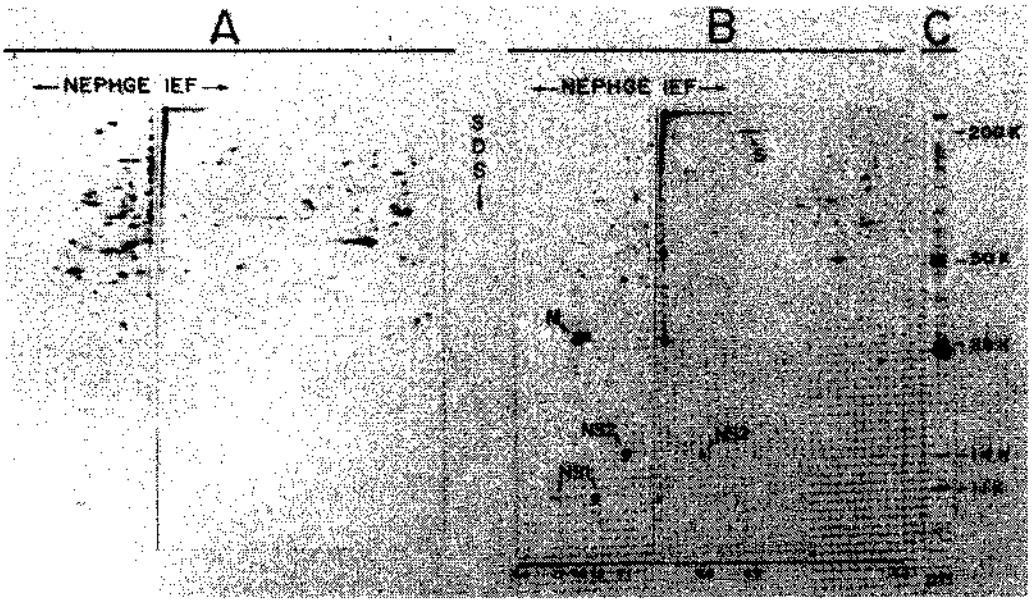

Fig. 2. Two-dimensional analysis of uninfected (A) and TGEV-infected (B) ST cells labeled with 500 μCi/ml of ^{35}S-methionine for 2 hours (8-10 hpi in B). Proteins marked as NS1 and NS2 in B correspond to the characterized as only one protein each by one-dimensional electrophoresis (C). N protein was not observed using the pH gradient shown in the foot of pannel B.

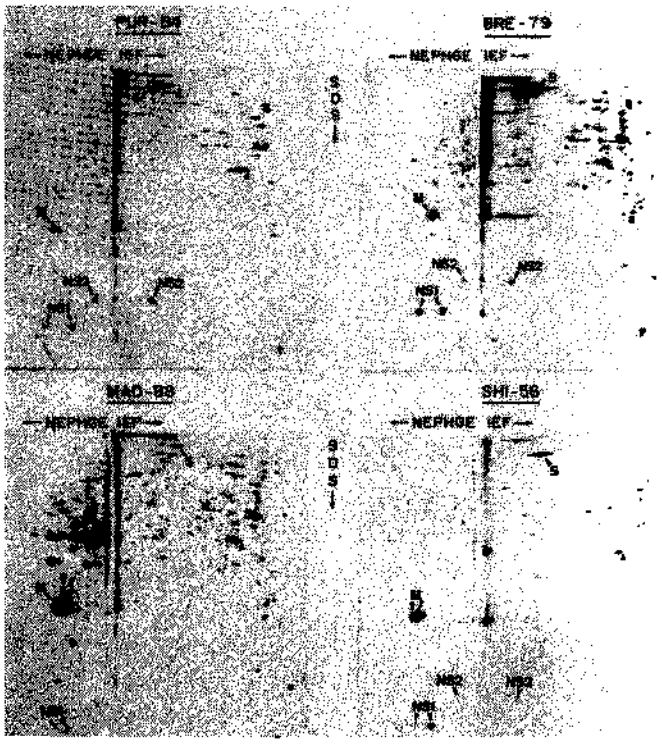

Fig. 3. Two-dimensional electrophoresis of infected cells by three attenuated (PUR-54, BRE-79 and SHI-56) and one virulent (MAD-88) TGE viruses labeled with ^{35}S-methionine (500 μCi/ml). ST cells were infected at moi 10 with all the viruses and labeled for 2 hours at 8 hpi. Letter A in the acidic gels indicate the position of the cellular actin.

Third, two non-structural proteins were detected by two-dimensional electrophoresis in FIPV infected cells. These proteins, with a MW of about 12K, showed different isoelectric points (Fig. 4). In CCV infected cells two polypeptides with MW of 12K and 14K were also detected (Fig. 4).

Discussion

The above results demonstrated that more than one virus-specific mRNA encoding for non-structural proteins are translationally active in TGEV-infected cells. We have characterized at least four polypeptides that corresponded with the number of non-structural mRNA species previously characterized (12, 18). Other authors, using conventional one-dimensional electrophoresis have largely failed to identify more than one non-strutural polypeptide in TGEV-infected cells. However, the mRNA species that code for those proteins have been detected easily (8, 12, 17). Two-dimensional electrophoresis could distinguish more efficiently between cellular and virus-induced proteins, as well as between proteins with similar MW. Some evidence of the existence of more than one non-structural protein with similar MW has been described (15). These authors showed that a major intracellular polypeptide of 17 K and later sized as 14 K (16) was reproducibly resolved as a doublet.

The predicted MW of proteins assigned for ORFs A (7.7 K), B (18.8 K), C (9.2 K) and D (9 K) are not coincident with the MW of the polypeptides that we characterized above. Similar differences in the predicted MW of TGEV-induced non-structural proteins and those observed in the infected cells have been described (7, 15). After sequencing of A and B non-structural genes, potential N-glycosylation sites are present in both polypeptides (17). Attempts to label with ^{3}H-glucosamine the protein of 17 K detected in infected cells have failed (15). We have not observed changes in the MW or isoelectric points of any of the non-structural proteins detected after tunycamicin tre-

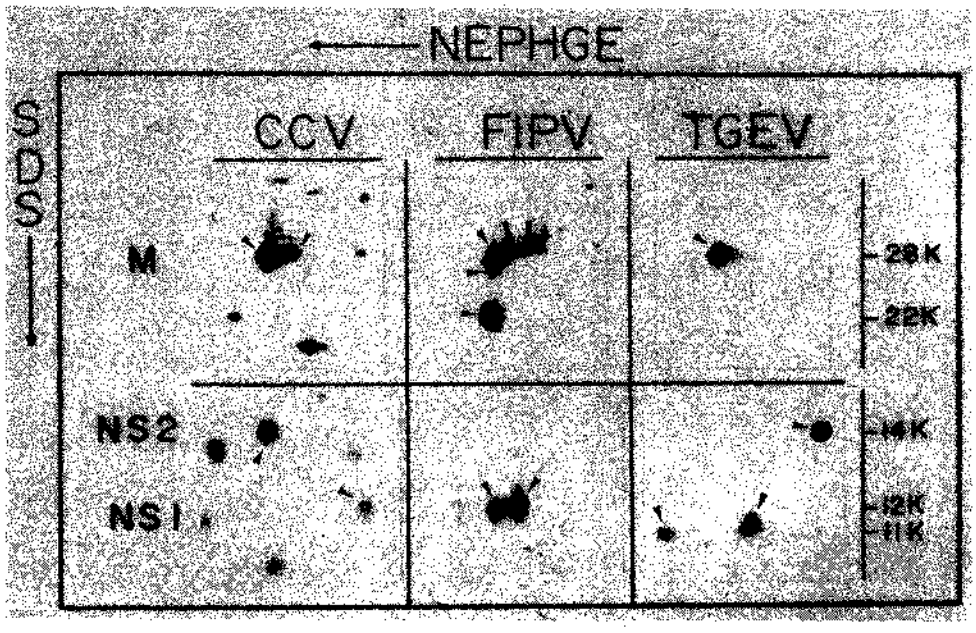

Fig. 4. Comparison of transmembrane (M) and non-structural (NS) proteins induced by Pur-54 strain of TGEV, 1146 strain of FIPV and 353716 strain of CCV in infected cells using two-dimensional electrophoresis. In this figure it is shown the NEPHGE gel fragments comprising the proteins with MW between 10 K to 15 K and between 20 K to 35 K. The labeling and moi conditions for the three viruses compared were the same used in Figure 3.

atment. No explanation can be hypothesized at present for the differences in the predicted and observed MW of the non-structural proteins. Pulse-chase experiments also showed no processing of non-structural proteins. Since we have detected antibodies against one of the NS1 proteins in serum of an infected sow, we could assume that one of these proteins may be the same described with a MW of 14K (7, 16).

We have shown differences in the protein patterns obtained by infection with attenuated and virulent strains. The most important difference was found in the NS2 proteins which were not detected in the virulent strain (Fig. 3). Differences in subgenomic RNAs 3 and 4 were previously described between attenuated and the parent virulent virus (18). Our finding contrasts with the previously described, since those authors found that deletions in the A and B ORFs lead to attenuation. However, we have found less number of TGEV-induced polypeptides in cells infected with the virulent virus. Experiments about definitive coding assigments of the four non-structural polypeptides detected in TGEV-infected cells are in course. The use of antisera against synthetic peptides derived from the sequence of non-structural genes or direct sequencing of the detected polypeptides could clarify the relationship between these polypeptides and the subgenomic RNAs induced by TGEV.

Differences in structural and non-structural proteins induced by TGEV, CCV and FIPV have also been shown. The different number of amino acids between TGEV and FIPV M proteins seems to be compensated by further glycosylation in FIPV, since the final MW of this protein in both viruses was the same. On the other hand, the number of partially glycosylated forms of M protein found at the same time after infection in the infected cells by the three viruses was also different. Some of these forms in FIPV seem to be incorporated into the virion (5). Finally, differences in the non-structural proteins were also observed in the three viruses. A comparison between the genetic organization of FIPV and TGEV demonstrated that the number of non-structural genes detected in the FIPV genome was lower than in the TGEV genome (5). This observation may correlate with our findings because we have detected only two non-structural proteins in cells infected by FIPV.

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Quantification of alphafetoprotein (AFP) endocytosis by PHA-activated peripheral blood mononuclear cells (PBMC) from HIV-infected individuals. A useful test of predictive value for the progression of AIDS

José J. Aguilar[*1], Antonio Macho[1], Marianne Semmel[2] and José Uriel[1]

[1] *Laboratoire de Chimie des Protéines. Institut de Recherches Scientifiques sur le Cancer (CNRS), Villejuif (France).*
[2] *Laboratoire d'Oncologie Appliqueé (Unité INSERM 268), Villejuif (France).*
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Summary

Serum alphafetoprotein (AFP) is actively taken up, through receptor-mediated endocytosis, by many embryo-fetal cells during ontogenic development but also by neoplastic cells, as well as by normal peripheral T lymphocytes after mitogenic activation. We have previously shown that the ability to internalize AFP is impaired in mitogen-activated T cells from several groups of (HIV$^+$)-seropositive individuals and that this expression rouhgly correlates with the progression of the disease. It is not clear whether this impaired AFP-endocytosis results from an HIV-mediated inhibition of AFP-receptor expression or if it is the consequence of a target signal transduction impairment due to HIV-infection. In the present work we have explored both possibilities by studying AFP-endocytosis in peripheral blood mononuclear cells (PBMC) from seropositive (HIV$^+$)-asymptomatic heterosexual individuals and in *in vitro* HIV-infected PBMC from healthy donors, either quiescent or stimulated with phytohemagglutin (PHA). Our results suggest that this novel abnormality of T-cells associated with HIV-infection reflects an unusual proliferative response of PBMC to mitogenic stimuli.

Key words: Human Immunodeficiency Virus (HIV), seropositive (HIV$^+$)-asymptomatic individuals, peripheral blood mononuclear cells (PBMC), alphafetoprotein (AFP) endocytosis.

Resumen

La alfafetoproteína (AFP) es una proteína sérica activamente captada por células que han sufrido transformación tumoral, así como por linfocitos T tras su estimulación mitogénica. En nuestro laboratorio hemos demostrado que tal captación tiene lugar por endocitosis mediada por receptor y que el proceso está alterado en individuos infectados por el virus del SIDA, tanto más cuanto más avanzada se encuentra la infección. El presente trabajo trata de descubrir el origen de esta disfunción del linfocito T, considerando la posibilidad de que obedezca a la acción directa y específica del

(*) Current address: Departamento de Microbiología. Facultad de Ciencias (Universidad de Córdoba). Córdoba (Spain).

virus de la inmunodeficiencia humana (VIH) sobre el sistema AFP-receptores de la AFP en células infectadas, o que sea el resultado de una actuación más general del VIH en el organismo invadido: la génesis de señales de traducción defectuosas. Nuestros resultados indican que la incapacidad de las células mononucleares de sangre periférica (CMSP) para sufrir una adecuada transformación blástica bajo la acción de la fitohemaglutinina (PHA), y con ello de que ocurra una óptima expresión de receptores de AFP, podría por sí sola explicar el origen de la defectiva endocitosis de AFP detectada en CMSP infectadas —tanto *in vivo* como *in vitro*— por el VIH.

Introduction

Alphafetoprotein (AFP) is a serum protein that reversibly binds fatty acids (22), the active molecules involved in structural and/or metabolic processes indispensable for cell growth, proliferation and survival. Cell uptake of fatty acids seems to be regulated by a carrier protein —AFP— through cell-surface receptors specific for AFP (16). T-cells possess the ability to actively endocytose AFP during a transitory period of several days after mitogen activation. This property is greatly diminished or absent in quiescent lymphocytes (17).

The Human Immunodeficiency Virus (HIV), the agent etiologically related to the Acquired Immunodeficiency Syndrome (AIDS) (3, 6), infects preferentially both CD4-positive human T-lymphocytes (4) and monocytes (12). After the initial infection with HIV, patients may remain asymptomatic for years before the onset of Lymphoadenopathy Syndrome (LAS) or AIDS (7, 15).

Recently, in our laboratory, we have used PHA-activated peripheral blood mononuclear cells (PBMC) from HIV-1 seropositive (HIV^+) individuals to test the expression of AFP-receptors by measuring their ability to endocytose AFP. Preliminary results (23) showed an impaired endocytosis of AFP by PBMC from HIV-infected individuals, which rouhgly correlated with the progression of disease (1, 23). We concluded that the quantification of the defective AFP-uptake by T-cells from HIV-infected individuals could serve as a quantitative functional assay.

On the other hand, it is known that HIV may act as a mitogen (25) and can induce partial activation of PBMC (13). *In vivo* this effect determines that PBMC from seropositive (HIV^+) individuals are continuously subjected to active signals (8), resulting in partial activation (2) and unresponsiveness to further mitogenic stimulation of T-cells (9).

Taken together the above mentioned observations, it is noteworthy that the worsening in the different clinical status of HIV infection is simultaneously accompanied by a more progressive deterioration in both T-cell properties —proliferative response to mitogen (10) and in the ability for AFP-endocytosis (23)—. According to Fuchs *et al.* (5), the prior stimulation of PBMC could prevent or decrease the specific proliferative response of T-cells. This can explain the defective uptake of AFP by PHA-activated peripheral blood lymphocytes found in patients with AIDS and related syndromes.

In the present study we have reexamined the endocytosis of AFP by PBMC (the PBMC came from a wider group of seropositive (HIV^+)-asymptomatic heterosexual individuals and we used healthy donors as a control) before and after activation with PHA and the expression of interleukin-2 receptors (IL-2r) upon PHA activation of PBMC, as a well documented control of T-cell response to mitogenic stimulation. In addition we have analyzed the time course of AFP-endocytosis by PHA-activated PBMC *in vitro* HIV-infected and mock-infected, to elucidate whether the expression and function of AFP-receptors is really impaired in these cells or whether the changes observed result from the time elapsed between T-cell infection and the examination of this funcional defect in PBMC (2).

As a conclusion we propose a unifying mechanism for this functional defect in HIV-infected T-cells, by considering the possibility that the abnormal AFP-endocytosis could be another qualitative alteration due to a HIV-induced signal transduction defect. The same mechanism, not necessarily related to the presence of HIV in the cell (13), can explain not only the results presented in this work but also our earlier observations with T8 lymphocytes (23) —one T-cell subset apparently resistant to HIV-infection (4)—.

Materials and methods

Isolation and PHA-activation of peripheral blood mononuclear cells (PBMC)

Heparinized peripheral blood samples were obtained from twenty-five seropositive (HIV^+)-asymptomatic heterosexual individuals, supplied by Dr. Didhiero (Institute Pasteur, France), and from fifty healthy blood donors (Service de Transfusion Sanguine, Hôpital Kremlin-Bicêtre, France).

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque (Pharmacia, Sweden) density centrifugation. The PBMC were washed twice with Hank's balanced salt solution and immediately resuspended at a concentration of 10^6 cells/ml in RPMI 1640 medium (GIBCO), supplemented with heat-inactivated fetal calf serum (10%), penicillin (250 units/ml), streptomycin (250 µg/ml) and L-glutamine (2 mM). PBMC were activated by adding phytohemagglutinin (PHA)-M (Sigma, UK) to the culture medium at a final concentration of 2 µg/ml, and incubated for 72 h at 37° C in a humidified atmosphere of 5% CO_2, 95% air.

Virus Stocks

The experiments of *in vitro* HIV infection of PBMC were performed with the BRU strain of HIV-1 (3). The virus stocks were prepared by collecting cell-free supernatant from HIV-1 infected CEM cells and diluted to a concentration of 10^6 cpm/ml of reverse transcriptase (RT) activity.

HIV in vitro *infection, anti-IF treatment and culture of activated T-lymphocytes*

Half of each PBMC preparation from five healthy donors (randomly chosen) was simultaneously activated with PHA and infected with the virus stock (5×10^3 cpm of RT activity per ml), in the presence of 1 µg/ml of polybrene (Sigma, UK) and 20 U/ml of serum against human interferon alpha —SAIF— (Miles, France). The other half was cultured under the same conditions and mock-infected. After 3 days cells were washed in the Hank's solution, the medium was replaced with fresh culture medium containing 1 U/ml of recombinant interleukin 2 (rIL-2, Cetus Corp., USA) instead of PHA. Cells were maintained 14 days in the medium which was changed twice a week.

Assessment of HIV-1 replication

To assess the efficiency of *in vitro* HIV infection, reverse transcriptase (RT) activity and the presence of infectious virions were measured in supernatants harvested at different times after infection (ten and fourteen days for RT activity and fourteen days for infectivity).

Mg^{2+}-dependent RT activity of each supernatant was tested according to Jonassen (11). The presence and concentration of infectious virions were followed by the β-galactosidase assay develo-

ped by Rocancourt *et al.* (18). The results are given as cpm of RT activity/ml of supernatant or as the number of β-gal$^+$ syncytia/ml after infection with 0.1 ml of cell free supernatant.

Protein

Human alphafetoprotein (AFP) was a kind gift from Dr. Tecce (Sclabo Lab., Sienna, Italy). The fluorescent derivative of AFP (FITC-AFP) was prepared by conjugation with fluorescein isothiocyanate (FITC) as described elsewhere (21). Fluorescein to protein molar ratios ranged between 2.5 and 3.5.

AFP-endocytosis and IL-2r expression analysis by flow cytofluorometry

Prior to AFP-endocytosis analysis, PHA-treated PBMC were preincubated 30 min at 37° C in serum-free RPMI-1640 medium to deplete cells of any endogenous bovine AFP that could have been internalized from the culture medium: cells were then incubated at 37° C for 90 min in serum-free medium containing 0.5 % ovalbumin (OVA) and 150 μg/ml of FITC-AFP (without FITC-AFP for each PBMC control of cell autofluorescence) (20). To assess IL-2r expression, 10^6 PHA-treated PBMC were incubated in 1 ml RPMI-1640 medium containing 5 % heat-inactivated fetal calf serum and anti-Tac monoclonal antibodies fluoresceinated (Immunotech, France) at 1/100 dilution for 30 min at 4° C. After incubation, cells were washed three times with ice-cold phosphate buffered saline (PBS) and fixed with a solution of 1 % paraformaldehyde in PBS at a final density of 10^6 cell/ml. As previously described (14), cells fixed under these conditions maintain the fluorescence labelling for approximately 1 week.

The amount of FITC-AFP taken up by PBMC was determined by fluorescence analysis in a FACS 440 cell sorter, using appropriate standards which allowed us to determine absolute values of labelling. For each stained sample, after subtracting the value obtained for control (i.e., the autofluorescence), the percentage of fluoresceinated cells and the total FITC molecules taken up by 10^4 cells were determined. This value divided by the FITC/protein ratio allowed us to calculate the total number of protein molecules which had seen internalized. Results are expressed as the average number of AFP molecules fixed per cell. For IL-2r expression, the results are given as the percentage of fluoresceinated Tac$^+$ cells.

Results and discussion

All the experiments were performed on two independently grown batches of each PBMC preparation.

Table 1 shows RT activity and production of β-gal$^+$ syncytia/ml of supernatants harvested at various times after HIV-infection of five different human lymphocytes cultures. Data confirm that PBMC from all subjects infected *in vitro* became permissive for HIV and producers of infectious virions. These results are in agreement with previous studies, corroborating either the differential susceptibility of PBMC to infection by HIV-1 (24, 26) or the nonexistence of a good positive correlation between measures of RT activity and infectivity (18).

Comparative data of AFP endocytosis (percentage of FITC-AFP$^+$ PBMC and quantitation of AFP-uptake) by nonstimulated and PHA-activated PBMC from healthy donors and seropositive (HIV$^+$) asymptomatic individuals are summarized in Table 2. In this table we also show the results for IL-2r expression (% Tac$^+$ cells) upon PHA-activation. Time courses of AFP-uptake by PHA-activated (72 h) noninfected and *in vitro* HIV-infected PBMC are illustrated in Table 3.

TABLE 1
REVERSE TRANSCRIPTASE (RT) ACTIVITY, AFTER 10 AND 14 DAYS OF CULTURE (PARENTHESES), AND β-GAL$^+$ SYNCYTIA FORMED AFTER INFECTION OF HT4 LAC Z1 CELLS WITH 0,1 ML OF SUPERNATANT OBTAINED FROM PHA-ACTIVATED PBMC AFTER 3 DAYS OF *IN VITRO* INFECTION BY HIV-1

PBMC preparations	RT activity ($\times 10^6$ cpm/ml)		β-gal$^+$ syncytia/ml supernatant
	10 ds	14 ds	14 ds
1	0,25	(4,80)	485
2	ND	(0,50)	73
3	0,08	(0,90)	90
4	0,40	(5,10)	315
5	0,18	(2,54)	370

ND = Undetected.

TABLE 2
AFP-UPTAKE BY PBMC FROM SEROPOSITIVE (HIV$^+$)-ASYMPTOMATIC INDIVIDUALS AND HEALTHY DONORS EITHER NONACTIVATED OR PHA-ACTIVATED (72 H)

	(AFP molecules/cell) $\times 10^{-3}$[a]		% T cells AFP$^+$[b]		% T-cells
	Unstimulated	PHA-activated	Unstimulated	PHA-activated	Tac$^+$[c]
Control (HIV$^-$) n = 50[d]	25,6 ± 7,6 p < 0,01	49,0 ± 11,2 p < 0,01	25,3 ± 8,7 p < 0,01	41,8 ± 8,4 p < 0,01	56,1 ± 18,4 p < 0,01
Seropositive (HIV$^+$) n = 25	48,5 ± 23,5 p < 0,01	65,3 ± 26,7 p < 0,01	35,0 ± 14,5 p < 0,01	43,1 ± 11,9 p < 0,01	24,5 ± 15,8 p < 0,01
HIV$^+$-1	26,2	47,9	18,2	37,3	23
HIV$^+$-2	27,4	36,5	22,5	36,5	8
HIV$^+$-3	53,0	108,5	37,4	58,2	58
HIV$^+$-4	34,8	97,8	35,6	54,0	41
HIV$^+$-5	31,7	56,8	25,6	35,6	27
HIV$^+$-6	49,9	58,6	27,8	38,6	19
HIV$^+$-7	84,9	67,2	56,1	41,0	17
HIV$^+$-8	83,1	44,1	56,6	40,3	12
HIV$^+$-9	45,6	55,8	35,6	46,4	16

[a] Average number of AFP-FITC molecules endocytosed per cell after fluorescence intensity analysis of 10^4 cells. Mean ± SE.
[b] Percentage of PBMC AFP-FITC$^+$. Mean ± SE.
[c] Percentage of PBMC anti-Tac-FITC$^+$ upon PHA-activation. Mean ± SE.
[d] n = number of samples.

Contrary to the common belief, cell autofluorescence was not similar in each population of PBMC tested nor in the same population analyzed at different times of culture (not shown). In HIV-infected PBMC the background of fluorescence was higher than in noninfected controls, and this parameter increased with the culture age, the change being more pronounced for infected

TABLE 3
TIME-COURSES OF AFP-UPTAKE BY PHA-ACTIVATED (72 h) PBMC UNINFECTED AND *IN VITRO* INFECTED BY HIV-1

	(AFP molecules/cell) × 10^{-3}[a]						
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Mean	SD
Controls							
Cultured 2 d	31,1	26,5	30,6	37,1	30,3	31,1	3,8
Cultured 3 d	49,6	42,8	47,5	55,1	49,8	49,0	4,4
Cultured 5 d	25,7	32,1	36,3	37,6	22,1	30,7	6,7
Cultured 7 d	7,8	8,1	12,3	13,9	6,1	9,6	3,3
HIV-infected							
Cultured 2 d	36,5	28,2	29,5	44,3	33,6	34,4	6,5
Cultured 3 d	73,0	54,6	62,0	83,5	75,2	69,7	11,4
Cultured 5 d	17,5	27,6	15,3	14,2	15,3	18,0	5,5
Cultured 7 d	5,8	8,2	6,5	3,0	5,6	5,8	1,9

[a] Average number of AFP-FITC molecules endocytosed per cell after fluorescence intensity analysis of 10^4 cells.
SD = Standard deviation.

T-cells. These data suggest the importance of checking autofluorescence prior to FACS analysis of each sample to be tested.

The lower expression of IL-2r upon T-cell activation associated with T lymphocytes from seropositive (HIV^+) asymptomatic heterosexuals (24.5 ± 15.8 vs. 56.1 ± 18.2) (Table 2) confirms an impaired response of PBMC to PHA. These results agree with our observations in seropositive (HIV^+) asymptomatic homosexuals (23).

The highest AFP-uptake values were observed in cells from seropositive (HIV^+) asymptomatic individuals, both nonstimulated (48,500 molecules/cell) and PHA-activated (65,300 molecules/cell), in comparison to noninfected controls (25,600 and 49,000 molecules/cell, respectively) (Table 2). Data for quiescent, non activated PBMC, seem to confirm an inital status of preactivation: a relative constant feature associated with HIV mitogenic action (25). On the other hand, changes in the ability of AFP-uptake after PHA-activation (measured as percentage of FITC-AFP^+ T cells) are less important for HIV-infected patients than for healthy donors (35.0 to 43.1 vs. 25.3 to 41.8), despite the greater absolute values of AFP-uptake measured in HIV^+-seropositive individuals vs. uninfected controls. This impairment might probably be related either to the transient expression of AFP-receptors by T-cells (16) or to the unresponsiveness of HIV-preactivated T cells to a second signal of activation (stimulation with PHA) (5). We think that the postulated depressed synthesis of AFP-receptors, upon blastic transformation of these cells (19), is not directly due to the infection by HIV but the consequence of an abnormal mitogenic response of PBMC connected with the viral presence. This alteration (25) and its consequences (23) are obviously more important in later stages of HIV-infection.

These results contrast with earlier data obtained in our laboratory with PBMC from asymptomatic homosexual men (23), the discrepancy being probably due either to the limited number of cases tested in the earlier study or to the heterogeneity of AFP-endocytosis of the patients. The high standard deviation of their average values as well as the measurements obtained from some of the HIV^+-seropositive individuals (see Table 2) seem to confirm such a conclusion.

The wide range of AFP-uptake values observed in the group of seropositive (HIV^+) asympto-

matic individuals (Table 2) deserves some comments. This variability may simply reflect differences in the time elapsed between primary infection and T-cell examination for AFP endocytosis (2). It may also reveal differences in susceptibility to T-cell disfunction following HIV-infection (24, 26). Contrary to some HIV^+-seropositive individuals, with levels of AFP-endocytosis in the range of those of healthy donors (cases n° 1, 2 and 5), we have analysed others that did not display this behavior. For example, in cases 7 and 8, the highly defective endocytosis of AFP after PHA-treatment was in agreement with their initial status of preactivation. AFP-uptake even dropped after PHA-treatment. On the other hand, cases 3 and 4, exhibited upon PHA-stimulation stronger AFP-endocytosis capability than PHA-treated noninfected cells.

The same explanations are valid for *in vitro* infected PBMC (Table 3). In spite of the limited number of cases studied, the time courses were clearly different for infected and for noninfected controls. The values obtained from healthy donors agreed with other reported data (19).

Although these observations need to be extended to other clinical groups of HIV-infected patients, the data presented herein strongly suggest that quantification of AFP-endocytosis can be used as a functional assay that reflects both the status of T-cells «activation» associated with HIV-infection and their degree of responsiveness to mitogenic stimulation. The great variability of AFP-uptake values, from one individual to another, in response to mitogenic stimuli makes them of poor clinical significance. For a single individual, however, the quantification of AFP-endocytosis by PBMC upon mitogen stimulation appears as a useful predictor of the progression of the disease even at early stages of HIV- infection. The assay described in this paper has the advantage of detecting anomalies which would pass unnoticed by other methods.

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An automated RNA extraction procedure and application for 16S rRNA sequencing of *Leuconostoc amelobiosum*

A. J. Martínez-Murcia

Department of Microbiology, AFRC Institute of Food Research, Reading Laboratory, Shinfield, Reading RG2 9AT, UK.
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Summary

The determination of 16S ribosomal ribonucleic acid (16S rRNA) primary structures by *in vitro* reverse transcription requires the extraction of rRNA in pure form. Although a number of high reliable techniques have been developed for the purpose most are fairly complex, involving numerous steps and the wasting of large volumes. It describes here a RNA extraction and purification method, suitable for automatic extractors, which consistently yields reasonable amounts of pure total RNA from prokaryotes, free of DNA and RNases. The rRNA from the type strain *Leuconostoc amelobiosum* was isolated using this procedure. Its 16S rRNA sequence was determined and a comparative analysis with those from all currently described leuconostocs, including several atypical lactobacilli, revealed very high sequence homology with *Leuconostoc citreum* confirming *Leuconostoc amelobiosum* is phylogenetically a member of the genus *Leuconostoc sensu stricto.*

Key words: RNA extraction; Leuconostoc; *Phylogeny; 16S rRNA.*

Resumen

La determinación de estructuras primarias del ácido ribonucleico ribosómico 16S (ARNr 16S) mediante transcripción reversa *in vitro* requiere la extracción de ARNr en forma pura. A pesar de que para ello se han desarrollado numerosas técnicas de alta fidelidad, la mayoría son bastante complejas, implicando numerosos pasos y desperdicio de grandes volúmenes. Aquí se describe un método de extracción y purificación de ARN adaptable a extractores automáticos que, de forma consistente, rinde cantidades razonables de ARN total puro a partir de procariotas, libre de ADN y ribonucleasas. Siguiendo este procedimiento se aisló el ARNr de la cepa tipo de *Leuconostoc amelobiosum.* Se determinó la secuencia de su ARNr 16S y un análisis comparativo con las de todos los leuconostocs actualmente descritos, incluyendo varios lactobacilos atípicos, reveló una homología de secuencia muy alta con *Leuconostoc citreum,* confirmando que, filogenéticamente, *Leuconostoc amelobiosum* es un miembro del género *Leuconostoc sensu stricto.*

Introduction

Sequencing of 16S rRNA using reverse transcriptase is a rapid and powerful technique of elucidating the natural relationships of microorganisms (18-20). Nevertheless, sequence determinations can present reading problems in part due to high protein contamination in the RNA samples. Use of denaturants (1) and proteinase K digestion (10) followed by several phenol extractions, have considerably improved RNA purification. However, Gram-positive cell walls are very difficult to break using only detergents. Use of a Braun homogenizer followed by lysozyme treatment was beneficial. This protocol can be adapted for use with an automatic DNA/RNA extractor which processes eight RNA preparation samples in approximately five hours. The 16S rRNA sequencing method has done much to clarify the classification of *Leuconostoc* spp. (13, 20) and other lactic acid bacteria (2, 17). Recently, the species *Leuconostoc amelobiosum* has been described on the bases of nucleic acid pairing studies (16). The 16S rRNA sequence of *L. amelobiosum* is here reported and a comparative analysis with the other *Leuconostoc* spp. confirmed this is a member of the genus *Leuconostoc sensu stricto* and exhibits a specific relationship with *Leuconostoc citreum.*

Material and methods

Bacterial cultures and chemicals

Details of the test strains examined are listed in Table 1. All *Leuconostocs* and *Lactobacillus* strains were obtained from, or were deposited in, the National Collection of Food Bacteria (NCFB) and the National Collection of Dairy Organisms (NCDO), England, UK. *Aeromonas hydrophila* was obtained from the NCIMB, Ltd., Scotland, UK. Strains of lactobacilli were grown statically in MRS Broth (Oxoid Manual), whereas the leuconostocs were cultivated in YGP Broth (9), both at 30° C. Cultures were checked for purity, harvested in mid-log phase by centrifugation (at x3000 g) and washed in distilled water.

RNA extraction

10 ml of sampling buffer (100 mM Tris-HCL; 10 mM Ethylenediaminetetraacetic Acid (EDTA); 1% Sodium Dodecylsulphate (SDS); pH 8.0) were added to 2 g of wet cells and transfered to a Braun bottle containing 30 g of glass beads. The sample was homogenised for 5 sec and stored on ice. The homogenate was transferred to a fresh tube using a Pasteur pippete and dispensed into Eppendorf tubes and centrifuged for 3-5 sec. One ml of the sample was added to each vessel of the DNA/RNA Extractor Model A340 (Applied Biosystems, Ltd., Warrington, UK) which was programmed according to the manufacturer's instructions. The program included; addition of 0.6 ml 1% lysozyme in 20 mM Tris-HCl buffer, pH 7.5, 12 min at 37° C. Two ml of lysis solution (4 M Guanididinium Thiocyanate (GTH); 0.25 M Sodium Citrate (NaCi); 0.1 M 2-mecarptoethanol; 0.5% Sarcosyl; 1% SDS; 0.1% Antifoam A) were added and the vessels shaken for 10 min at 50° C. Previous dilution of the GTH by three consecutive additions of 2 ml dilution buffer (0.25 M NaCi; 1% SDS; 0.5% Sarcosyl; 0.1% Antifoam A; pH 7.5) the homogenate was digested by addition of 0.5 ml 2% proteinase K in 20 mM Tris-HCl buffer, pH 7.5, for 45 min at 50° C. The pH was lowered with 0.7 ml Sodium Acetate, pH 4.5. RNA was extracted twice with 10 ml 70% phenol-TES satured (50 mM Tris-HCl; 50 mM NaCl; 5 mM EDTA; pH 8.0) at ambient temperature for emulsing steps and

TABLE 1
HOMOLOGY VALUES FOR A 1340 NUCLEOTIDE REGION OF 16S rRNAs OF *LEUCONOSTOC* AND OTHER TEST STRAINS

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Lactobacillus confusus* NCDO 1586	100	95.9	96.1	96.2	91.4	90.7	90.8	91.5	90.8	91.5	85.5	96.0	91.5
2. *Lactobacillus kandleri* NCDO 2753		100	96.0	97.0	90.6	90.4	90.2	90.6	90.4	90.9	85.9	94.6	90.8
3. *Lactobacillus minor* NCDO 1973			100	98.4	90.1	89.3	90.1	90.2	89.8	90.8	86.3	95.2	90.4
4. *Lactobacillus viridescens* NCDO 1655				100	90.2	89.8	90.8	89.8	90.3	90.7	86.8	95.6	90.6
5. *Leuconostoc amelobiosum* NCFB 2787					100	97.4	99.1	97.8	98.4	97.7	86.8	90.9	97.4
6. *Leuconostoc carnosum* NCFB 2776						100	97.5	98.3	97.2	97.5	86.7	89.3	97.2
7. *Leuconostoc citreum* NCDO 1837							100	97.1	98.9	97.8	86.8	90.5	97.6
8. *Leuconostoc gelidum* NCFB 2775								100	97.5	97.6	85.9	90.4	97.4
9. *Leuconostoc lactis* NCDO 533									100	97.8	86.5	90.9	97.3
10. *Leuconostoc mesenteroides* NCDO 523										100	86.0	90.1	99.6
11. *Leuconostoc oenos* NCDO 1674											100	86.2	86.8
12. *Leuconostoc paramesenteroides* NCDO 803												100	90.3
13. *Leuconostoc pseudomesenteroides* NCDO 768													100

at 55° C for the phase separations which occur in 10 min. Equal volume of chloroform was added to remove the residual phenol and the RNA was then precipitated with 6.5 ml isopropanol, collected on a filter-cartridge and washed with 8.5 ml ethanol. Filters were removed from the cartridge using sterile forceps and placed into sterile Eppendorf tubes. The filter was dryed in a vacuum centrifuge (V. A. Howe & Co. Ltd., London, UK) for 5 min and resuspended into sterile 20 mM Tris-HCl buffer, pH 7.5.

Spectrophotometric and agarose gel assays

Optic density readings (OD) were made on the preparations at 230, 260 and 280 nm to determine the quantity and quality of the samples obtained. The samples were analysed by horizontal 1 % agarose minigel electrophoresis using 16S and 23S rRNA from *Escherichia coli* (Boehringer) as molecular weight marker. Eight µl of a mixture containing 0.3 µg/µl RNA and bromophenol-xylene cyanol dye (10:1 v/v) were loaded onto the gel. Electrophoresis were at 30 mA for two hours and the gel was stained with ethidium bromide (0.5 µg/ml) and photographed using Kodak 667 film.

Sequence determination and analysis of the RNA sequence data

Nucleotide sequences were determined by the dideoxynucleotide method (15) using AMV reverse transcriptase (Life Science). The sequences of oligonucleotides primers and their 16S rRNA target sites were as described by Lane *et al.* (12) and Embley *et al.* (4) except for a DNA primer of sequence 5' TCAGTCTCTCAACTCGGCTA, which was employed for the ca. 300 region (complementary to positions 287 to 306; *E. coli*). The sequencing products were separated on 55 cm wedge-shaped (0.2-0.6 mm) 6% (w/v) polyacrylamide denaturing (7 M urea) gels at 55° C using an LKB Macrophor 2010 sequencing unit operated al 50 W per gel. The sequences were aligned and homology values determined using the Beckman Microgenie program (14). Nucleotides substitution rates (Knuc values) were calculated (11) and an unrooted phylogenetic tree produced using the algorithm of Fitch and Margoliash (8) contained in a program written by Felsenstein (5) (PHYLIP version 3.1) for the IBM PC.

Results and discussion

A new protocol for RNA extraction from prokaryote cells has been described. Due to the difficulties in breaking Gram-positive walls, a short physical pre-treatment was necessary. This made the cells susceptible to lysis by a denaturant agent and digestion with lysozyme. The presence of ribosomal proteins can interfere with the reverse transcriptase reaction and RNases can degrade the template producing high background and sequence ambiguities. The use of strong denaturants such as guanidine hydrochloride for RNA extractions was first decribed by Cox *et al.* (3) but GTH was much more efficient (1). The protein denaturation rate is maximized by the combined use of this denaturant (in which both cation and anion are potent chaotropic agents) and a reductant to break protein disulphide bonds (1). Other protocols include a digestion by proteinase K which assures RNA samples free of ribonucleases (7) and has proved to be stimulated by SDS (10). The use of hot phenol extraction (6) offers a clean phase separation in 10 min and so, centrifugation steps can be omitted. Because this protocol is suitable for an automated extractor whose system is sealed, sample contamination due to multiple transfers is minimized and eight RNA preparations have been obtained in five hours. The above method has been used to extract RNA from 'Lactic Acid' bacteria yielding RNA templates of consistently high quality for sequencing: ratios OD^{260}/OD^{280} between 1.8 and 2.0 whereas ratios OD^{230}/OD^{260} were 0.4-0.5. Their purity was checked by means of 1% agarose gel electrophoretic separation (Fig. 1) which showed two bands aligned with those of the 16S and 23S molecular markers from *E. coli*. Four samples (lanes 4 to 7) were compared with those obtained following a manual protocol (lanes 1 to 3) as described by Embley *et al.* (4). RNA extraction products from *Aeromonas hydrophila* were included as a Gram-negative control. The quantity of the samples was always more than enough to achieve the complete set of sequence reactions determining ca. 1500 bases of the total primary sequence. In several cases, the use of templates extracted by this method offered higher quality of data resolving a large number of sequence ambiguities.

A recent molecular taxonomic study of the genus *Leuconostoc* and some heterofermentative lactobacilli, which phenotypically resemble leuconostocs, was performed by comparative analysis of their 16S rRNAs (13) showing that the *Leuconostoc* spp. fall into three distinct groups/lines; *Leuconostoc mesenteroides* cluster (designated *Leuconostoc sensu stricto*), *Leuconostoc paramesenteroides* cluster (containing the atypical lactobacilli) and the acidophilic species *Leuconostoc oenos* which possessed a long and monospecific stalk. The species *L. amelobiosum* (formely *L. mesenteroides* subsp. *amelobiosum*) has been described on the basis of DNA-DNA and DNA-rRNA hybridization studies (16). In the present study, the RNA sequence from its small ribosomal subunit was de-

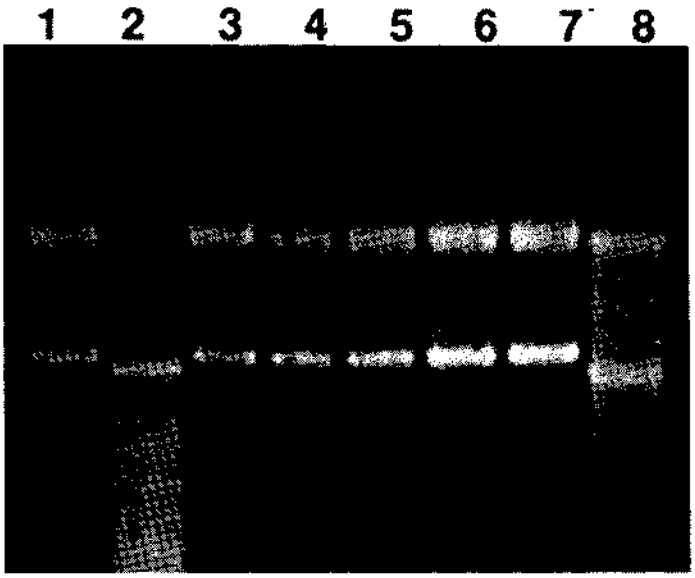

Fig. 1. 1% agarose gel electrophoresis showing rRNA samples for *Leuconostoc amelobiosum* (lane 4), *Lactobacillus kandleri* (lane 5), *Vagococcus salmoninarum* (lane 6) and *Carnobacterium piscicola* (lane 7) all obtained following the above protocol. rRNA from *L. amelobiosum* (lane 1), *Leuconostoc mesenteroides* (lane 3) and *Aeromonas hydrophila* (lane 2) were extracted using a manual protocol as described by Embley *et al.* (4). Lane 8 contains 23S rRNA (upper band) and 16S rRNA (lower band) from *E. coli.*

5'
UAAAUUGAGAGUUUGAUCCUGGCUCAGGAUNAACGCUGGCGGCGUGCCUAAUACaUGCAAGUCGAACCGC
AGCGAGAGGUGCUUGCACCuuUCNAGCGAGAUGGCGAACGGGUGAGUaAcNCGUGGAUaACCUGCCUcaA
GGCUGGGGAUaACaUUUGGAAACaGAUGCUNAUACCGAAUNAAACUUAGUAUCGCAUGAUACUAAGUUNA
AAGGCGCUACGGCGUCUCCUAGAGAUGGAUCCGCGGUGCAUUAGUUAGUUGGUGGGGUaAAGGCUUACCA
AGACGAUGAUGCgUAGCCGAGUUGAGAGACUNAUCGGCCACAUUGGGACUGAGACACGGCCCNAACUCCU
ACGGGAGGCUGCAGUAGGGAAUCUUCCACAAUGGGCGCAAGCCUGAUGGAGCaACGCCGCGUGUGUGAUG
AAGGCUUUCGGGUCGUAAAGCACUGUUGUAUGGGAAGAAAUGCUAAAAUAGGGAAUGAUUUUAGUUUGAC
GGUACCAUACCAGAAAGGGACGGCUNAAUACGUGCCAGCAGCCGCGGUNAUACGUAUGUCCCNAGCGUUA
UCCGGAUUUAUUGGGCGUAAAGCGAGCGCAGACGGUUGAUUAAGUCUNAUGUGAAAGCCCGGAGCUCAAC
UCCGGAAUGGCAUUGGAAACUGGUUAACUUGAGUGUUGUAGAGGUAAGUGGAACUCCAUGUGUAGCGGUG
GAAUGCGUAGAUAUAUGGAAGAACACCAGUGGCGAAGGCGGCUUACUGGACAACAACUGACGUUGAGGCU
CGAAAGUGUGGGUAGCAAACAGGAUUAGAUACCCUNGUAGUCCACACCGUAAACGAUGAAUACUAGGUGU
UAGGAGGUUUCCGCCUCUUAGUGCCGAACUAACGCAUUAAGUAUUCCGCCUNGGGAGUACGACCGCAAGG
UUGAAACUCAAAGGAAUUGACGGGGACCGCACNAGCGGUGGAGCAUGUGGUUUAAUUCGAAGCAACGCGA
AGAACCUUACCAGGUCUUGACAUCCUUUGAAGCUUUUAGAGAUAGAAGUGUUCUCUUCGGAGACAAAGUG
ACAGGUGGUGCAUGGUCGUCGUCAGCUCGUGUCGUGAGAUGUUGGGUUAAGUCCCGCAACGAGCGCAACC
CUNAUUGUUAGUUGCCAGCAUUCAGUUGGGCACUCUAGCGAGACUGCCGGUGACAAACCGGAGGAAGGCG
GGGACGACGUCAGAUCAUCAUGCCCCUUAUGACCUGGGCUACACACGUGCUACAAUGGCGUAUACAACGA
GUUGCCAACCUGCGAAGGUGAGCUAAUCUCUUAAAGUACGUCUCAGUUCGGACUGCAGUCUGCAACUCGA
CUGCACGAAGUCGGAAUCGCUAGUAAUCGCGGAUCAGCACGCCGCGGUGAAUACGUUCCCGGGUCUUGUA
CACACgGCcCGUCACACCAUGGGAGUUUGUAAUGCCCAAAGCCGGUGGCCUAACCUUCGGGAGGGAGCCG
UCUAAGGCAGGACAGAUGAC
3'

Fig. 2. 16S rRNA sequence from *Leuconostoc amelobiosum* NCFB 2787. The first and last nucleotide are analogous to position 1 and 1486 on the *E. coli* numbering system. N, undetermined nucleotide.

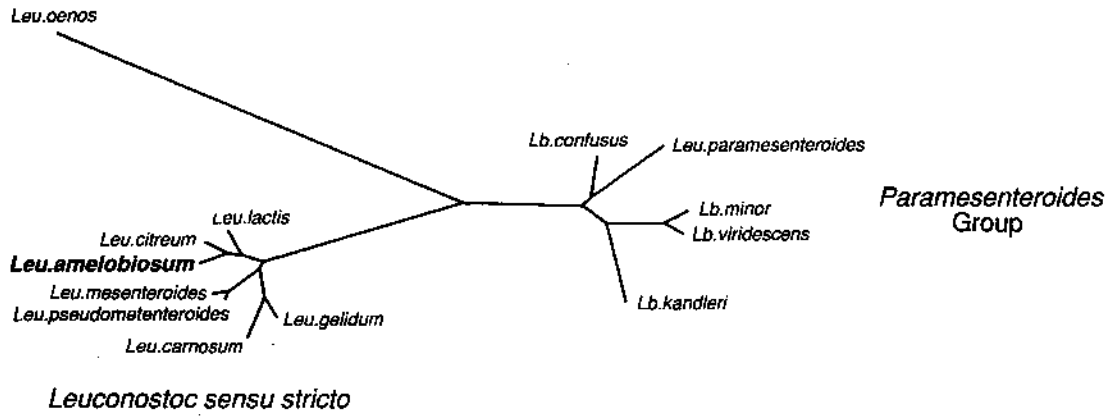

Fig. 3. Unrooted phylogenetic tree showing the relationship of *L. amelobiosum* to other *Leuconostoc* and related *Lactobacillus* species.

termined for a continuos stretch of 1490 bases (ranging from positions 1 to 1486 on the *E. coli* numbering system) (Fig. 2). Sequence homologies with all those species of the three phylogenetic *Leuconostoc* lines were also determined (Table 1). Unrooted tree constructed from calculated evolutionary distance values is shown in Figure 3. It is evident from the tree (branching pattern and evolutionary distances) that *L. amelobiosum* occupies a position into the *Leuconostoc sensu stricto* group, displaying higher sequence homology with *L. citreum* (99.1 %). *L. amelobiosum* was more related to *L. lactis, L. gelidum, L. mesenteroides, L. carnosum, L. pseudomesenteroides* (97.4 to 98.4 %) rather than to *L. paramesenteroides, L. confusus, L. kandleri, L. minor and L. viridescens* (90.1 to 91.4 %) constituting the so-called 'Paramesenteroides Group'. Finally, it was totally unrelated to the phylogenetically isolated *L. oenos* (86.8 %). These findings are in accordance with the DNA-rRNA hybridization studies of Schillinger *et al.* (16) confirming *L. amelobiosum* represents a new species of the genus *Leuconostoc.*

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Inhibition of the degranulation and myeloperoxidase activity of human polymorphonuclear neutrophils by *Brucella melitensis*

Antonio Orduña*, Carlos Orduña, José María Eiros, Miguel Angel Bratos, Purificación Gutiérrez, Pedro Alonso and Antonio Rodríguez Torres

Departamento de Microbiología. Hospital Clínico Universitario. Facultad de Medicina. Universidad de Valladolid. Ramón y Cajal, s/n. 47005 Valladolid (Spain).

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Summary

The degranulation and myeloperoxidase-H_2O_2-halide activities of human polymorphonuclear leukocytes from healthy donors were tested after co-incubation with either *Brucella melitensis* 16M, *Staphylococcus aureus* or *Staphylococcus aureus* in presence of lipopolysaccharide, protein fraction, native hapten and soluble fractions released at 65 °C from smooth strain of *Brucella melitensis* 16M. The degranulation and myeloperoxidase activities of polymorphonuclear leukocytes were significantly higher when co-incubated with *Staphylococcus aureus* than with *Brucella melitensis.* The presence of lipopolysaccharide, protein fraction, and native hapten did not cause significant modification of either degranulation or myeloperoxidase activities of polymorphonuclear leukocytes against *Staphylococcus aureus.* Soluble fraction released al 65 °C produced a significant reduction in the myeloperoxidase activity but did not alter the degranulation of polymorphonuclear leukocytes triggered by *Staphylococcus aureus.*

Key words: Brucella, *polymorphonuclear neutrophils, intracellular survival.*

Resumen

Se estudia la desgranulación y la actividad mieloperoxidasa-H_2O_2-haluro de polimorfonucleares de personas sanas sobre *Brucella melitensis* 16M, sobre *Staphylococcus aureus* y sobre *Staphylococcus aureus* en presencia de lipopolisacárido, de fracción proteica, hapteno nativo y de fracción soluble liberada a 65 °C de *Brucella melitensis* 16M en fase lisa. La desgranulación y la actividad mieloperoxidasa de los polimorfonucleares neutrófilos fueron significativamente superiores durante la ingestión de *Staphylococcus aureus* que durante la ingestión de *Brucella.* La presencia de lipopolisacárido, fracción proteica y hapteno nativo no originaron alteraciones significativas sobre la

(*) Corresponding author.

Abbreviations: MPO: myeloperoxidase-H_2O_2-halide; NH: native hapten; PF: protein fraction from *B. melitensis;* PMN: polymorphonuclear leukocytes; SFR: soluble fraction released at 65 °C from *B. melitensis;* S-LPS: lipopolysaccharide antigen from smooth cells.

desgranulación ni sobre la actividad mieloperoxidasa frente a *Staphylococcus aureus*. La fracción soluble liberada a 65 °C originó una reducción significativa en la actividad mieloperoxidasa, pero no modificó la desgranulación de los polimorfonucleares durante la ingestión de *Staphylococcus aureus*.

Introduction

Polymorphonuclear neutrophils (PMN) constitute one of the host defense mechanisms effectors able to protect certain microbial infections. However, certain microorganisms are able to survive and multiply within the phagocytic cells of the host, behaving like facultative intracellular pathogens.

Intracellular bacteria use different mechanisms to counteract the bactericidal activity of the phagocytic cells. Several microorganisms such as *Rickettsia tsutsugamushi* (15, 16) are able to escape from the phagocytic vacuola; *Mycobacterium tuberculosis* seems to resist the bactericidal action of phagocytes by producing sulfatides which prevent phagosome-lysosome fusion (8) and *Toxoplasma gondii* mantains its intracellular viability by blocking the respiratory burst of phagocytes (22).

The mechanisms used by *Brucella* to resist the bactericidal system of PMN are not well understood. *Brucella* sp resists the bactericidal action of phagocytic cells better than other bacteria and this resistance is greater in the case of *Brucella melitensis* than of *Brucella abortus* and greater in smooth strains than in rough ones (23, 20, 17). Morris (14) showed that ingestion of *Brucella* induces degranulation of PMN, although this is less than that produced after ingestion of other bacteria, such as *Staphylococcus epidermidis* (17). Several authors (1, 5) have shown the existence of nucleotides in *Brucella* which have the ability to inhibit degranulation and in this way block the activity of the myeloperoxidase-H_2O_2-halide (MPO) system of PMN.

On the other hand, Riley and Robertson (17, 18) showed that *Brucella* resists better than other bacteria to the oxygen-dependent and oxygen-independent bactericidal systems present in the lysosomal granules of PMN.

In this work the influence of different components of *B. melitensis* 16M on degranulation and MPO activity of human PMN from healthy donors is studied.

Materials and Methods

Bacterial strains and growth conditions

B. melitensis 16M and *S. aureus* ATCC 25923 were obtained by cultivation in Isosensitest broth (Oxoid) at 37 °C with agitation. After growth the bacteria were killed with 0.5 % phenol and washed in distilled water.

Opsonization of the killed bacteria

The killed bacteria were suspended in PBS and the concentration was adjusted to OD_{650} 0.500 for *B. melitensis* and OD_{650} 0.250 for *S. aureus*. Normal human plasma from healthy donors was added (final concentraction 10 % v/v) to the bacterial suspensions which were incubated at 37 °C for one hour. The opsonized bacteria were washed in PBS and were resuspended in a volume of RPMI 1640 1/20 of the initial suspension.

Preparation of B. melitensis *fractions*

To obtain each of the brucellar fractions a suspension of *Brucella* equivalent to 4×10^{14} CFU was used.

Lipopolysaccharide antigen (S-LPS) of *B. melitensis* was obtained following the hot phenol-water method modified for *Brucella* as described by Leong *et al.* (11). The bacteria were suspended in distilled water (5 g/170 ml, ratio dry weight/volume). The suspension was diluted 1/2 in phenol at 66 °C and was agitated vigorously for 15 minutes. The phenol fraction obtained by centrifugation was filtered and precipitated at –20 °C in three volumes of methanol supplemented with 1 % of methanol saturated with sodium acetate. The precipitate was dissolved in distilled water and the S-LPS was obtained by cold precipitation in four volumes of methanol with 1% of sodium acetate saturated methanol. The S-LPS was purified according to the method of Moreno *et al.* (13) by precipitation with 4 M NaI diluted in dimethyl-sulfoxide. The purified S-LPS was dialysed and freeze-dried.

The method used to obtain the protein fraction (PF) was previously described by Bhongbhibhat *et al.* (2). Acetone-dried *Brucella* was resuspended at 5 % (dry weight/volume) in 2.5 % NaCl and maintained at 4 °C for 48 hours with constant stirring. The suspension was centrifuged and the protein fractions were obtained by precipitation of the supernatant with three volumes of ethanol at –20 °C. The precipitate obtained by centrifugation was dissolved in distilled water, dialysed and freeeze-dried.

The native hapten (NH) was extracted from *B. melitensis* killed with phenol following the method of Díaz *et al.* (6). The *Brucella* were suspended at 30 % (weight/volume) in distilled water and the suspension was autoclaved at 120 °C for 30 minutes. The suspension was centrifuged and the proteins of the supernatant were eliminated by precipitation at 4 °C with three volumes of ethanol. The NH was isolated from the supernatant by precipitation al –20 °C with two additional volumes of ethanol. The NH was purified by filtration in Sephadex G200 (Pharmacia F. Ch.). The harvested fractions were analysed by the anthrone assay for the hexoses.

The soluble fraction, obtained from *B. melitensis* killed with phenol and washed with distilled water, was released after heating at 65 °C for one hour. The suspension was centrifuged and the supernatant was freeze-dried.

PMN function tests

The tests were performed using PMN from 26 healthy donors obtained by sedimentation in Dextran T500 (Pharmacia F. Ch.) at 2 % at 37 °C for 35 minutes.

The degranulation assay of PMN was carried out determining the activity of the β-glucuronidase released (21) by the PMN after ingestion of opsonized *B. melitensis* 16M, opsonized *S. aureus* or opsonized *S. aureus* in the presence of *Brucella* fractions. Test tubes containing 0.8 ml (10^7 cells), 0.1 ml of bacterial suspension (*B. melitensis* or *S. aureus*) and 0.1 ml of RPMI 1640 were incubated at 37 °C for 30 minutes. After centrifugation, 0.1 ml of the supernatant was incubated with 0.9 ml of p-nitrophenil-β-glucuronide 0.0315 % in 0.05 M sodium acetate acid buffer pH 5 with 0.1 % of Triton X100 for 18 hours at 37 °C. The reaction was stopped with 1 ml of 0.1 M NaOH and the OD_{410} was determined. The results are expressed as picomoles of β-glucuronidase released per hour by 10^7 PMN.

To study the MPO activity of the PMN the protein-iodination test was used (9). The standard reaction mixture contained 0.1 ml of PMN (10^7 cell/ml), 0.1 ml of ^{125}I labelled NaI (10 μCi/40 nmol/ml), 0.1 ml of bacterial suspension (*B. melitensis* or *S. aureus*) and 0.2 ml of RPMI 1640 were mixed in test tubes. After incubation (37 °C, 30 minutes) the reaction was stopped by adding 0.1 ml of 0.1 M sodium thiosulphate. The proteins were precipitated with 10 % of trichloroacetic acid and

TABLE 1
COMPARISON OF THE DEGRANULATION OF PMN AGAINST OPSONIZED *BRUCELLA MELITENSIS* AND AGAINST OPSONIZED *STAPHYLOCOCCUS AUREUS* IN PRESENCE OF *BRUCELLA* FRACTIONS VS *STAPHYLOCOCCUS AUREUS*. PICOMOLES OF β-GLUCURONIDASE RELEASED (MEAN ± S.D.)

		Statistical significance
S. aureus	49.73 ± 15.53	
B. melitensis	27.38 ± 10.18	$p < 0.001$
S. aureus-S-LPS Bm*	48.76 ± 14.48	Not significant
S. aureus-PF Bm	46.78 ± 13.31	Not significant
S. aureus-NH Bm	45.62 ± 14.77	Not significant
S. aureus-SFR Bm	47.18 ± 16.69	Not significant

* Bm: *B. melitensis*; S-LPS: Lipopolysaccharide antigen; PF: Protein fraction; NH: Native hapten; SFR: Soluble fraction released at 65 °C.

the readioactivity present in the precipitate was measured. The results are expressed as percentage of radioactivity incorporated in the precipitated proteins.

To standardize the tests, double dilutions of the 20x of initial concentration of bacterial suspensions were tested by the protein iodination test. In the subsequent tests the concentration which originated the highest protein iodination was used. (10x of initial concentration of *B. melitensis* and *S. aureus*.)

The effect of the *Brucella* fractions on the functions of PMN against *S. aureus* was studied. RPMI 1640 (50 µl) was substituted for S-LPS (11.3, 1.13, 0.113 µg/ml) PF (3.5, 0.35, 0.035 µg/ml, NH (5.5, 0.55, 0.055 µg/ml) or SFR (28.2, 2.82, 0.282 µg/ml).

Wilcoxon's test was used for the statistical analysis.

Results

Degranulation

As it is seen in Table 1, the amount of β-glucuronidase released by the PMN after ingestion of *B. melitensis* 16M (27.38 ± 10.18 picomoles) was significantly less ($p < 0.001$) than that released after ingestion of *S. aureus* (49.73 ± 15.53 picomoles).

The presence of S-LPS and SFR did not significantly modify the degranulation of PMN produced by *S. aureus*. PF and NH decrease the degranulation, although the differences were not statistically significant ($p > 0.1$) (Table 1).

Activity of the myeloperoxidase system

As it is seen in Table 2, the activity of the MPO system of the PMN, measured as percentages of protein iodination was significantly less ($p < 0.001$) with *B. melitensis* 16M (2.15% ± 0.60) than with *S. aureus* (2.78% ± 0.71).

Significant differences were not observed when comparing the results of the iodination assay against *S. aureus* in the presence of S-LPS (2.75% ± 0.78), PF (2.87% ± 0.81) or NH (2.88% ± 0.76), with the results obtained against *S. aureus* in the absence of *Brucella* fractions (Table 2). Neit-

TABLE 2
COMPARISON OF THE MPO ACTIVITY OF PMN AGAINST OPSONIZED *BRUCELLA MELITENSIS* AND AGAINST OPSONIZED *STAPHYLOCOCCUS AUREUS* IN PRESENCE OF *BRUCELLA* FRACTIONS VS *STAPHYLOCOCCUS AUREUS*. PERCENTAGE OF RADIOACTIVITY INCORPORATED TO THE PRECIPITATED PROTEINS (MEAN ± S.D.)

		Statistical significance
S. aureus	2.78 ± 0.71	
B. melitensis	2.15 ± 0.60	$p < 0.001$
S. aureus-S-LPS Bm*	2.75 ± 0.78	Not significant
S. aureus-PF Bm	2.87 ± 0.81	Not significant
S. aureus-NH Bm	2.88 ± 0.76	Not significant
S. aureus-SFR Bm	2.58 ± 0.79	$p < 0.01$

* Bm: *B. melitensis*; S-LPS: Lipopolysaccharide antigen; PF: Protein fraction; NH: Native hapten; SFR: Soluble fraction released at 65 °C.

TABLE 3
INFLUENCE OF THE CONCENTRATION OF *BRUCELLA* FRACTIONS ON MPO ACTIVITY OF PMN AGAINST OPSONIZED *STAPHYLOCOCCUS AUREUS*. PERCENTAGE OF RADIOACTIVITY INCORPORATED TO THE PRECIPITATED PROTEINS (MEAN ± S.D.)

	Fraction dilution*		
	1/1	1/10	1/100
S. aureus-S-LPS Bm	2.75 ± 0.78	2.75 ± 0.75	2.72 ± 0.74
S. aureus-PF Bm	2.87 ± 0.81	2.79 ± 0.83	2.79 ± 0.85
S. aureus-NH Bm	2.88 ± 0.76	2.73 ± 0.80	2.71 ± 0.79
S. aureus-SFR Bm	2.58 ± 0.79	2.75 ± 0.80	2.76 ± 0.79

* Initial concentration of S-LPS 11.3 μg/ml; PF 3.5 μg/ml; NH 5.5 μg/ml; SFR 28.2 μg/ml.

her were the results significantly modified on altering the concentrations of the *Brucella* fractions (Table 3).

However, the presence of SFR significantly reduced (2.58% ± 0.79, $p < 0.01$) the protein iodination and this decrease was proportional to the concentration of the SFR present in the reaction (Table 3).

Discussion

The intracellular survival and multiplication of *Brucella* has been studied by numerous authors. In 1958 Braun *et al.* (3) observed differences in the intracellular survival in macrophages between the smooth and the rough strains of *B. abortus*. In 1964, several authors (12, 20) suggested that the existing differences in the intracellular survival between the virulent and non-virulent strains of *Brucella* were due to the different chemical composition of the cell wall.

Previous studies indicate that *Brucella* is ingested by PMN in a similar way to other extracellu-

lar bacteria and that ingestion of *Brucella* is dependent upon the presence of normal fresh human serum, even in the absence of specific antibodies (23).

Morris (14) found that *Brucella* produces degranulation of human PMN similar to that caused after ingestion of *E. coli* or latex particles. However, Riley and Robertson (17), using electron microscope techniques, showed that degranulation of human and bovines PMN is lower after ingestion of *B. abortus* than after phagocytosis of *S. epidermidis.* In our study, we show that degranulation of human PMN produced against *B. melitensis* is significatly lower than that produced by *S. aureus,* releasing almost double the amount of β-glucuronidase against *S. aureus* than against *B. melitensis.*

Furthermore we observed that the activation of the MPO system of PMN is significantly less after ingestion of *B. melitensis* than *S. aureus.* This decreased activity of the MPO system of PMN may be due to the inhibition of phagolysosomal fusion or, in accordance with Kreutzer *et al.* (10), it may be due to a blocking of respiratory burst.

Riley and Robertson (17) found that the intracellular survival of the smooth strains of *Brucella* is greater than that of the rough strains. On the basis of different composition of the cell wall, they considered that the LPS might be responsible for the greater resistance of smooth strains to the bactericidal action of PMN.

Frenchick *et al.* (7) obtained a soluble extract of *B. abortus* (2308 strain) by vigorous agitation of live bacteria. This extract inhibited phagolysosomal fusion of macrophages, suggesting that the extract consists largely of surface material such as carbohydrate, glycopeptides or lipids since the extraction process does not affect the viability of the bacteria.

Bertran *et al.* (1) and Canning *et al.* (4, 5) isolated from *B. abortus* two nucleotides, adenine and 5'guanosine monophosphate, which inhibited degranultion of PMN. However, these fractions were inactive on the horse-radish peroxidase system. They concluded that one of the mechanisms used by *Brucella* to survive within the phagocytes is the release of nucleotides, which are able to inhibit the phagosome-lysosome fusion. These studies appear to indicate that the resistance to the bactericidal action of PMN is due to the ability of *Brucella* to block the degranulation, inhibiting the activation of the MPO system.

However, Riley and Robertson (17, 18) showed that *Brucella* resists the batericidal effect of the oxygen-independent systems of the lysosomal granules, and that the oxygen-dependent brucellicidal effect of the granules is less against the smooth strains of *Brucella* than the rough ones.

In our work we found that S-LPS alters neither the degranulation nor the MPO activity of PMN against *S. aureus* supporting the findings of Frenchick *et al.* (7), which did not observe differences in the degranulation of macrophages treated with LPS from virulent and attenuated strains of *B. abortus.* Furthermore, our sudies show that NH and PF do not influence either the degranulation or the activation of the MPO system.

However, while the SFR does not modify the degranulation of PMN, it significantly decreases the activity of the MPO system and this inhibition is dose dependent.

Acknowledgments

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Estudio taxonómico de levaduras de la Denominación de Origen «Vinos de Madrid», aisladas en el transcurso de la fermentación espontánea de mostos y vinos

Pilar Hidalgo*[1] y Margarita Flores[2]

[1] *Unidad de Viticultura y Enología. Servicio de Investigación. Comunidad de Madrid. Finca «El Encín». Apdo. 127. 28000 Alcalá de Henares (Madrid).*

[2] *Departamento de Microbiología I. Facultad de Biología. Universidad Complutense de Madrid. 28040 Madrid.*

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Summary

The natural yeast flora of musts and wines elaborated in wineries of the three Madrid viticultural areas, has been taxonomically analyzed. Two hundred and seventy yeast strains belonging to eighteen species, have been isolated, and *Sacch. cerevisae* strains are the most frequent.

The chemical characterization of samples with enological parameters defines the yeast fermentative ecosystem.

Key words: must, wine, yeast, fermentation, taxonomy.

Resumen

Analizada taxonómicamente la flora de levaduras de mostos y vinos elaborados en bodegas de la Denominación de Origen «Vinos de Madrid», aislamos 270 cepas pertenecientes a 18 especies, siendo *Sacch. cerevisiae* la más frecuente. La caracterización química de las muestras mediante parámetros enológicos nos definen el ecosistema fermentativo de las levaduras estudiadas.

La Denominación de Origen «Vinos de Madrid», con una superficie de viñedo alrededor de 30.000 hectáreas y una producción media anual de 500.000 hl de vino, engloba a tres subzonas vitivinícolas tradicionales: Arganda, Navalcarnero y San Martín de Valdeiglesias, situadas al sur de la provincia de Madrid, en la que se elaboran distintos tipos de vinos: blancos, rosados y tintos.

En la elaboración tradicional del vino la fermentación del mosto se desarrolla espontáneamente gracias a las levaduras que se encuentran de forma natural, junto con otros microorganismos (bacterias y mohos) en el hollejo de la uva, teniendo en cuenta además la eventual intervención de la flora contaminante de los elementos de transporte, de la bodega, su maquinaria y otros instrumentos de elaboración.

A lo largo de la fermentación alcohólica intervienen varias especies de levaduras, sucediéndose

(*) A quien debe dirigirse la correspondencia.

gradualmente en el transcurso del proceso (1, 2, 4, 8, 9), por una serie de mecanismos de selección, debido a factores intrínsecos y extrínsecos de naturaleza física, química y biológica.

De una manera general, los vinos de las zonas vitivinícolas en estudio son elaborados con fermentaciones espontáneas de los mostos, aun cuando actualmente se inicia la tendencia a realizar fermentaciones dirigidas mediante el uso de levaduras seleccionadas de la propia zona, o de levaduras secas activas (LSA) comerciales.

En el presente trabajo se realizó el análisis taxonómico de las levaduras que intervienen de un modo natural en el proceso fermentativo de los vinos de Madrid, recogiéndose las muestras *in situ* en los mismos depósitos de fermentación de cada bodega en estudio, y se determinó la evolución, variabilidad y predominio de las cepas en el transcurso de la fermentación alcohólica.

Estos datos, junto con los análisis químicos de las muestras, nos permiten tener un conocimiento real de los vinos en estudio, con una importante proyección a la hora de solucionar posibles problemas que pudieran plantearse en la elaboración de vinos con garantía de calidad.

En la vendimia de 1984 se tomaron 54 muestras de 11 bodegas representativas de la Denominación de Origen «Vinos de Madrid», pertenecientes a ocho localidades: Arganda del Rey, Valdilecha, Colmenar de Oreja, Navalcarnero, El Alamo, Cadalso de los Vidrios, Cenicientos y Villa del Prado.

El muestreo se llevó a cabo al inicio, mitad y final de la fermentación, de elaboraciones en blanco, rosado o tinto, atendiendo al tipo de vinificación llevado a cabo en cada una de las bodegas en estudio. Se realizó la caracterización química de las muestras, mediante la determinación del pH, acidez total, azúcar, grado alcohólico, anhídrido sulfuroso libre, anhídrido sulfuroso total y acidez volátil (6).

El aislamiento de levaduras se efectuó mediante la técnica de diluciones sucesivas y siembra en placa sobre agar extracto de levadura-malta (LM) (0,3 % extracto de levadura, 0,3 % extracto de malta, 0,5 % Bacto-peptona, 1 % glucosa, 2 % agar).

Después de incubadas a 25° C, durante 72 h, se eligieron, a partir de cada placa, 5 ufc de morfología diferenciada. Los cultivos puros fueron sembrados en estría, en tubos con agar LM y mantenidos a 4° C.

Posteriormente se llevó a cabo el análisis taxonómico de las cepas aplicando una clave simplificada de clasificación, por nosostros propuesta, basada en el «Manual de Taxonomía», de Kreger van Rij (1984), seleccionando las pruebas de identificación más discriminatorias para las levaduras vínicas (3).

Del conjunto de muestras estudiadas se aislaron 270 cepas de levadura pertenecientes a 18 especies diferentes (Tabla 1), siendo dominante *Saccharomyces cerevisiae,* levadura fermentadora por excelencia, como acontece en otros estudios realizados en el campo de la microbiología enológica (1, 2, 4, 8, 9), aislándose un total de 167 cepas, lo que representa un 61,89 % del total de levaduras aisladas.

Dentro del género *Candida* se encontró la mayor variabilidad de especies, coincidiendo con otras situaciones (1).

A su vez, el conjunto de cepas desglosadas en las tres fases fermentativas, inicio, mitad y final, se representa gráficamente mediante histogramas (Fig. 1) en los que se refleja el número y la frecuencia de cepas diferentes aparecidas.

Se observa que la mayor diversidad de especies se encuentra en mosto, aislándose 15 especies diferentes, con porcentajes poco elevados para cada una de ellas, siendo las más frecuentes *Saccharomyces cerevisiae, Torulaspora delbrueckii* y *Rhodotorula minuta.* Esta última tiene un poder fermentativo nulo (7, 8), por lo que son las otras especies las que de una forma mayoritaria iniciarán la fermentación alcohólica.

En mitad de la fermentación la variabilidad de especies es menor, siendo diferentes 10 de ellas, entre las que *Saccharomyces cerevisiae* es la que domina.

TABLA 1
ESPECIES DE LEVADURAS AISLADAS

Especies	Cepas aisladas	
	N.°	%
C. cantarellii	3	1,11
C. castelli	1	0,37
C. glabrata	6	2,22
C. parapsilosis	5	1,85
C. stellata	5	1,85
C. vini	1	0,37
H'spora valvyensis	1	0,37
H. anomala	6	2,22
H. subpelliculosa	1	0,37
Kl. apiculata	7	2,59
K. thermotolerans	6	2,22
M. pulcherrima	5	1,85
P. membranaefaciens	3	1,11
Rh. glutinis	6	2,22
Rh. minuta	12	4,44
Sacch. cerevisiae	167	61,89
Sacch. exiguus	20	7,40
T'spora delbrueckii	15	5,55
TOTAL	270	100,000

Al final de la fermentación aparecen únicamente cuatro especies, con un predominio casi absoluto de *Saccharomyces cerevisiae,* siendo la levadura que prácticamente finaliza el proceso.

Ruiz *et al.* (1986), en el estudio de «Los agentes de fermentación de los mostos de uva de la Comunidad de Madrid», realizado sobre la base de fermentaciones obtenidas en laboratorio con muestras de mosto representativas de la zona, obtuvieron menor número y variabilidad de especies de levaduras según el sistema de clasificación propuesto por Kreger van Rij (1984).

Ello puede deberse a varias causas, siendo la principal y fundamental el que nosotros planteásemos el estudio microbiológico tomando las muestras directamente de los depósitos en fermentación de las bodegas estudiadas, como se especificó anteriormente, y no de las fermentaciones llevadas a cabo en laboratorio, lo que aproxima más los resultados a la realidad del proceso en las industrias vinícolas.

También ha de considerarse la variabilidad anual en el tiempo y localización de las bodegas, así como el que nosotros tomáramos un mayor número de muestras, consiguiendo una mayor representatividad.

En los vinos de las zonas vitivinícolas en estudio las especies aisladas que aparecen únicamente en mosto y que no lo hacen en las fases fermentativas posteriores son: *Candida parapsilosis, Candida stellata, Hanseniaspora valvyensis, Metschnikowia pulcherrima, Pichia membranaefaciens, Rhodotorula glutinis* y *Rhodotorula minuta,* tratándose de levaduras de nulo o escaso metabolismo fermentativo (7, 8, 9).

De las especies que aparecen al final de la fermentación, *Saccharomyces cerevisiae* va aumentando en número en cepas del inicio al final del proceso; *Kluyveromyces thermotolerans* también aumenta a lo largo de la fermentación, pero aparece en escasa cuantía respecto al total de cepas aisladas. Por último, la especie *Saccharomyces exiguus* disminuye su número de cepas a lo largo de la fermentación alcohólica.

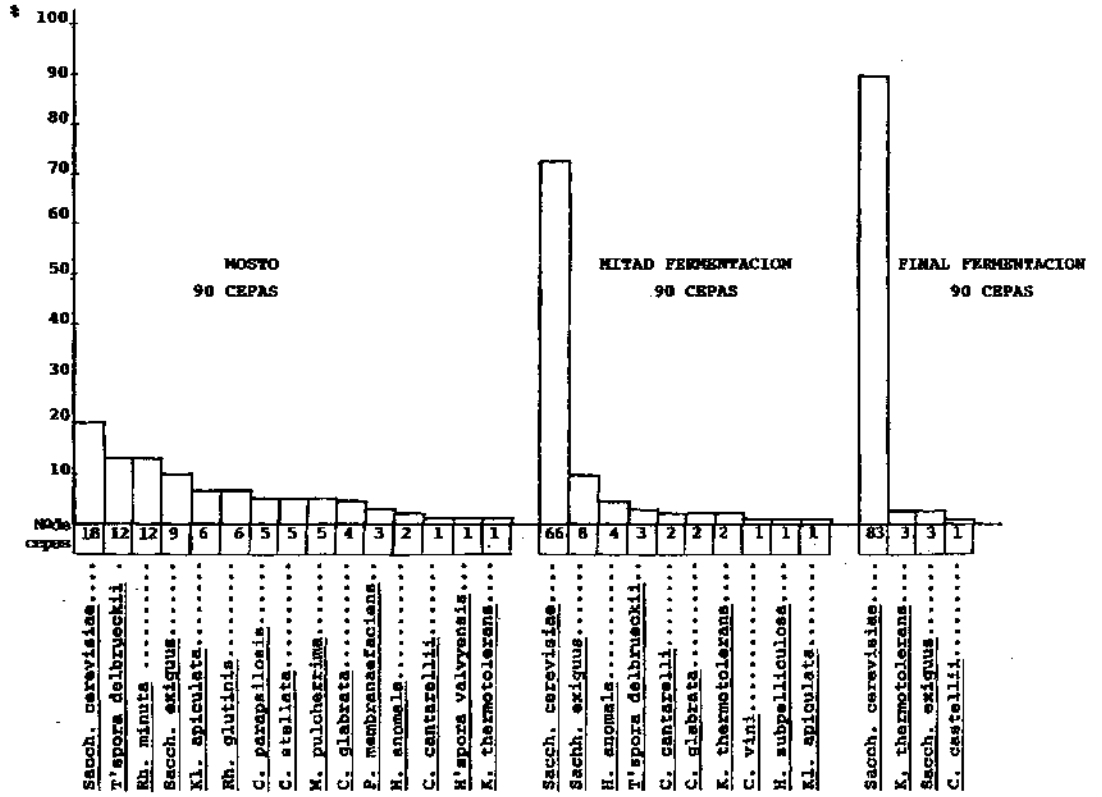

Fig. 1. Número y frecuencia de las cepas de levadura, aisladas en mosto, mitad y final de la fermentación.

En resumen y como era de esperar, se tiende a una menor diversidad de géneros y especies, conforme avanza la fermentación alcohólica, hasta llegar al final de la misma.

Completamos el estudio presentando la caracterización química de las muestras, reflejada en la Tabla 2, que define el ecosistema enológico de las tres subzonas que comprende.

De una manera general, siempre se observan en los vinos pH elevados: valores más altos para los obtenidos en San Martín de Valdeiglesias, intermedios para las elaboraciones de Navalcarnero y más bajos para los de Arganda, que se corresponden de una manera natural con una acidez total siempre baja, pero creciente respecto a la ordenación de las tres subzonas establecidas.

En todos los casos los vinos son secos, como consecuencia de fermentaciones completas, con riquezas alcohólicas decrecientes en San Martín, Navalcarnero y Arganda, llegando a valores destacadamente elevados en la primera zona.

Los valores anteriormente citados de acidez total y grado alcohólico son consecuencia natural de los contenidos en acidez y azúcar de los mostos que se toman como base para la elaboración de estos vinos.

Los valores de acidez volátil de los vinos son bajos y siempre dentro de los límites legales permitidos, pudiendo ser debido a que las levaduras que intervienen en el proceso fermentativo producen pequeñas cantidades de estos ácidos, y en todo caso a que se han llevado las fermentaciones de una forma correcta, evitándose alteraciones microbianas indeseables que pudieran dar origen a valores elevados.

Los contenidos de anhídrido sulfuroso total en todas las muestras son bajos, y dentro de los lí-

TABLA 2
ANALISIS QUIMICOS DE LAS MUESTRAS

Bodega*	Muestra[a]	pH	Acidez total meq/l	Azúcar g/l	Alcohol % Vol.	SO_2 libre mg/l	SO_2 total mg/l	Acidez volátil meq/l
A	MT	3,66	78,76	238,78	—	28,3	105,0	—
	MFT	3,61	77,33	15,30	12,8	1,6	31,2	8,33
	FFT	3,60	76,00	2,32	13,3	1,6	53,6	5,00
B	MT	3,40	88,93	204,10	—	32,1	121,7	—
	MT	3,55	82,66	227,28	—	26,3	107,0	—
	MR[b]	3,55	82,66	227,28	—	26,3	107,0	—
	MFT	3,47	80,93	12,70	12,5	3,2	72,1	6,66
	MFT	3,39	80,93	13,10	12,4	3,2	59,1	8,33
	MFR	3,65	81,33	10,40	11,9	3,2	60,9	5,00
	FFT	3,69	74,66	2,06	13,2	1,6	53,1	5,00
	FFT	3,55	68,93	2,75	13,0	1,6	39,8	5,00
	FFR	3,50	70,00	2,86	12,5	1,6	47,2	3,33
C	MB	3,50	82,66	210,00	—	31,9	95,0	—
	MFB	3,55	79,33	18,30	11,2	3,2	82,9	8,33
	FFB	3,50	80,00	2,20	12,3	1,6	47,2	3,33
D	MB	3,43	86,00	210,33	—	28,9	85,3	—
	MT	3,52	77,83	226,70	—	33,1	90,4	—
	MFB	3,57	60,93	7,40	11,0	3,2	57,8	5,00
	MFT	3,53	54,93	11,50	11,6	1,6	35,6	5,00
	FFB	3,45	60,93	2,50	11,5	1,6	63,8	1,66
	FFT	3,54	58,93	2,41	12,1	1,6	53,4	1,66
E	MT	3,70	70,00	268,00	—	42,9	147,6	—
	MR[b]	3,70	70,00	268,00	—	42,9	147,6	—
	MFT	3,63	68,66	15,70	14,3	3,2	77,3	8,33
	MFR	3,67	84,13	11,20	13,7	3,2	92,1	6,66
	FFT	3,60	76,93	3,10	15,1	1,6	83,2	5,00
	FFR	3,52	68,66	2,50	14,2	1,6	72,9	5,00
F	MT	3,60	78,66	261,68	—	37,9	135,6	—
	MFT	3,50	81,33	16,50	14,3	1,6	47,1	6,66
	FFT	3,69	78,00	2,60	15,4	1,6	55,2	5,00
G	MT	3,80	70,00	273,48	—	39,8	129,8	—
	MR[b]	3,80	70,00	273,48	—	39,8	129,8	—
	MFT	3,70	71,60	16,32	13,6	1,6	51,2	10,00
	MFR	3,80	68,66	11,21	14,8	3,2	61,1	6,66
	FFT	3,75	64,00	3,30	14,9	1,6	40,8	8,33
	FFR	3,70	68,00	2,73	15,3	1,6	32,8	5,00
H	MT	3,82	74,00	279,03	—	47,1	143,1	—
	MFT	3,73	54,66	17,20	15,2	3,2	85,3	8,33
	FFT	3,80	60,26	2,60	16,3	1,6	56,5	5,00
J	MT	3,80	78,66	262,55	—	37,2	127,9	—
	MFT	3,75	64,00	10,50	14,8	3,2	45,6	8,33
	FFT	3,79	74,66	2,57	15,3	1,6	37,1	6,66
K	MT	3,71	81,46	250,85	—	29,1	110,2	—
	MR[b]	3,71	81,46	250,85	—	29,1	110,2	—
	MFT	3,69	84,26	10,42	14,2	1,6	42,1	10,00
	MFR	3,61	78,66	8,21	13,2	3,2	67,9	5,00
	FFT	3,60	88,00	2,68	14,7	1,6	40,6	6,66
	FFR	3,59	77,73	2,50	14,0	1,6	39,9	5,00
L	MT	3,73	56,26	249,70	—	37,1	125,1	—
	MR[b]	3,73	56,26	249,70	—	37,1	125,1	—
	MFT	3,57	82,00	8,42	14,0	3,2	83,1	6,66
	MFR	3,61	70,66	13,21	13,8	3,2	57,6	6,66
	FFT	3,60	76,00	2,94	14,6	1,6	45,1	5,00
	FFR	3,68	90,00	3,00	14,4	1,6	49,2	3,33

[a] M: mosto. MF: mitad fermentación. FF: final fermentación. B: blanco. R: rosado. T: tinto.

[b] MR: mosto tinto para elaboración en rosado.

* Bodega A y B: Arganda del Rey, Bodega C: Valdilecha, Bodega D: Colmenar de Oreja — Subzona de Arganda
Bodega E y F: Navalcarnero, Bodega G: El Alamo — Subzona de Navalcarnero
Bodega H: Cadalso de los Vidrios, Bodega J y K: Cenicientos, Bodega L: Villa del Prado — Subzona de San Martín de Valdeiglesias

mites permitidos por la legislación vigente. El contenido máximo admitido para vinos tintos secos es de 150 mg/l y para vinos blancos y rosados secos de 200 mg/l.

Agradecimientos

Expresamos nuestro agradecimiento al Consejo Regulador de la Denominación de Origen «Vinos de Madrid», y a las bodegas pertenecientes a la misma, por facilitarnos la toma de muestras.

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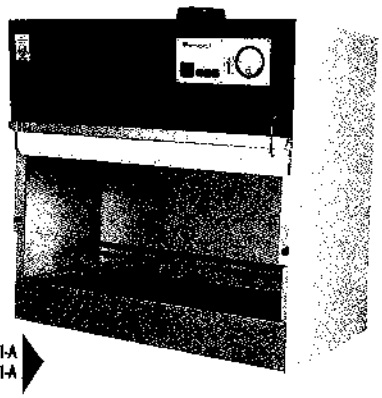
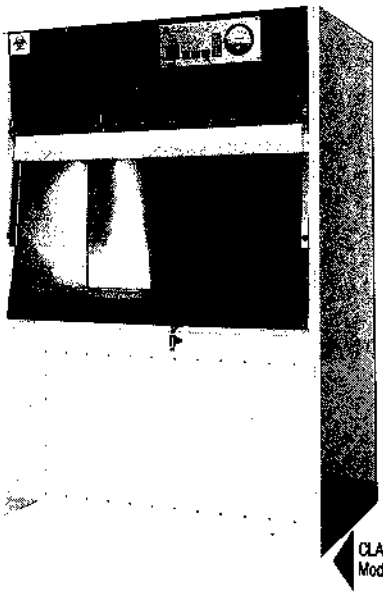
Cabinas de seguridad biológica «Biohazard»



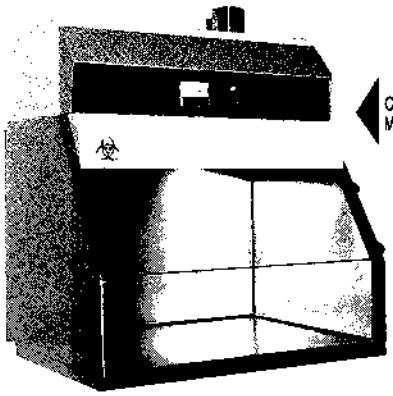
CLASE II-A
Mod. BIO-II-A

PROTECCION TOTAL DEL OPERADOR (ZONA TRABAJO EN DEPRESION), DEL AMBIENTE (FILTRO HEPA EN LA EXPULSION DEL AIRE), Y DEL PRODUCTO.

EL FACTOR DE PROTECCION DE ESTAS CABINAS, CUMPLE LAS ESPECIFICACIONES EXIGIDAS POR LA NORMA BRITISH STANDARD, BS 5726, LA N.S.F. STANDARD 49 Y LAS G.L.P. PARA CABINAS **BIOHAZARD**



CLASE II-B
Mod. BIO-II-B



CLASE I
Mod. DEP-I

INDICADAS PARA MANIPULACIONES BACTERIOLOGICAS DE MICROORGANISMOS PATOGENOS.

telstar s.a.

José Tapiolas, 120 • 08226 TERRASSA • Tel. 785 28 00 • Télex: 56101 LIOF E • Telefax: 785 93 42
Delegación: Amado Nervo, 15 • 28007 MADRID • Tel. 433 72 96 - 433 73 46 • Télex: 43542 LIOF E

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