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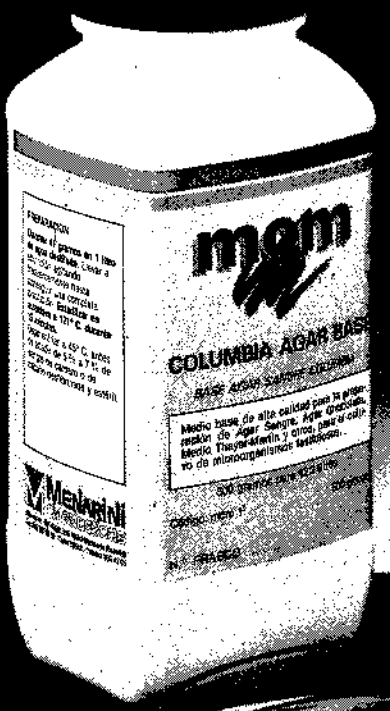

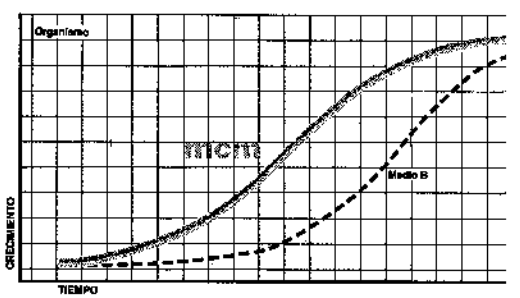

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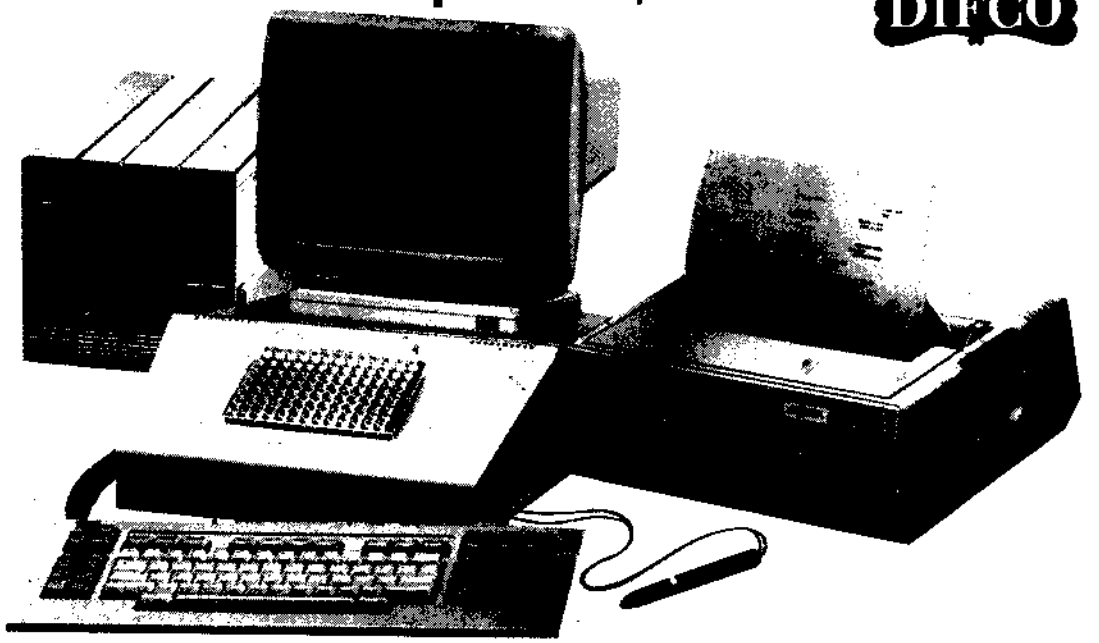

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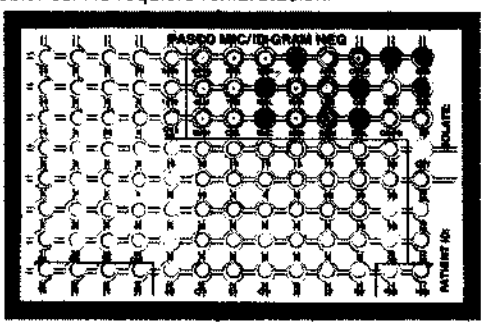

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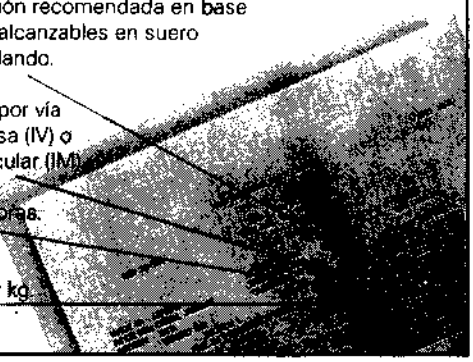

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MICROBIOLOGIA SEM 5 (1989), 69-77 **MINIREVIEW**

Transcription in vaccinia virus

Richard Kent

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(Received June 6, 1989)

Summary

Transcription of poxvirus genes is temporally regulated and takes place in the cytoplasm of the infected cell. Virus-encoded enzymes produce capped, methylated and polyadenylated mRNA. The virus transcriptional apparatus recognises promoter sequences within the virus genome which bear little resemblance to either eukaryotic or prokaryotic promoter regions. mRNA's produced late in infection possess 5'poly (A) sequences which are not encoded in the genome. The process of transcription in poxviruses is reviewed using vaccinia virus as the prototype.

Key words: Poxvirus, vaccinia, transcription.

Resumen

La transcripción de los genes de poxvirus está regulada a lo largo del ciclo de multiplicación y tiene lugar en el citoplasma de la célula infectada. Las enzimas codificadas por el virus producen m-RNA poliadenilados, metilados y con «cap». El aparato transcripcional del virus reconoce las secuencias promotoras contenidas en el genoma vírico que muestran poca semejanza con las regiones promotoras eucarióticas o procarióticas. Los m-RNAs producidos en la fase tardía de infección contienen secuencias 5' poli (A) que no están codificadas en el genoma. El proceso de transcripción en poxvirus se estudia utilizando como prototipo el virus vaccinia.

Introduction

Vaccinia is the prototypical and most studied member of the orthopoxvirus genus (reviewed in 45). Other members of this genus include buffalopox, camelpox, cowpox, ectromelia and variola. All of these viruses are closely related, both genetically (37) and antigenically (23). The latter relationship has allowed both cowpox and vaccinia to be used for successful prophylactic immunisation against variola (smallpox) infection (reviewed in 25). Although the origin of vaccinia virus is unknown, it is clear from genetic analysis that it is unlikely to have arisen from simple mutation of, or recombination between, either variola or cowpox viruses (37).

Vaccinia has an ovoid virion of over 200 nanometers in length and a complex morphology. The majority of virions remain intracellular and are surrounded by a single lipoprotein bilayer. Extracellular virions, which are probably of greater importance in the *in vivo* spread of infection (51), have an additional outer lipoprotein envelope which is acquired from the Golgi membranes of the host-

cell (30). Within the inner envelope of the virion is a biconcave core which contains virus DNA and many enzymes (reviewed in 45). Among these core-associated enzymes are a DNA-dependent RNA polymerase (1) and enzymes capable of capping, methylating and polyadenylating mRNA (41, 46). Within the concavities of each *vaccinia* virion core lie two structures of unknown function called lateral bodies.

The vaccinia genome is a linear double-stranded DNA molecule of over 180 kilobase pairs whose two strands are covalently linked by single-stranded hairpin loop structures (2). The genome possesses an inverted terminal repetition of about 10 kilobase pairs within which are short, direct tandem repeats (76). The central portion of the orthopoxvirus genome is well conserved between different members of the genus; variation occurs primarily at the ends of the genome (37).

Replication of the virus genome occurs in the cytoplasm of the host-cell and is believed to involve a self-priming strand displacement mechanism by a virus-encoded DNA polymerase (22) following the introduction of nicks close to the termini of the genome.

Knowledge of the vaccinia genome and its structure has been obtained from analysis of restriction endonuclease fragments of virus DNA (20, 37), transcriptional analysis (e. g. 18) and more recently from nucleotide sequence analysis of large segments of the genome (e. g. 49, 52, 56, 57, 68, 72). These studies have revealed that *vaccinia* mRNA's are collinear with their genes which are closely packed, sometimes overlapping with each other. Transcription occurs in each direction of both strands of the genomic DNA.

Poxvirus gene expression is temporally regulated. Immediately upon infection transcription early genes are expressed. After the onset of DNA replication transcription of most early genes is suppressed and expression of the late genes begins. The control of expression of these two major classes of genes will be discussed in some detail (see below). Recently a novel class of *vaccinia* gene has been described (69) which is expressed after DNA replication has begun but before the expression of the majority of the late genes. At present little more is known about these intermediate genes: they will not be discussed further in this review.

Interest in vaccinia virus and the regulation of poxvirus gene expression has been stimulated by the recent development of recombinant vaccinia viruses which have been used to express a variety of foreign genes in eukaryotic cells (38). Such viruses can infect a wide range of mammalian cell types and foreign DNA sequences of up to 25 kilobase pairs in length can be stably integrated into non-essential regions of the vaccinia genome, such as the thymidine kinase gene, without loss of virus infectivity (62). Recombinant vaccinia viruses have a number of possible applications, they can be considered as candidate live vaccines with potential veterinary and medical applications (e. g. 7, 47, 63) and as a means of investigating the immune response (e. g. 34) or protein function (e. g. 24). However, the expression of foreign genes in cells infected with recombinant vaccinia viruses is dependent upon the presence of poxvirus transcriptional control sequences upstream of the heterologous open reading frames (39).

Methods of investigating transcription

At the same time as heightening interest in poxvirus transcription the development of vaccinia as an expression vector has also provided a powerful technique for its study. For example, putative promoter elements can be isolated, manipulated and reintroduced into the vaccinia genome upstream of a gene with an easily assayable product. The effect of any particular mutation on the activity of that promoter region can be assessed by assaying the level of the reporter gene product in cells infected with the recombinant virus. Vaccinia virus promoters which drive the expression of non-essential genes can be manipulated and reintroduced into their normal position within the genome (e. g. 19). However a similar technique can be employed for any promoter sequence. The

promoter sequence is maintained at its original position while a copy, or mutated derivative, is introduced into a non-essential region of the genome upstream of a suitable reporter gene. This method has been used to identify important regulatory sequences in both early (e. g. 74) and late (e. g. 4) vaccinia promoters. The amount and kinetics of transcription from unmutated translocated promoter sequences are similar to those observed from the same sequences at their authentic genomic position.

A more rapid technique for studying late promoter activity without the need to construct recombinant viruses is the vaccinia virus-dependent transient expression system (15). Plasmids containing a vaccinia late promoter element are transfected into vaccinia-infected cells. The transfected promoter element is recognised by the vaccinia transcriptional apparatus in the infected cell and the reporter gene is expressed. The level of expression of the reporter gene provides an estimate of promoter strength. The expression of the reporter gene is dependent upon the presence of an upstream vaccinia late promoter; neither vaccinia early promoters nor eukaryotic promoters are effective. The inactivity of vaccinia early promoters in this system is presumed to be due to the close association of the early transcriptional apparatus with the virion cores during the early stages of infection. The relative activities of late promoters in this system are consistent with those observed in cells infected with the corresponding recombinant viruses (73).

Extracts of vaccinia virus particles (28, 54) or vaccinia-infected cells (26, 53) will transcribe DNA from early vaccinia virus promoters. Such systems have provided a means of identifying some of the factors involved in transcription (13, 60) including a transcription factor which interacts with vaccinia early promoter regions in electrophoretic mobility shift assays and DNase I footprint analysis (78). An extract of vaccinia-infected cells has also been shown to be capable of transcribing DNA from late vaccinia promoters (58, 77).

Transcription

The majority of DNA-containing viruses replicate within the nucleus of the infected cells. Transcription of virus genes is frequently performed by host-cell RNA polymerase II and initiates downstream of regions within the virus genome which show sequence homology to cellular RNA polymerase II promoters (e. g. 3, 16, 71). Vaccinia virus, however, replicates exclusively within the cytoplasm of infected cells and encodes many of the polysephides required for transcription of its genes (1, 32, 64).

The vaccinia DNA-dependent RNA polymerase shares a number of features with eukaryotic class II RNA polymerases. Both are multipartite enzymes of about 500 kiloDaltons with two large subunits of over 100 kiloDaltons as well as a number of smaller components. The eukaryotic enzyme has at least eight of these smaller polypeptides which range in size from 40 to 10 kiloDaltons. Vaccinia RNA polymerase has at least seven subunits of less than 40 kiloDaltons which, like the two larger polypeptides, are encoded within the central conserved region of the vaccinia genome (32). The nucleotide sequence of the gene which encodes the 147 kiloDaltons component of the vaccinia polymerase has been determined and reveals regions of extensive homology with the largest subunits of eukaryotic RNA polymerases II and III, and slightly less homology with the prokaryotic RNA polymerase (11). Like its eukaryotic counterpart, the purified vaccinia enzyme is unable to transcribe double-stranded DNA in the absence of protein extracts of virions or virus-infected cells (26, 28, 53, 54).

Vaccinia transcripts have 5' cap structures, 7methyl-G-p-p-p-(A/G)-p-RNA, similar to those of normal eukaryotic mRNA's. Nascent transcripts are processed by an enzyme complex of over 100 kiloDaltons. The enzyme consists of at least two subunits, the largest of which has been shown to be virus encoded (43). The enzyme has a series of activities; RNA triphosphatase, guanylyltransferase

and guanine-7-methyltransferase (41, 70) and may also perform a function in the termination of early vaccinia transcription (see below).

Despite its cytoplasmic replication, vaccinia is dependent on the host-cell nucleus for productive infection; maturation of vaccinia virus is blocked in enucleated cells (31). This dependence may result from the need of vaccinia to selectively recruit components of the nucleus for successful virus maturation (reviewed in 48). One of these components appears to be the largest subunit of RNA polymerase II. This nuclear polypeptide is translocated to the cytoplasm of cells infected with rabbit poxvirus and copurifies with rabbit poxvirus RNA polymerase isolated from virions (44).

Further evidence for the involvement of cellular RNA polymerase II in the replication comes from studies involving an inhibitor of the enzyme, α-amanitin (to which the virus RNA polymerase is relatively resistant); the usual inhibition of poxvirus replication by α-amanitin can be by-passed in cells which are resistant to the effects of the drug (61). The inhibition of poxvirus replication by α-amanitin can also be by-passed by mutation of the virus. The region of the genome which encodes the gene(s) responsible for this resistance has recently been sequenced (65). However, the mechanism through which virus α-amanitin resistance is acquired, and exact nature of the role of the host-cell nucleus in vaccinia replication, have yet to be determined.

Transcription of early genes

Following virus adsorption and penetration the virus cores are uncoated and released into the cytoplasm of the host-cell. Transcription of about 100 genes, distributed throughout the virus genome, begins immediately. These genes, transcribed prior to DNA replication, are designated «early». The cores contain all the enzymes necessary to generate capped (41), methylated (70) and polyadenylated (46) transcripts of the early class of genes. Consequently, transcription of early genes is not affected by inhibitors of either protein or DNA synthesis. Virus cores are capable of transcribing these genes in cell-free systems (17). Many of the enzymes involved in this transcription, including components of the virus RNA polymerase and the mRNA capping enzyme, are virus encoded (32, 43). As might be expected for a virus which replicates within the cytoplasm, there is no evidence for splicing of vaccinia virus early transcripts and the 5' terminus of the mRNA is believed to correspond to the transcriptional start site.

Transcription of each early gene initiates at a single discrete site both *in vivo* (36) and in cell-free systems (53). Eukaryotic RNA polymerase II promoters have conserved sequence motifs upstream of the transcriptional start sites. These are the TATA or Goldberg-Hogness box and the CAAT sequence which normally occur about 30 and 70 nucleotides (respectively) upstream of the transcriptional start site. Despite the similarities between the vaccinia RNA polymerase and the eukaryotic class II polymerases, examination of the nucleotide sequences upstream of virus early transcriptional start sites reveals no homology to known eukaryotic (or prokaryotic) promoter elements. This is consistent with the observation that cell-free extracts of uninfected cells, unlike infected-cell extracts, are incapable of transcribing vaccinia DNA (53).

Like prokaryotic and eukaryotic RNA polymerase II-mediated transcription, expression of vaccinia early genes is controlled by sequences upstream of the mRNA start sites. Temporally controlled expression of foreign genes in recombinant vaccinia viruses can be obtained when the foreign open reading frame is placed just downstream of the transcriptional start site of a vaccinia virus early gene (e. g. 19). Deletion mutagenesis of such early promoter regions has demonstrated that the sequences which control transcription can be contained within a short region, less than 40 base pairs, upstream of the RNA start site (10, 14, 19, 74). Vaccinia virus genes are closely packed within the genome, therefore, in some cases, the early promoter of one gene lies within of open reading frame of another.

TABLE 1
MINIMUM SEQUENCE REQUIREMENTS FOR CORRECT TEMPORAL REGULATION OF TRANSCRIPTION FROM VACCINIA PROMOTERS

The 5' flanking sequences from several vaccinia genes have been investigated using deletion mutagenesis. The sequences shown have been shown to correctly regulate expression of foreign genes in recombinant viruses. The major RNA start sites are marked by asterisks (*) and the initiation codons for late genes are underlined (ATG).

a) *EARLY PROMOTER SEQUENCES*

Gene		Reference
TK	CGAATAAAGTGAACAATAATTAATTCTTTATT*GTC	74
7.5kDa	CGTAAAAGTAGAAAATATATTCTAATTTATT*GCA	14

b) *LATE PROMOTER SEQUENCES*

Gene		Reference
11kDA	GTTTTTTTCTATGCTATA*AATG	5
28KDa	CACAAAAAAAACTTCTCTA*AATG	75

Vaccinia early promoters contain conserved sequence motifs:

$$TAAA\text{-}N_{0\text{-}4}\text{-}TAAAAA\text{-}N_{0\text{-}2}\text{-}TAA\text{-}A/T\text{-}A/T$$

which are centred approximately 20 nucleotides upstream of the transcriptional start sites (40, 66). These motifs have been demonstrated to interact with at least one early transcription factor to allow initiation of transcription by the virus RNA polymerase. This early transcription factor is probably a heterodimer of 82 and 77 kiloDalton subunits and has been demonstrated to have a DNA-dependent ATPase activity (12, 13). Mutations which decrease the ability of an early vaccinia promoter to bind this factor decrease the transcriptional activity of that promoter region (78).

Early transcription terminates at discrete sites about 50 base pairs downstream of a signal sequence TTTTTNT, or, as it occurs within the nascent transcript, UUUUUNU (55). This termination is dependent upon another transcription factor associated with the RNA polymerase and which copurifies with, and may be identical to, the *vaccinia* mRNA capping enzyme (60).

Polyadenylation of vaccinia mRNA's, unlike that of cellular transcripts, does not appear to require any specific signal sequences as prematurely terminated mRNA's possess poly(A) tails (27).

Between two and five hours after infection with *vaccinia* virus, replication of the virus genome begins. At the same time there is a profound change in the pattern of transcription and gene expression. Most of the early virus proteins are not produced after DNA replication although *in vitro* translation of RNA from infected cells suggests that significant amounts of functional early mRNAs are still present at this time (67). Prior to DNA replication about half the *vaccinia* genome, corresponding to the early genes, is transcribed. After DNA synthesis the whole of the genome is transcribed (8). A new class of genes is expressed from these transcripts; these genes are designated «late genes». The factors controlling this switch in gene expression are largely unknown although it can be prevented by inhibitors of DNA synthesis.

Transcription of late genes

Nuclease protection experiments which map the 5'termini of late vaccinia mRNA's suggest that transcription of each late gene initiates at a discrete site which is usually just upstream of the

start of the open reading frame (e. g. 35, 56, 57). Examination of the sequences which surround these late transcriptional start sites reveals no homology to previously observed transcriptional control signals, not even those of the vaccinia virus early promoters.

Late vaccinia gene expression is regulated by short sequences preceding and including the transcriptional start site. High level expression of heterologous open reading frames in vaccinia-infected cells can be directed from short sequences which normally precede late open reading frames (4, 5, 14, 29, 42, 75). The length of these late promoter sequences can be less than 20 base pairs.

Few areas of sequence homology between different late promoter elements have been reported. The most obvious conserved sequence is the motif TAAAT which occurs at the transcriptional start site of a number of late vaccinia genes. Frequently this motif overlaps with the translational start site, resulting in the motif TAAATG, and has been proposed to play a crucial role in the initiation of late transcription (56). Support for this proposal has come from the results of mutagenesis experiments involving the deletion of, or multiple base pair substitutions within, the area of this motif. Both of these procedures abolish the promoter activity of the sequence which precedes the 11 kiloDalton polypeptide open reading frame (5, 29). In addition, *in vitro* transcription from this promoter in extracts of vaccinia-infected cells is abolished by conversion of the motif into the sequence TGAAT. The introduction of a similar mutation into the TAAAT motif of another late promoter, from a 28 kiloDalton polypeptide gene, has been reported to abolish its *in vivo* activity (77).

Those vaccinia virus early genes which continue to be expressed at late times possess upstream sequences which are characteristic of both early and late promoter elements. Prior to DNA replication, transcription occurs from the early promoter region; after DNA replication, transcription initiates from within the late promoter element (14).

One peculiar characteristic of late poxvirus transcripts, whether generated *in vivo* or *in vitro,* is the presence of 5' poly (A) sequences for up to 35 nucleotides upstream of the start of the mRNA as defined by S1 or mung bean nuclease protection experiments (6, 21, 50, 58, 59, 77). The 5' termini of the poly (A) heads generated *in vivo* are capped and methylated (59).

The mechanism by which these poly (A) heads are generated is unclear. *In vitro* experiments (58) are consistent with a model in which transcription initiates at the second residue of the TAAAT motif but in which the transcriptional complex repeatedly slips in a 3'-5' direction before progressing from the position of the motif. A similar mechanism has been proposed to account for 5'poly (A) sequences generated on *in vitro* transcripts from bacteriophage T4 late promoters (33). However, it is not yet possible to exclude the possibility that transcription is initiated from a poly (A) primer. The biological functions of the poly (A) heads are unknown.

There has been one report (6) which suggests that the 5' poly (A) sequences of some late transcripts may be preceded by longer sequences, perhaps several thousand nucleotides long, which are derived from transcription of separate regions of the vaccinia genome. However, this observation has not been confirmed by other workers and may have been an artefact resulting from infidelity of the reverse transcriptase during cloning procedures.

Another unusual feature of late poxvirus transcription is the lack of discrete termination. Transcripts arising from a late promoter may continue for several kilobases beyond the end of the first open reading frame (18). Early transcription termination signals are not recognised late in infection. This phenomenon accounts for the presence of large amounts of double-stranded RNA present in cells late in infection wich vaccinia viruses (9).

Lack of precise termination of transcription does not appear to be universal for late poxvirus genes. Patel & Pickup (50) report that transcripts of the 160 kiloDalton polypeptide gene of cowpox virus are a defined length. Since the gene is not close to either of the termini of the genome this finding suggests that the normal 3'terminal heterogeneity of late messages does not arise from a failure of the RNA polymerase to recognise termination signals, but rather to an absence of such signals at the 3'terminus of most late genes.

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Levaduras asociadas a procesos de fermentación espontánea en vinos de Ribeiro. Análisis del homo/heterotalismo y sistema killer de las cepas de *S. cerevisiae*

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Summary

A taxonomic analysis has been carried out concerning the different yeast species involved in spontaneous fermentations of Ribeiro wine. The results revealed the presence of 15 species belonging to 7 genera: *Saccharomyces, Torulaspora, Dekkera, Saccharomycodes, Debaryomyces, Kloeckera* and *Candida.* Within the genus *Candida* a high number of species was detected. The 28 isolated strains of *S. cerevisiae* showed with some exceptions, a remarkable sporulation ability and viability of the meiotic products. All the strains were homothallic. Among all *S. cerevisiae* strains, 6 exhibited killer K_2 activity with higher isolation percentages in intermediate fermentations than in late fermentations.

Key words: Wine, yeasts, sporulation, killer activity.

Resumen

Se ha llevado a cabo un estudio taxonómico de las poblaciones de levaduras implicadas en fermentaciones espontáneas de vinos de Ribeiro. Los resultados indicaron la presencia de 15 especies englobadas en siete géneros: *Saccharomyces, Torulaspora, Dekkera, Saccharomycodes, Debaryomyces, Kloeckera* y *Candida.* Dentro del género *Candida* se encontró un elevado número de especies. Las 28 cepas de *S. cerevisiae* aisladas mostraron, salvo excepciones, una notable capacidad de esporulación y viabilidad de los productos meióticos. En todos los casos se trató de cepas homotálicas. Se detectaron seis cepas de *S. cerevisiae* con actividad killer K_2 con porcentajes de aislamiento más elevados en fases intermedias que en fases finales de fermentación.

Introducción

La identificación y clasificación de las especies de levaduras que intervienen en la fermentación de un mosto resulta de gran utilidad en la industria vitivinícola, ya que permite conocer las posibilidades enológicas reales de un vino en función de la microflora propia de dicho mosto. El estudio de la dinámica poblacional de levaduras y su distribución durante las sucesivas etapas del proceso fermentativo puede, a su vez, contribuir a resolver los problemas que a menudo se plantea la industria de vinificación (17).

(*) A quien debe dirigirse la correspondencia.

En general puede afirmarse que la flora blastomicética implicada en las fermentaciones espontáneas del mosto está representada, en las diferentes comarcas vitivinícolas, por unas 100 especies de levaduras. Su distribución en la viña y en la bodega, así como su evolución en las fermentaciones, responde a un esquema común (16).

En Galicia existen aportaciones descriptivo-taxonómicas importantes en el campo de las levaduras con interés enológico y que cubren diferentes comarcas vitivinícolas [Longo, E., Agrelo, D. y Villa, T. G. (1987). XI Congr. Nac. Microbiol., pp. 866, 868; 9, 15].

En levaduras industriales en general y en vínicas en particular la mayoría de las cepas de *S. cerevisiae* responsables del proceso fermentativo son homotálicas (21), siendo este carácter bien conocido desde el punto de vista genético (8). Su presencia complica el análisis y estudio de características susceptibles de manipulación genética.

Por otra parte, el fenotipo killer se encuentra ampliamente distribuido en levaduras, incluyendo las especies del género *Saccharomyces*. En *S. cerevisiae* se han descrito diversos tipos de carácter killer, destacando el K_1 y el K_2. El K_1 fue el originariamente descrito (12) y se encuentra principalmente en cepas de laboratorio, mientras que el K_2 se describió en cepas vínicas (13). Las cepas portadoras del fenotipo killer se caracterizan por secretar una toxina de naturaleza glicoproteica que mata a las cepas sensibles pero no a sí mismas y está asociado a la presencia en el citoplasma de ds-RNA (4).

En el presente trabajo se describe el aislamiento e identificación de levaduras vínicas asociadas a fermentaciones espontáneas de vinos de Ribeiro, así como la sucesión de poblaciones que se establece durante las diferentes fases del proceso de fermentación. Además se tuvo también en cuenta el carácter killer, homo/heterotalismo, porcentaje de ascosporulación y viabilidad de los productos meióticos, en base a la realización de una caracterización de las cepas de *S. cerevisiae* implicadas en las fermentaciones que permitiese la selección de aquellas con mayor potencialidad de manipulación genética.

Materiales y métodos

Método de muestreo

El muestreo se llevó a cabo en dos bodegas representativas de la comarca objeto de estudio en cuanto a las variedades de uva empleadas y a la metodología de vinificación. Las cubas 1, 2 y 3 corresponden a vino blanco, mientras que la 4 es de vinificación en tinto. Los depósitos de fermentación fueron en todos los casos de cemento recubiertos con resina Epoxi, con volúmenes de 25.000 l por depósito. La uva de vinificación fue Jerez en un 95 % en cuanto a los vinos blancos y 100 % de Alicante en los tintos. El mosto entró en las cubas con una densidad media de 1.075 g/l y pH 3,2; fue sulfitado en las cubas 1 y 2 con 80 mg/l de metabisulfito potásico y con 200 mg/l en las cubas 3 y 4, siendo la temperatura media de las bodegas de 19° C.

Durante los sucesivos períodos de fermentación se recogieron asépticamente muestras de 100 ml a una altura media de la cuba de fermentación correspondiendo a mosto sulfitado, mosto en fermentación, mosto-vino y vino, transportándose rápidamente al laboratorio en recipientes refrigerados a una temperatura de 4° C.

Aislamiento de levaduras

Las muestras fueron diluidas o concentradas en función de la densidad celular. En el primer caso se efectuaron diluciones seriadas en agua destilada estéril (10^{-1} a 10^{-6}), mientras que en el segun-

do se procedió a la filtración a través de membranas de 0,22 µm que fueron incubadas sobre placas Petri con medio YPD-agar (extracto de levadura, Difco, 1 %; peptona, Difco, 2 %; glucosa, Difco, 2 %; agar, Difco, 2 %) ajustado a pH de 5,7.

A partir de la dilución más conveniente en cada muestra, el aislamiento de levaduras se efectuó inoculando por extensión alícuotas de 0,1 ml sobre placas de Petri con medio YPD-agar. Después de 3 a 7 días de incubación a 25° C, la presencia de colonias de diferente morfología se hizo evidente, por lo que se procedió a su aislamiento en placas con medio YM-agar (extracto de malta, Difco, 0,3 %; extracto de levadura, 0,3 %; peptona, 0,5 %; glucosa, 1 %; agar, 2 %) ajustado a pH 5,7. Los cultivos puros así obtenidos se conservaron en tubo inclinado sobre medio de conservación YPD a 4° C.

Análisis taxonómico

La identificación de las cepas aisladas se llevó a cabo según los criterios de Barnett y col. (3).

Morfología celular. Se realizó sembrando los cultivos en medio YM-agar y observando las características morfológicas en el microscopio de contraste de fases a los 3 ó 5 días de crecimiento a 25° C. Estas pruebas se completaron con la observación de la morfología de las colonias, así como su textura y pigmentación.

Pruebas bioquímicas: Fermentación de glucosa; asimilación de nitrato y nitrito potásicos; asimilación de compuestos carbonados (pentosas: L-arabinosa, D-arabinosa, ribosa, xilosa; hexosas: glucosa, galactosa, sorbosa; disacáridos: sacarosa, maltosa, celobiosa, trehalosa, lactosa, melibiosa; trisacáridos: rafinosa, melezitosa: polisacáridos: almidón, inulina; alcoholes: glucitol, eritritol, manitol, mio-inositol; ácidos orgánicos: succínico, cítrico y láctico). Los diferentes medios de cultivo para la asimilación de los compuestos carbonados contenían 0,67 % de YNB (Difco-yeast nitrogen base), agar al 2 % (Difco) y la fuente de carbono correspondiente (Merck) al 1 %. Para la asimilación de nitrato y nitrito potásicos, el medio contenía 1,17 % YCB (Difco-yeast carbon base), agar al 2 % (Difco) y 1 % de la fuente de nitrógeno (Merck).

Pruebas fisiológicas: Resistencia a la cicloheximida (Sigma: entre 0,01 y 0,1 %); resistencia a altas presiones osmóticas (YNB-glucosa al 50 y 60 %); resistencia a la temperatura (YNB-glucosa incubado a 37° C); reproducción asexual (simétrica o asimétrica); caracteres de reproducción sexual (porcentaje de ascosporulación y número de esporas por asca. Las cepas se sembraron en agar-acetato (11) y agar-Gorodkova (10) a 30° C, observando en el microscopio de contraste de fases la presencia de ascas y ascosporas a los 3, 7, 15 días y posteriormente cada 3 días hasta los 30); la formación de seudomicelio se observó sembrando las cepas en estría sobre portaobjetos con medio patata-glucosa-agar (10), colocados sobre un tubo en U dentro de una placa de Petri con un papel de filtro impregnado con agua estéril.

Caracteres de crecimiento en mosto: Se realizaron en tubos Durham y con mosto natural estéril de Ribeiro. Los inóculos se adicionaron a una concentración final de 10^6 cel/ml y se estudió la capacidad fermentativa, formación de velo, anillo y sedimento.

Las cepas se sembraron con un replicador automáico en grupos de 21. La lectura de los resultados se llevó a cabo después de 3, 7 y 15 días de incubación a 25° C, anotando la respuesta a cada ensayo en función de la ausencia o presencia de crecimiento, comparando éste con el obtenido en medio con 0,67 % YNB (Difco-yeast nitrogen base) y 2 % agar (Difco) utilizado como control (3).

Para confirmar la exactitud del método de identificación empleado y asegurar así la veracidad de los resultados obtenidos se incluyeron cepas patrón de diferentes especies: *Saccharomyces cerevisiae* CECT 1170, *Kluyveromyces marxianus* CECT 1121, *Candida utilis* CECT 1061, *Hansenula anomala* CECT 1107, *Pichia carsoni* CECT 1129, *Kloeckera apiculata* CECT 1120, y *Saccharomycodes ludwigii* CECT 1382.

Disección de ascas y determinación de homo/heterotalismo

Las cepas esporuladas de *S. cerevisiae* fueron tratadas con una solución de zimoliasa-5000 (Seikagaku Kogyo Co., Ltd. Japón) durante 1 ó 2 horas a 30° C y las ascosporas se aislaron por micromanipulación (7). En todos los casos se disecaron un mínimo de 6 ascas por cepa, determinando posteriormente la viabilidad de los productos meióticos que a su vez se conjugaron con cepas a y α heterotálicas de *S. cerevisiae*, observándose la formación de zigotos (20). Al mismo tiempo, en clones derivados de ascosporas aisladas por micromanipulación se investigó su capacidad ascosporogénica.

Determinación del carácter killer

Las cepas a ensayar fueron sembradas sobre césped de la cepa sensible de *S. cerevisiae* 5 × 47 (a/α, his1/+, trp1/+, ura3/+, K^-) sembrada en medio YPD suplementado con azul de metileno (30 mg por cada 100 ml de medio) y tamponado a pH 4,5 con tampón citrato-fosfato 0,2M (12). Las cepas portadoras del fenotipo killer se manifestaron por la producción de un halo de inhibición en el césped de la cepa sensible. Para determinar si el carácter killer observado en las cepas vínicas era de tipo K_1 o K_2, se sembraron a su vez sobre céspedes de cepas de referencia: 1385 (α, lys1, ura1, K_2^+) y T158c (α his 4c-864, K_1^{++}), teniendo en cuenta que las cepas portadoras del sistema killer K_1 inhiben el crecimiento de las K_2 y viceversa (22).

Resultados

Muestreo

En total se tomaron 15 muestras correspondientes a mosto sulfitado, mosto en fermentación y vino joven, de las cuales se aislaron 93 cultivos de levaduras, 82 en las cubas 1, 2 y 3 (vino blanco) y 11 en la cuba 4 de vino tinto. Resultó una media de 6,2 aislamientos por muestra. Los porcentajes de aislamiento en cada una de las fases de muestreo se muestran en la Tabla 1.

Identificación de levaduras

Los resultados derivados del análisis taxonómico de las cepas de levaduras aisladas a lo largo de la vinificación para las cuatro cubas, se encuentran expuestos en la Tabla 2. Se observa la presencia de 145 especies correspondientes a 7 géneros, 5 de los cuales son esporulados y 2 no esporulados *(Candida y Kloeckera)*. Asimismo se puede verificar la evolución de cada una de las especies aisladas a lo largo de las fases de fermentación, representado en porcentaje de aislamiento respecto al total de cepas aisladas de la misma especie. Los valores X representan los valores promedio de los tres tanques muestreados de vino blanco.

Dentro del grupo de levaduras no esporógenas, con metabolismo predominantemente oxidativo, destaca la presencia de 9 especies del género *Candida (C. apicola, C. diversa, C. glabrata, C guilliermondii, C. lambica, C. pulcherrima, C. stellata, C. solani* y *C. valida)*. La mayoría de las especies se aislaron en P. I. (período de inducción), mientras que en F.T. (fermentación tumultuosa) se aislaron *C. apicola, C. glabrata, C. pulcherrima* y *C. valida*. Finalmente, en F.L. (fermentación lenta) se recuperaron *C. stellata, C. glabrata, C. pulcherrima, C. valida* y *C. guilliermondii. Dekkera intermedia* únicamente se aisló en una cuba de blanco en P.I. y F.T.; *Saccharomycodes ludwigii* se

TABLA 1
NUMERO DE CEPAS DE LEVADURAS Y PORCENTAJE DE AISLAMIENTO EN CADA FASE DE MUESTREO DURANTE LA VINIFICACION EN LAS CUATRO CUBAS DE FERMENTACION

Cuba	Fase de muestreo *						Total
	1.ª	2.ª	3.ª	4.ª	5.ª	6.ª	
1	9 (33,3)	5 (18,5)	4 (14,8)	—	9 (33,3)	—	27
2	8 (29,6)	—	8 (29,6)	6 (22,2)	5 (18,5)	—	27
3	5 (17,9)	—	8 (28,5)	—	7 (25)	8 (28,5)	28
4	3 (27,3)	—	5 (45,4)	—	3 (27,3)	—	11

* Período de inducción (1.ª y 2.ª fases), fermentación tumultuosa (3.ª y 4.ª fases), fermentación lenta (5.ª y 6.ª fases).

encontró en P.I. del tanque 3 para luego desaparecer. *Kloeckera apiculata* se recuperó en P.I. de todos los tanques excepto en el 3. *Torulaspora delbrueckii* presentó un aislamiento elevado en las tres cubas de blanco y en todas las fases excepto en P.I. del tanque 2 (en el tanque 4 se aisló sólo en F.T.). Por último, *S. cerevisiae*, la especie fermentiva más importante responsable del proceso de vinificación se aisló en la cuatro cubas estudiadas y en todas las fases, a excepción de P.I. de las cubas 3 y 4.

En cuanto a los resultados medios en las tres cubas de blanco, *Candida,* arrojó un porcentaje medio de aislamiento variable según la especie de que se tratara. Así, destacan *C. glabrata* y *C. valida:* la primera presentó un mayor porcentaje de aislamiento en F.L., mientras que en P.I. y en F.T. los valores fueron idénticos; la segunda mostró un porcentaje de aparición mayor en P.I., para disminuir en F.T. y volver a aumentar al final de la fermentación. *K. apiculata* y *S. ludwigii* solamente se aislaron en P.I., mientras que *Debaryomyces hansenii* sólo lo fue en F.L. *D. intermedia* apareció tanto en P.I. como F.T. *T. delbrueckii* se aisló en porcentaje idéntico en F.T. y F.L., siendo los valores algo inferiores en P.I. Por último, *S. cerevisiae* mantuvo un progresivo aumento a lo largo de la fermentación, pasando de un 16 % en P.I. a un 36 % en F.T., para terminar en un 48 % en F.L. En la cuba de tinto ha sido, sin embargo, más elevado el porcentaje de aislamiento en F.T. (66 %) que en F.L. (33 %). Es importante destacar que un 60 % de las cepas aisladas de esta especie presentaron crecimiento positivo en medio con 50 % de D-glucosa.

Características de esporulación y homotalismo en las cepas de S. cerevisiae

En todas las cepas de *S. cerevisiae* vínicas aisladas a lo largo de las sucesivas fases de fermentación (28 cepas) se estudió su capacidad de esporulación, así como la viabilidad de las esporas; finalmente se evaluó si se trataba de cepas homo- o heterotálicas (Tabla 3). La mayoría de las cepas mostraron una capacidad de esporulación igual o superior al 50 %, llegando en algunos casos a valores máximos, de 80 a 85 % (SMR1, SMR6, SMR19 y SMR21); también existen cepas con muy bajo índice de esporulación (1-20 %), como son SMR3, SMR13, SMR16, SMR25 y SMR27.

Un dato complementario al del porcentaje de esporulación lo constituye el número de ascosporas más frecuente que se encuentra en los cultivos esporulados. En la mayoría de las cepas existe un predominio de ascas con dos y cuatro ascosporas; sin embargo, se detectaron algunas cepas que presentaban esporulaciones atípicas, predominantemente con ascas de dos (SMR8, 25 y 27) y tres ascosporas (SMR13).

TABLA 2
FRECUENCIAS DE AISLAMIENTO DE LEVADURAS EN DIFERENTES CUBAS Y FASES DE FERMENTACION

Especies	Fase de muestreo *	Cuba 1	Cuba 2	Cuba 3	X **	Cuba 4
Saccharomyces cerevisiae	PI	9,1	42,4	—	16,0	—
	FT	27,3	28,8	57,1	36,0	66,7
	FL	63,6	28,8	42,9	48,0	33,3
Torulaspora delbrueckii	PI	33,3	—	33,3	25	—
	FT	33,3	50	33,3	37,5	100
	FL	33,3	50	33,3	37,5	—
Debaryomyces hansenii	PI	—	—	—	—	—
	FT	—	—	—	—	—
	FL	100	—	—	100	—
Kloeckera apiculata	PI	100	100	—	100	100
	FT	—	—	—	—	—
	FL	—	—	—	—	—
Saccharomycodes ludwigii	PI	—	—	100	100	—
	FT	—	—	—	—	—
	FL	—	—	—	—	—
Dekkera intermedia	PI	—	50	—	50	—
	FT	—	50	—	50	—
	FL	—	—	—	—	—
Candida apicola	PI	100	—	—	50	—
	FT	—	100	—	50	—
	FL	—	—	—	—	—
Candida diversa	PI	—	—	100	100	—
	FT	—	—	—	—	—
	FL	—	—	—	—	—
Candida glabrata	PI	100	—	—	25	50
	FT	—	50	—	25	50
	FL	—	50	100	50	—
Candida guilliermondii	PI	—	—	50	50	—
	FT	—	—	—	—	—
	FL	—	—	50	50	—
Candida lambica	PI	—	100	—	100	100
	FT	—	—	—	—	—
	FL	—	—	—	—	—
Candida pulcherrima	PI	100	100	—	100	—
	FT	—	—	—	—	50
	FL	—	—	—	—	50
Candida solani	PI	100	—	—	100	—
	FT	—	—	—	—	—
	FL	—	—	—	—	—
Candida stellata	PI	—	—	—	—	—
	FT	—	—	—	—	—
	FL	—	—	100	100	—
Candida valida	PI	100	50	33,3	57,1	—
	FT	—	—	33,3	14,3	—
	FL	—	50	33,3	28,6	100

* PI: período de inducción; FT: fermentación tumultuosa y FL: fermentación lenta.
** Valores media para las cubas 1, 2 y 3 (vino blanco); cuba 4 (tinto).

TABLA 3
CARACTERISTICAS DE ESPORULACION, HOMOTALISMO Y FENOTIPO KILLER DE LAS CEPAS VINICAS DE *S. CEREVISIAE*

Cepa/muestreo *	Esporulación ** (%)	Ascosporas por asca ***	Viabilidad Ascosporas (%)	Homotalismo	Killer ****
SMR1/PI	80	2, 4	64	+	S
SMR2/FT	65	2, 4	68	+	K_2
SMR3/FT	20	2, 4	25	+	S
SMR4/FT	35	2, 4	100	+	K_2
SMR5/FL	60	2, 4	75	+	K_2
SMR6/FL	85	4	90	+	S
SMR7/FL	30	2, 4	100	+	S
SMR8/FL	53	2	100	+	S
SMR9/FL	70	2, 4	81	+	K_2
SMR10/FL	50	2, 4	86	+	S
SMR11/FL	25	1, 2, 4	42	+	S
SMR12/PI	75	2, 4	87	+	S
SMR13/PI	1	3	—		S
SMR14/PI	50	2, 4	100	+	S
SMR15/FT	60	4	50	+	S
SMR16/FT	20	4	56	+	S
SMR17/FL	30	2, 4	82	+	S
SMR18/FL	50	4	—		S
SMR19/FT	80	2, 4	50	+	S
SMR20/FT	50	2, 4	68	+	K_2
SMR21/FT	80	2, 4	55	+	K_2
SMR22/FT	55	2, 3	69	+	S
SMR23/FL	50	2, 4	96	+	S
SMR24/FL	50	4	75	+	S
SMR25/FL	15	2	—		S
SMR26/FT	75	2, 4	93	+	S
SMR27/FT	10	2	—		S
SMR28/FL	58	4	100	+	S

* PI: período de inducción; FT: fermentación tumultuosa, y FL: fermentación lenta.
** Sobre 200 células en cámara de Neubauer.
*** Número de esporas más abundante por asca.
**** K_2: fenotipo killer del tipo 2; S: fenotipo sensible.

Todos los cultivos de *S. cerevisiae* que presentaron índices de esporulación adecuados, así como ascas con los cuatro productos meióticos, fueron micromanipulados para estudiar las características de viabilidad de las ascosporas. El porcentaje de viabilidad se efectuó en todos los casos sobre un mínimo de 24 ascosporas (6 tétradas) por cultivo. Los resultados obtenidos muestran valores de viabilidad altos para la mayoría de las cepas, siendo en todos los casos mayores o iguales al 50 %, salvo para las cepas SMR3 y SMR11, con valores del 25 y 42 %, respectivamente. Cinco cepas presentaron un 100 % de viabilidad de los productos meióticos: SMR4, 7, 8, 14 y 28.

Los cultivos de las esporas obtenidas por micromanipulación para cada cepa se conjugaron con cepas heterotálicas a y α, no observándose en ningún caso la formación de zigotos. Por otra parte, los cultivos procedentes de las esporas se sembraron en medio de esporulación, verificándose en la práctica totalidad de los casos la formación de ascas. Estos nuevos cultivos esporulados fueron mi-

cromanipulados de nuevo, y los cultivos obtenidos inducidos a esporular, lo que ocurrió en la práctica totalidad de los casos.

Fenotipo killer en las cepas de S. cerevisiae

En las cepas de *S. cerevisiae* se realizó un test de actividad killer cuyos resultados se expresan en la Tabla 3. En aquellas que no presentaron dicha actividad se estudió si se trataba de cepas sensibles o resistentes al carácter killer. Para ello se sembraron en lugar de la cepa sensible de colección, y sobre ellas se inocularon cepas killer K_1 y K_2. En todos los casos se verificó la aparición de halos de inhibición de crecimiento, por lo que se trató de cepas sensibles a la toxina killer. La cepas que mostraron actividad killer fueron probadas para determinar qué tipo de carácter killer poseían. Los resultados demostraron que se trataba de cepas killer K_2. En cuanto a la distribución del fenotipo killer a lo largo de la fermentación (Tabla 4), se observa que se aislaron en total 6 cepas killer K_2 (SMR2, 4, 5, 9, 20 y 21), que representan un 21 % del total de *S. cerevisiae* aislados; de estas 6 cepas no se aisló ninguna correspondiente al período de inducción, mientras que 4 corresponden a la fase de fermentación tumultuosa y 2 a la fase lenta de fermentación. Por tanto, el porcentaje de aislamiento es significativamente más elevado en F.T. que en F.L. (un 36 % en la primera por un 15 % en la segunda).

TABLA 4
DISTRIBUCION DEL FENOTIPO KILLER DURANTE LA FERMENTACION

Muestreo	N.°de cepas de *S. cerevisiae*	N.° de cepas killer (%)
PI	4	0 (0)
FT	11	4 (36)
FL	13	2 (15)
TOTAL	28	6 (51)

Discusión

La población de carácter fermentativo elevado está representada únicamente por *S. cerevisiae.* Dentro de esta especie se han detectado hasta 10 grupos, que pueden diferenciarse en base a la asimilación de ciertos compuestos carbonados (L-lactato, melezitosa y succínico) y otras pruebas como crecimiento a 37° C y en presencia de glucosa al 50 %, siendo para este último carácter (importante en los procesos de fermentación), un porcentaje elevado de cepas las que muestran un resultado positivo. Otros géneros, como *Torulaspora, Debaryomyces* o *Dekkera,* presentaron un poder alcohológeno y fermentativo débil (16). Esta distribución de especies parece estar relacionada con una cierta lentitud de estas fermentaciones, ya observada por otros autores (9). *K. apiculata* aparece únicamente en fases primarias de fermentación, como cabía esperar (18), desapareciendo en fases posteriores debido a su baja resistencia al etanol. Destaca la considerable proporción de levaduras del género *Candida* que presentan un carácter predominantemente oxidativo y productor de ácido acético (1). Su presencia puede deberse por una parte a una escasa higiene en bodega, pues estas especies son aisladas fácilmente entre la flora de bodega (6, 16), y por otra al mal estado de la vendimia, dado el elevado índice de pluviosidad de la cosecha.

Salvo casos aislados, la mayoría de la cepas aisladas esporularon apreciablemente, llegando en algunas a valores del 80 %. De todas formas hay que indicar que los datos de esporulación se refieren a una temperatura de incubación de 30° C, y dado que se trata de un proceso termosensible, probablemente los porcentajes observados mostrarían variaciones si la incubación se llevara a cabo a otras temperaturas. Por otra parte, las esporas de una cepa vínica poseen una viabilidad que oscila entre un 15 y 90 % (21), valores que se obtienen en el presente trabajo y que para algunas cepas llega a ser del 100 %. En cuanto a este aspecto, existen diferencias con respecto a las cepas de cervecería (2). Ello puede ser debido a la existencia en estas cepas de formas triploides, poliploides o aneuploides, que se encuentran genéticamente desbalanceadas (5).

La práctica totalidad de los cultivos de esporas derivados de las levaduras aisladas en el presente estudio fueron capaces de esporular, pero no conjugaron con las cepas a y α de colección. Este hecho sugirió que se trataba de cepas poliploides u homotálicas. Las cepas tetraploides son relativamente frecuentes en la naturaleza, pero no así las pentaploides o hexaploides. Para eliminar la posibilidad de poliploidía, los cultivos esporulados derivados de las esporas fueron sometidos a análisis de tétradas, y los clones derivados de éstas volvieron a esporular, con lo cual, de tratarse de cepas poliploides, deberían ser al menos octaploides, por lo que cabe concluir que se trata de cepas homotálicas. El hecho de que la amplia mayoría de las cepas de levaduras vínicas sean homotálicas puede significar que esta característica sirve como un mecanismo conservativo que contribuye a la estabilidad de la cepa y, por tanto, una característica deseable para ella (21).

En cuanto al fenotipo killer, aquellas cepas que resultaron killer-positivo, mostraron ser el tipo 2. Aunque la actividad killer manifiesta actividades máximas a valores de pH en torno a 4,5 (14, 19), se ha encontrado que en fermentación vínica a pH de mosto (3, 5) también se manifiesta, aunque en menor medida, la actividad killer sobre cepas sensibles (19). Cabría esperar, por tanto, que a lo largo de la fermentación se fuese mostrando una predominancia de cepas killer sobre el resto de las cepas, a menos que existiesen cepas killer resistentes (que no hemos encontrado). Sin embargo, no hemos detectado un dominio acusado en la fermentación de cepas de *S. cerevisiae* de fenotipo killer. Además, los porcentajes significativamente más elevados de aislamiento de cepas killer en fases de fermentación tumultuosa, que en fermentación lenta pueden ser explicados en base a que la producción de toxina se mantiene paralela al crecimiento durante la fase exponencial, comenzando su degradación o inactivación al iniciarse la fase estacionaria de crecimiento (Bergillos, L., 1987. Tesis Doctoral, Universidad de Oviedo). De esta forma la menor actividad de la toxina coincidiría con un aumento de poblaciones de cepas sensibles de *S. cerevisiae*.

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Ultraestructura del desarrollo intracelular del bacteriófago ØC31

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Summary

The intracelullar development of the bacteriophage ØC31 in thermally induced cultures of the lysogen *Streptomyces coelicolor* 01 changes remarkably its cell structure. At 10 min post-induction, a big number of mesosomes are shown by the cells. At 30 min post-induction, the cytoplasm contains capsids which are still empty. At the end of the latent period mature virions are shown and immediately after, cell lysis occurs through the tip of germinative tubes. In old cultures (10 h or more) no viral progeny is detected. However, when the amino acid glycine is added to the culture medium, new virions are seen, but in smaller number than in germinating cultures. These results seem to indicate that the lysis happens at the tip of the germinative tubes probably because this is an area weakened by the preferential growth that takes place on it.

Key words: Bacteriophage, ØC31, ultrastructure, Streptomyces.

Resumen

El desarrollo intracelular del fago ØC31 en cultivos de la cepa lisogénica *Streptomyces coelicolor* 01 inducidos térmicamente, provoca cambios notables en la estructura celular. Dichos cambios ya son evidentes 10 min después de la inducción, momento en el que las células muestran abundancia de mesosomas; a los 30 min son visibles en el citoplasma celular cápsidas virales vacías en su mayor parte; al final del período de latencia se observan viriones maduros; 60 min después de la inducción las células ya han empezado a lisarse, produciéndose la ruptura en el ápice de los tubos germinativos. En cultivos viejos (10 h o más), no se detecta progenie viral. Sin embargo, cuando el lisógeno crece en presencia de glicina, la cual debilita las paredes celulares, se observa la aparición de nuevos viriones, aunque en menor número que en cultivos germinantes, lo cual parece indicar que, en estos últimos, la salida de partículas fágicas se produce a través del ápice de los tubos germinativos por ser la zona más débil de la pared al producirse allí el crecimiento de forma preferencial.

Introducción

El estudio del desarrollo lítico del bacteriófago ØC31 de *Streptomyces* presenta la limitación de su baja capacidad infectiva, lo cual determina que los ciclos líticos sean asincrónicos (9). Para su-

(*) A quien se dirigirá la correspondencia.

perar este problema hemos construido un lisógeno termoinductible. Este permite la iniciación del desarrollo fágico en todas las células simultáneamente cuando se exponen a cultivos de esporas germinantes a 40° C durante 5 min (12). Los períodos de eclipse y latencia en estas condiciones son de 20 y 45 min respectivamente, habiéndose observado que únicamente se produce progenie viral cuando el cultivo es joven. No obstante, en cultivos viejos se produce la muerte celular como consecuencia de la inducción térmica. El desarrollo viral va a alterar profundamente el modelo de biosíntesis macromolecular en las células. Así, la síntesis de ARN celular se inhibe casi inmediatamente después de la inducción, existiendo datos que sugieren la modificación de las ARN polimerasas, que de este modo realizarán la transcripción preferencial del ADN viral, cuya replicación se inicia a los 10 min post-inducción (12, 13). Por otro lado se ha realizado un mapa de transcripción viral mediante la detección y localización en su genoma de las secuencias complementarias a 16 especies de ARN, de las cuales 12 son de transcripción temprana y vida media muy corta, siendo solamente detectados hasta 10-15 min post-inducción, y los 4 restantes son de transcripción tardía. Entre estos últimos se ha detectado un ARN policistrónico de unas 13 Kb (14).

En el presente artículo presentamos resultados que muestran los cambios morfológicos que sufren las células de la cepa lisogénica tras la inducción del ciclo de desarrollo del fago ØC31, así como datos que permiten explicar la ausencia de progenie viral exocelular en cultivos viejos.

Materiales y métodos

Organismos y métodos microbiológicos

El estudio del desarrollo intracelular del fago ØC31 se llevó a cabo en cultivos inducidos de *Streptomyces coelicolor* 01, cepa lisogénica para el mutante termoinducible ØC31 cts (10), en medio GAE fortificado (12). La enumeración de la progenie viral se realizó en medio agar común suplementado con $MgSO_4$ 10 mM y Ca $(NO_3)_2$ 8 mM, utilizando como cepa indicadora *Streptomyces lividans* 66, cuyas esporas se incorporaron en la capa superior de agar semisólido. Las condiciones de esporulación, obtención de esporas, almacenamiento, etc., han sido publicadas previamente (12).

Microscopía electrónica

Las muestras fueron tratadas según se ha descrito (6, 7), con la única excepción de que los cortes ultrafinos se contrastaron mediante una solución de acetato de uranilo al 2 % en etanol al 50 %, durante 5 min, lavándose a continuación con agua destilada. Seguidamente se realizó un tratamiento con citrato de plomo en las condiciones descritas con anterioridad (11). La observación se realizó en un microscopio Zeiss EM 109 a 50 Kv, y se utilizó película Kodak grano fino 5 302 de 35 mm para la obtención de microfotografías.

Resultados y discusión

A las 7 horas de incubación a 28° C y en agitación en medio GAE fortificado, los cultivos iniciados con esporas durmientes de *S. coelicolor* 01 están compuestos por hifas de 5-8 µm de largo, en uno de cuyos extremos es visible aún la espora a partir de la cual se originaron.

Su estructura es la característica de cultivos de actinomicetos creciendo activamente (6, 8). Así, es observable una pared de apariencia uniforme que rodea la membrana plasmática trilamelar, y un citoplasma en el que los ribosomas son abundantes y el ADN aparece muy laxo, indicando que existe una transcripción muy activa (Fig. 1 A). La inducción térmica de los cultivos provoca cam-

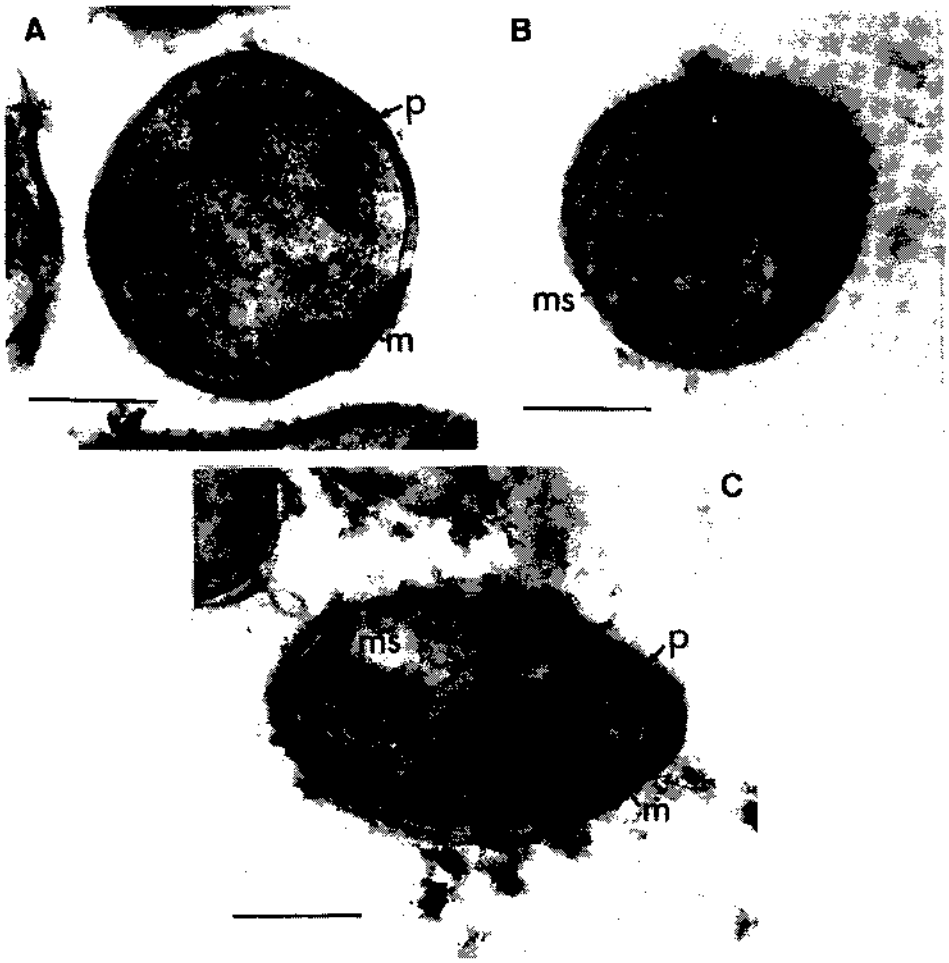

Fig. 1. Ultraestructura del desarrollo intracelular del fago ØC31: A) Sección transversal de célula de un cultivo de *Streptomyces coelicolor* 01 no inducido, a las 7 h de germinación. p: pared celular; m: membrana celular. B) Sección transversal de célula de un cultivo de *S. coelicolor* 01 10 min después de la inducción. La célula muestra formaciones membranosas de tipo mesosómico (ms) muy desarrolladas. C) Sección transversal de célula de *S. coelicolor* 01 30 min después de la inducción. Se distingue perfectamente la pared (p) y la membrana (m) celulares, y un mesosoma (ms) muy desarrollado. En este momento se hacen visibles las cabezas fágicas (c) vacías, poco densas a los electrones. La barra equivale a 200 nm.

bios importantes en la estructura de las células: a los 10 min post-inducción (Fig. 1 B), las células presentan multitud de mesosomas, que son también evidentes a los 30 min post-inducción (Fig. 1 C), momento en el cual ya se observan en el citoplasma cápsidas virales que en su mayoría están aún vacías, como se deduce de su baja densidad electrónica. A los 45 min post-inducción (Fig. 2 A), las cápsidas aparecen más densas a los electrones, indicando que ya se ha producido el ensamblaje de los viriones, y a los 60 min, prácticamente todo el cultivo aparece lisado (Fig. 2 B), siendo destacable el que la ruptura de las células ocurra a nivel del ápice de las hifas. El hecho de que en los organismos filamentosos el crecimiento de las hifas sea preferentemente apical, tanto en el caso de hongos (1, 4) como en *Streptomyces* (3), permite postular que dicho ápice es una zona especialmente débil de la pared celular, por lo que la lisis de las células ocurrirá preferencialmente en ese punto por efecto de la endolisina del fago.

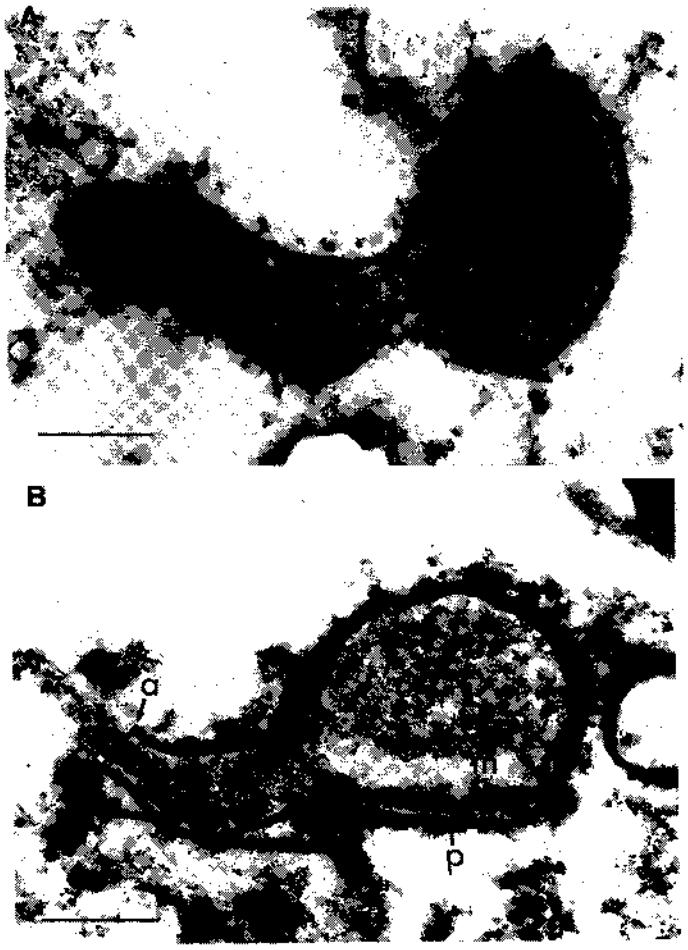

Fig. 2. Ultraestructura del desarrollo intracelular del fago ØC31. A) Sección longitudinal de un tubo germinativo de *S. coelicolor* 01 45 min después de la inducción. Aparecen cabezas fágicas (c) maduras, densas a los electrones, en las que ha penetrado del DNA fágico. B) Sección longitudinal de célula de *S. coelicolor* 01 60 min después de la inducción. En la fase de lisis, las células comienzan a lisarse por la zona apical (a) del tubo germinativo. La barra equivale a 200 nm.

Por otro lado, este último dato permite plantear la hipótesis de que sería posible que la no aparición de progenie fágica en cultivos inducidos después de 10 h de incubación, fuese debida, al menos en parte, a la incapacidad de los enzimas líticos virales para romper las paredes del micelio viejo que presenta su peptidoglicano modificado, ya que no es susceptible de digestión con lisozima (2). Para aclarar esta cuestión se incubaron esporas de *S. coelicolor* 01 en presencia de glicina al 1 % y en ausencia del aminoácido, dada la capacidad del mismo para debilitar las paredes de las células que crecen en su presencia (5). Alternativamente, los cultivos fueron sometidos a tratamiento con lisozima (10 mg/ml cf) durante 2 h a 28° C. Los cultivos se incubaron hasta alcanzar la fase estacionaria (15 h), momento en el que se sometieron a inducción térmica, incubándose entonces 2 h más para permitir la producción de progenie viral. Transcurrido este período se extrajo una alícuota para determinar la concentración de viriones, y al resto se añadió lisozima, prosiguiendo la incubación 2 h más. Los resultados (Tabla 1) indican que la progenie viral obtenida era significativamente mayor en los cultivos incubados en presencia de aminoácido, mientras que la presencia de lisozima

TABLA 1
RENDIMIENTO (1) EN PROGENIE VIRAL DE CULTIVOS DE *STREPTOMYCES COELICOLOR* 01, INCUBADOS EN PRESENCIA O AUSENCIA DE GLICINA, E INDUCIDOS TERMICAMENTE

Medio de cultivo	Momento de la inducción (h)	Rendimiento
GAEf (2)	7	500
GAEf	15	5,2
GAEf + Gly (1 %)	15	20
GAEf + lisozima (3)	15	5
GAEf + Gly (1 %) + lisozima	15	18,29

(1) Rendimiento: Relación entre el número de pfu/ml obtenido en cultivos inducidos con respecto al obtenido en cultivos controles.
(2) GAEf: Al medio fortificado se le añadieron 5 gr/l de extracto de levadura.
(3) La lisozima (10 mg/ml cf) se añadió a cultivos inducidos e incubados durante 2 h para permitir la formación de progenie viral, incubándolos durante 2 h más a 28° C.

no variaba el resultado. Además, en el microscopio de contraste de fases los cultivos crecidos en presencia de glicina e inducidos, aparecían lisados mientras que el resto no lo estaban. Sin embargo, el rendimiento en pfu/ml es menor que el obtenido en cultivos germinantes, en los que llega a ser 500 veces superior al mostrado por los cultivos controles (12) (Tabla 1). Es, pues, evidente que el estado fisiológico de las células influye en el tamaño de la progenie viral. Esto se confirma porque no aparecen cápsidas virales en secciones de células en fase estacionaria (dato no mostrado), probablemente por la escasez de las mismas en estas condiciones junto con la imposibilidad física de los viriones que se producen por inducción espontánea o térmica para salir de las células. Dicha imposibilidad se debe posiblemente a la incapacidad de las endolisinas virales para hidrolizar la pared celular de los cultivos viejos de *Streptomyces*.

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Prevalence of *Salmonella* serotypes isolated in Spain from human and non human sources (1983-1987)

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Summary

Salmonella serotypes over a five year period were studied in order to know their prevalence in Spain. The *Salmonella* Reference Centre received a total of 17,612 strains from 1983-1987. The majority (16,133) were of human origin and only 1,479 strains were isolated from non-human sources. The serotyping yielded 100 different serotypes, *Salmonella enterica* serotype Enteritidis (8) being the commonest in both groups, 61.18% of human origin and 31.91 % of non-human origin. *Salmonella enterica* serotype Typhimurium the commonest serotype in many countries, occupies second place in our results with the following percentages 11.87 % and 9.67 % respectively. Among the strains of human origin *Salmonella enterica* serotype Typhi occupies fourth place (3.24 %). This is very low compared with the high number of clinically diagnosed typhoid fever cases declared in the country: over 5,000 cases per year.

Key words: Salmonella, *serovars, prevalence.*

Resumen

Con objeto de conocer la prevalencia de los serotipos de *Salmonella* en España, se han estudiado en el Centro Nacional de Referencia de *Salmonella* las cepas recibidas durante 5 años (1983-1987). De las 17.612 cepas recibidas la mayor parte (16.133) fueron de origen humano y solamente 1.479 se aislaron de otras fuentes. El serotipado presentó 100 serotipos diferentes, siendo *Salmonella enterica,* serotipo Enteritidis (8) el más frecuente en ambos grupos, 61,18 % en las cepas de origen humano y 31,91 % en las de otros orígenes. *Salmonella enterica,* serotipo Typhimurium, que es en otras comunidades el serotipo más frecuente, en nuestros resultados ocupa el segundo lugar con un 11,87 % y 9,67 % respectivamente. En las cepas de origen humano el cuarto lugar corresponde a *Salmonella enterica,* serotipo Typhi con un porcentaje muy pequeño (3,24 %) comparado con el alto número de casos de fiebre tifoidea declarados por diagnóstico clínico en España (más de 5.000 casos por año).

Introduction

The *Salmonella* foodborne outbreaks represent a very important problem world wide, not only for its incidence in human gastroenteritis (in Spain it is the principal etiological agent of gastroente-

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ritis) (5) but also due to its importance in other economic sectors, such as the processed food industry, the tourist trade, etc. Serotyping is the most useful epidemiological marker for *Salmonella,* differentiating over 2,000 serotypes according to the Kauffmann-White Scheme (9).

The *Salmonella* laboratory of the National Centre for Microbiology of Majadahonda, Madrid, has received *Salmonella* strains for serotyping since 1979, acting as the National *Salmonella* Reference Centre for Spain since 1984. The serotyping results are published annually in the «Boletin Epidemiológico Semanal» (1) and in the «Boletin Microbiológico Semanal» (4).

In this study we examined 17,612 *Salmonella* strains submitted to the national Reference Centre from many diagnostic laboratories in Spain between 1983-1987 in order to know the serotype prevalence, its evolution throughout this period, its source of isolation and the seasonal, sex and age distribution of the isolates.

Materials and methods

Salmonella strains were isolated and identified by conventional methods in hospital and public health laboratories situated in different parts of the country, mainly from human sources (faeces, blood, etc.) and also from food (egg products, poultry, meat, etc.) environmental sources (river and sea water, waste water, etc.) and from sick animals (sheep, chickens, etc.).

On receipt at the Reference Centre, each culture was replated onto McConkey agar for a purity chek, before studying a set of biochemical characteristics, to confirm the presence of *Salmonella* (6).

The anigenic formula was established by slide agglutination and microagglutination (11) with both polyvalent and specific sera. Swarm agar plates incorporating appropriate antisera for the selection of alternative flagellar phases were used. Additional biochemical tests were set up if indicated. Strains that gave rise to diagnostic problems were sent to the World Health Organization (WHO) Collaborating Centre (Institut Pasteur. Paris) for serotyping. Serological reactions were studied using sera prepared in our laboratory according to the Guidelines for the preparation of *Salmonella* antisera (10).

Results

The National *Salmonella* Reference Centre for Spain received and confirmed a total of 17,612 *Salmonella* strains between 1983-1987. They were classified according to isolate sources into two groups:

I. Strains of human origin (16,133).
II. Strains of non-human origin (1,479).

The number of strains received for serotyping increased annually (Table 1). The isolates pertaining to serogroups B, C and D represent 93.82 % (Table 2). We found 100 different serotypes whose isolate numbers are presented in Tables 3 and 4, grouped by serogroups. Serotypes whose percentage is over 1 are represented in Tables 5 and 6. The more frecuently found serotypes in both groups are similar, but there are significant percentage differences. The particular evolution of Enteritidis, Typhimurium and Virchow serotypes is shown in Figures 1 and 2 respectively.

Group I

The seasonal distribution shows an increasing number of isolates during the summer and at the beginning of the autumn as was expected.

TABLE 1
ANNUAL DISTRIBUTION OF STRAINS RECEIVED 1983-1987

	1983	1984	1985	1986	1987	Total
Strains of human origin	2,343	2,548	3,126	3,788	4,328	16,133
Strains of non-human origin	172	84	170	396	657	1,479

TABLE 2
SEROGROUP DISTRIBUTION OF THE TOTAL NUMBER OF STRAINS RECEIVED 1983-1987

Serogroup	Number of strains	Frecuency (%)
A	2	0.01
B	2,864	16.26
C C-1	1,849	10.50
C-2	586	3.33
C-3	1	0.01
D D-1	11,222	63.72
E E-1	127	0.72
E-4	43	0.24
F	1	0.01
G	16	0.09
I	2	0.01
M	1	0.01
Autoaglutinable	441	2.50
Others	457	2.59
TOTAL	17,612	100.00

Faeces are the principal isolation source for strains (84.6 %) except for *Salmonella* ser. Typhi whose principal source is blood (58.3 %). There is no significant difference between sexes, and the age distribution shows a higher number of isolates in the group under 4 years. In the case of *Salmonella* ser. Typhi the adult group is more often implicated.

Group II

The principal strain isolation sources are food: poultry (30.5 %) and eggs (16.8 %) and of environmental origin: waste water (67.6 %). In 1987 the strains received from this group increased considerably: from 396 in 1986 to 657 in 1987.

Discussion

The annually increasing number of *Salmonella* strains serotyped by the National *Salmonella* Reference Centre for Spain, allows us to improve our knowledge regarding *Salmonella* prevalence in the country. *Salmonella* strains pertaining to serogroups B, C and D represent over 90 % of the isolates, this allows us to suggest to Clinical and Public Health Laboratories to use a small set of antisera groups to confirm biochemical identification.

TABLE 3
SEROTYPE DISTRIBUTION OF HUMAN ORIGIN STRAINS

Serogroup	Serotype	1983	1984	1985	1986	1987	Total
A	Paratyphi A	—	—	1	—	1	2
B	Agona	—	14	6	—	13	33
	Azteca	—	1	—	—	—	1
	Bochum	5	—	—	—	—	5
	Bradford	1	—	1	—	—	2
	Brandenburg	7	8	3	21	42	81
	Bredeney	23	31	52	41	26	173
	Canada	—	—	1	—	—	1
	Coeln	—	1	1	1	—	3
	Derby	—	—	1	1	—	2
	Fyris	14	8	1	—	1	24
	Hato	1	—	—	—	—	1
	Heidelberg	63	45	20	19	10	157
	Hessarek	—	—	1	1	—	2
	Java	2	—	2	4	—	8
	Lagos	1	—	1	—	—	2
	Limete	—	1	1	—	—	2
	Paratyphi B	4	26	3	1	8	42
	Remo	—	3	—	—	—	3
	Saintpaul	—	4	10	3	1	18
	Sofia (II)	3	—	2	1	11	17
	Schwarzengrund	1	—	—	—	—	1
	Thyphimurium	504	348	357	344	363	1,916
	Group B	—	—	—	17	14	31
C C-1	Bareilly	—	—	1	—	—	1
	Braenderup	—	4	4	1	—	9
	Brazzaville	1	—	—	—	—	1
	Colindale	—	—	—	1	—	1
	Galiema	1	—	3	—	1	5
	Hartford	—	1	—	2	—	3
	Infantis	94	105	52	49	39	339
	Isangi	—	—	—	1	—	1
	Livingstone	—	—	—	3	—	3
	Mbandaka	3	1	—	—	—	4
	Mikawasima	4	3	3	70	9	89
	Montevideo	15	15	12	5	4	51
	Ohio	127	78	72	65	61	403
	Oranienburg	1	1	—	—	—	2
	Postdam	1	—	—	—	—	1
	Richmond	—	—	—	1	—	1
	Thompson	11	1	—	1	—	13
	Virchow	12	81	99	159	278	629
	Group C-1	—	—	—	2	11	13
C-2	Blockley	63	53	22	40	47	225
	Bonaerensis	—	—	1	—	—	1
	Bovismorbificans	4	4	7	1	6	22
	Cremieu	—	—	1	2	1	4
	Goldcoast	2	8	14	5	13	42
	Hadar	—	10	15	14	13	52
	Litchfield	2	—	—	2	2	6
	Loanda	—	1	—	—	—	1
	Manchester	—	1	—	—	—	1
	Manhattan	3	—	—	—	—	3

TABLE 3
(CONTINUED)

Serogroup	Serotype	1983	1984	1985	1986	1987	Total
	Muenchen	4	4	—	13	25	46
	Newport	15	7	14	22	26	84
	Stourbridge	1	—	—	—	—	1
	Takoradi	—	1	2	—	—	3
	Group C-2	—	—	—	2	1	3
C-3	Hindmarsh	—	—	—	1	—	1
D D-1	Enteritidis	1,021	1,382	2,016	2,571	2,976	9,966
	Goettingen	—	—	—	2	1	3
	Kapemba	29	11	3	3	6	52
	Mendoza	1	—	—	—	—	1
	Panama	28	28	14	29	34	133
	Typhi	89	77	109	115	132	522
	Group D-1	—	—	—	13	6	19
E E-1	Amsterdam	—	—	1	—	—	1
	Anatum	2	—	1	6	11	20
	Binza	—	—	3	—	—	3
	Birmingham	—	—	—	—	1	1
	Folkensee	—	—	1	—	—	1
	Give	2	—	2	—	3	7
	London	—	—	3	4	16	23
	Meleagridis	—	—	—	1	—	1
	Newbrunswick	—	—	1	3	2	6
	Newington	—	—	—	2	—	2
	Orion	5	—	—	—	—	5
	Tournai	—	—	—	1	—	1
	Vejle	1	—	—	—	—	1
	Group E-1	—	—	—	—	3	3
E-4	Fareham	—	—	—	1	—	1
	Fulda	—	—	1	—	—	1
	Senftenberg	3	—	—	1	3	7
	Tilburg	—	—	1	3	6	10
	Westhampton	—	—	—	—	1	1
	Group E-4	—	—	—	2	—	2
G	Havana	—	—	—	—	1	1
	Ordonez	2	—	—	—	—	2
	Poona	—	—	—	1	—	1
	Putten	—	—	—	1	—	1
	Worthington	—	—	—	4	—	4
	Group G	—	—	—	—	2	2
I	Weston	—	—	—	1	—	1
	Arizonae	—	—	—	—	1	1
	Diarizonae	—	—	—	—	1	1
	Autoaglutinable	61	35	81	101	83	361
	Monophasic	95	62	—	—	—	157
	Non motil	11	7	—	—	—	18
	Sp	—	77	102	7	12	198
TOTAL		2,343	2,548	3,126	3,790	4,328	16,133

In many countries *Salmonella* ser. Typhimurium is the commonest serotype (2) (7), but our results here suggest that since 1982 the first place is occupied by *Salmonella* ser. Enteritidis (61.18 % of the human origin strains) and this percentage is increasing annually. In strains of non human origin, although *Salmonella* ser. Enteritidis is also the commonest serotype (31.91 %), its frequency is

TABLE 4
SEROTYPE DISTRIBUTION OF NON-HUMAN ORIGIN STRAINS

Serogroup	Serotype	1983	1984	1985	1986	1987	Total
B	Abortusovis	—	—	5	2	15	22
	Agona	6	1	—	4	14	25
	Brandenburg	—	—	—	9	9	18
	Bredeney	6	12	5	10	20	53
	Derby	—	—	—	2	2	4
	Fyris	1	1	—	—	—	2
	Heidelberg	4	1	1	1	4	11
	Java	—	—	—	1	2	3
	Paratyphi B	4	3	3	2	2	14
	Reading	—	—	—	—	4	4
	Saintpaul	—	—	2	2	1	5
	Salinatis	—	—	—	—	1	1
	Sofia (II)	—	—	1	4	20	25
	Typhimurium	22	7	30	31	53	143
	Group B	—	—	—	12	6	18
C C-1	Braenderup	—	—	1	1	1	3
	Infantis	8	6	9	20	9	52
	Livingstone	—	1	—	4	—	5
	Mbandaka	—	—	—	—	7	7
	Mikawasima	—	—	—	1	3	4
	Montevideo	1	2	5	4	13	25
	Ohio	15	2	8	8	29	62
	Oranienburg	—	—	—	—	3	3
	Othmarschen	—	1	—	—	—	1
	Rissen	—	—	—	2	—	2
	Thompson	—	—	—	1	1	2
	Virchow	—	1	1	34	68	104
	Group C-1	1	—	—	5	4	10
C-2	Blockley	9	1	4	6	13	33
	Bovismorbificans	3	—	—	5	—	8
	Cleveland	1	—	—	—	—	1
	Goldcoast	—	—	—	3	9	12
	Hadar	4	1	—	—	1	6
	Kottbus	1	—	—	—	—	1
	Lichtfield	—	—	—	—	10	10
	Manchester	1	—	—	—	—	1
	Manhattan	1	—	—	1	—	2
	Muenchen	—	1	—	—	5	6
	Newport	1	—	—	4	—	5
	Group C-2	1	—	—	—	5	6
D D-1	Enteritidis	24	21	71	171	185	475
	Goettingen	—	—	—	—	2	2
	Jamaica	1	—	—	—	—	1
	Kapemba	25	—	1	—	1	27
	Panama	5	—	2	2	9	18
	Group D	—	—	—	1	2	3
E E-1	Anatum	—	—	—	2	18	20
	Drypool	—	—	—	—	1	1
	Give	—	—	—	1	1	2
	London	3	—	1	1	13	18
	Meleagridis	—	—	—	2	—	2
	Newbrunswick	—	—	—	1	1	2

TABLE 4
(CONTINUED)

Serogroup	Serotype	1983	1984	1985	1986	1987	Total
	Newington	—	—	—	2	2	4
	Orion	—	—	1	—	—	1
	Group E-1	—	—	—	—	1	1
E-4	Llandof	—	—	1	—	—	1
	Senftenberg	—	—	—	2	4	6
	Tilburg	—	—	—	—	13	13
	Group E-4	—	—	—	1	—	1
F	Abaetetuba	—	—	1	—	—	1
G	Atlanta	1	—	—	—	—	1
	Havana	—	—	—	1	2	3
	Worthington	—	—	1	—	—	1
I	Welikade	—	—	—	1	—	1
M	Umbilo	—	—	—	1	—	1
	Autoaglutinable	5	3	7	24	41	80
	Monophasic	13	2	—	—	—	15
	Non motil	4	1	—	—	—	5
	Sp.	1	16	10	4	27	58
TOTAL		172	84	170	396	657	1,479

TABLE 5
MOST FREQUENTLY FOUND SEROTYPES OF HUMAN ORIGIN (1983-1987)

Serotype	Total number of strains	Frequency (%)
Enteritidis	9,966	61.18
Typhimurium	1,916	11.87
Virchow	629	3.90
Typhi	522	3.24
Ohio	403	2.50
Infantis	339	2.10
Blockley	225	1.39
Bredeney	173	1.07
Others	1,960	12.75
TOTAL	16,133	100.00

lower than among the strains of human origin. We can also appreciate a greater serotype dispersion within this group although our experience is still limited. Compared with the clinically diagnosed typhoid fever cases declared annually in Spain (3), mean 5,000, we have received very few *Salmonella* ser. Typhi strains (mean 100).

Salmonella ser. Mikawasima an uncommon serotype in Spain has suffered a considerable increase during 1986 due to an outbreak in Catalonia, that affected a large number of people. Finally we want to sign the progressive increase of *Salmonella* ser. Virchow, and the slight decrease of *Salmonella* ser. Infantis during the period studied.

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We thank Rafaela Gutiérrez for expert technical assistance.

TABLE 6
MOST FRECUENTLY FOUND SEROTYPES OF HUMAN ORIGIN (1983-1987)

Serotype	Total number of strains	Frequency (%)
Enteritidis	472	31.91
Typhimurium	143	9.67
Virchow	104	7.07
Ohio	62	4.22
Bredeney	53	3.60
Infantis	52	3.53
Blockley	33	2.24
Kapemba	27	1.83
Agona	25	1.70
Montevideo	25	1.70
Sofia (II)	25	1.70
Abortusovis	22	1.49
Anatum	20	1.38
Brandenburg	18	1.22
London	18	1.22
Panama	18	1.22
Others	362	24.30
TOTAL	1,479	100.00

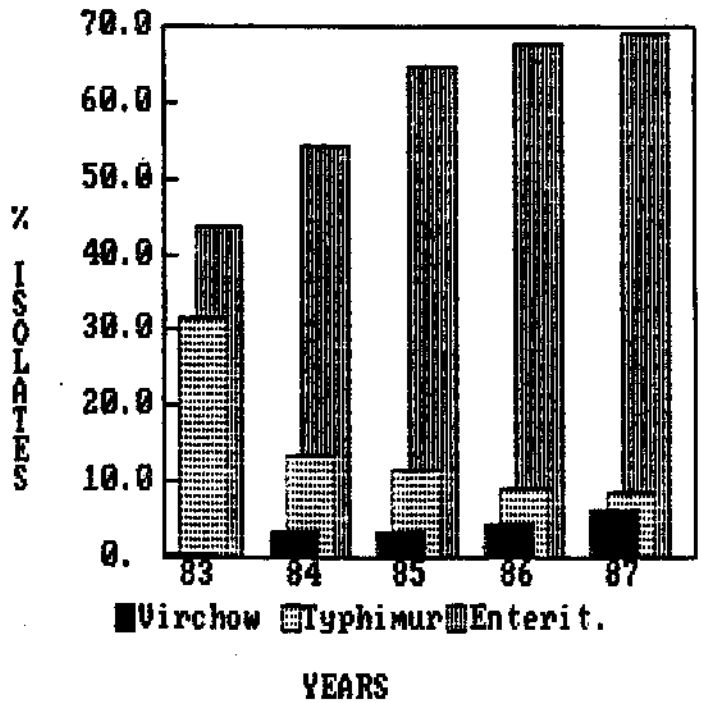

Fig. 1. Evolution of serotypes Enteritidis. Typhimurium and Virchow from human origin strains.

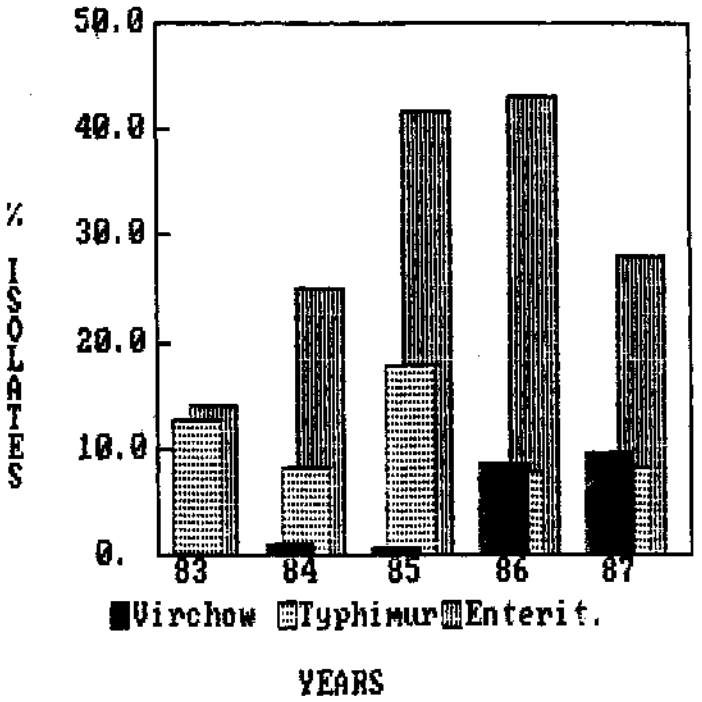

Fig. 2. Evolution of serotypes Enteritidis, Typhimurium and Virchow from non-human origin strains.

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Selected characteristics of several strains of *Lactobacillus plantarum*

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Summary

Several relevant characteristics have been studied in nine *Lactobacillus plantarum* strains isolated from Cabrales cheese. They fermented lactose efficiently and possessed both ß-galactosidase and ß-phosphogalactosidase activities, but no significant proteolytic activity was detected. Antimicrobial susceptibility tests revealed no high level resistance to any of the fourteen compounds tested. All the strains had a high content of extrachromosomal DNA of unknown function. A recombinant plasmid comprising pUC19 from *Escherichia coli* and a small cryptic plasmid from *L.plantarum* has been constructed and may be used to develop a shuttle vector for these species.

Key words: Lactobacillus plantarum, *Cabrales cheese, lactic acid bacteria.*

Resumen

Se han estudiado varias características relevantes en 9 cepas de *Lactobacillus plantarum* aisladas de queso de Cabrales. Todas ellas fermentaron eficientemente la lactosa y presentaron actividades ß-galactosidasa y ß-fosfogalactosidasa, pero no se encontró actividad proteolítica significativa. Los tests de susceptibilidad frente a 14 agentes antimicrobianos no revelaron resistencia a altos niveles de estos compuestos. Todas las cepas mostraron un alto contenido de DNA extracromosómico de función desconocida. Se ha construido un plásmido recombinante que comprende pUC19 de *Escherichia coli* y un pequeño plásmido críptico de *L. plantarum*. Este nuevo plásmido podría ser de utilidad en el desarrollo de un vector lanzadera para ambas especies.

Introduction

In recent years, there has been a growing interest in the genetics and phisiology of lactic acid bacteria that are used in a variety of fermentations of great economical importance. The research in this field may lead to the obtention of strains that would improve current processes or facilitate the development of new ones. Most studies, however, have dealt with species of genus *Lactococcus* (formerly lactic stroptococci) and the thermophylic strains of *Lactobacillus*.

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Lactobacillus species are among the most widely used lactic acid bacteria, participating in the production of food derivatives of diverse origin (meats, vegetables, milk, etc.). Due to the diversity in both strains and processes, there is an increasing trend towards the use of carefully selected strains with the most suitable characteristics for the different fermentations (3). One of the species belonging to this genus, *Lactobacillus plantarum,* is a rod-shaped bacterium frequently found in many cheese types. In Cabrales cheese, *L. plantarum* may account for up to 78 % of the total microflora at certain stages, suggesting that it may have a significant role on cheese maturation (25, 26). In the present study, we have performed the characterization of several relevant properties of nine strains of *L. plantarum* isolated from Cabrales cheese produced from raw milk, without the addition of starter bacteria. The results obtained may help to understand the role of these bacteria in food fermentations, where they often appear in significant numbers.

Materials and methods

Organisms

Eigth isolates of *L. plantarum* were obtained from Cabrales cheese at an early stage of maturation (5 d). The identification of strains was made according to Núñez and Medina (26) and Sharpe (31). *L. plantarum* INIA 169, also isolated from Cabrales cheese, was kindly provided by M. Núñez. *Escherichia coli* JM 83 (34) was donated by J. E. Suárez. Long term conservation of the strains without appreciable loss of properties was achieved by freezing concentrated suspensions of cells at –20° C in MRS medium (Oxoid) supplemented with 50 % glycerol.

Media and culture conditions

Lactobacilli were incubated as static cultures at 30° C in MRS medium, unless otherwise indicated. Lactose indicator agar (22) and milk agar medium (11) were used as test media for detection of lactose fermentation. *E. coli* cultures were prepared in LB medium .

Enzyme assays

For the determination of lactose hydrolyzing enzymes, cells were grown in lactose broth (21), harvested by centrifugation, washed twice with 0.1M phosphate buffer pH 7.0 and suspended in the same buffer. Crude extracts were prepared by mechanical disruption with glass beads (0.1 mm diameter) in a Vibrogen homogenizer, at 0° C for 2 min. After centrifugation (12,000 xg, 15 min, 4° C), the resulting supernatants were used as the enzyme source. ß-galactosidase (ß-Gal) activity was estimated according to Citti *et al.* (9) with the chromogenic substrate o-nitrophenyl-ß-D-galactopyranoside. ß-phosphogactosidase (ß-PGal) was assayed with o-nitrophenyl-ß-D-galactopyranoside-6-phosphate, following the procedure of McKay *et al.* (22). A unit of activity is defined as the amount of enzyme needed for the production of 1 µmol of o-nitrophenol per minute. The activities were normalized with the protein content of the crude extracts, measured by the method of Lowry *et al.* (18).

Proteolytic activity was determined in whole cells previously grown and inoculated in skim milk as described by Church *et al.* (7). After 24 h of incubation, the amino groups released during proteolysis were measured spectrophotometrically after derivatization with o-phthalaldehyde (8). Results were expressed as the increase in absorbance at 340 nm with respect to an uninoculated control.

Plasmid profiles

Cell lysis and extraction of plasmids were done as described by Anderson and McKay (1). Purification by CsCl-ethidium bromide gradients was also performed in some cases. For the determination of plasmid profiles in *E. coli* transformants, the procedure of Holmes and Quigley (15) was used. Electrophoresis were carried out in 0.7 % agarose gels.

Plasmid curing

L. plantarum LL2, that showed a high content of extrachromosomal DNA, was submitted to several treatments often employed to induce plasmid loss. For temperature-induced curing, cells were repeatedly subcultured at 45° C. Treatments with acriflavine, acridine orange and ethidium bromide were performed as described by Caro *et al.* (5). In every case, the cells were plated on lactose indicator agar, Elliker medium (12) supplemented with 20 µg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal), and citrate indicator medium (16).

Cloning techniques

Extraction of plasmids from *E. coli* was done by the method of Birnboim and Doly (2). Plasmids were obtained from agarose gels by electroelution (20). Enzymes employed in DNA manipulations were from Boehringer Mannheim. DNA restrictions and ligations were done according to manufacturer instructions. Transformation of *E. coli* JM83 was done as described by Hanahan (14). Transformants were selected in LB plates with ampicillin (100 µg/ml) and X-gal (20 µg/ml) (34).

Antimicrobial agent susceptibility

Disk tests and MIC determinations were done following standard procedures (17), with cells previously grown in MRS medium . The compounds tested and disk contents are listed in Table 3.

Results

All *L. plantarum* strains studied were lactose fermenters when tested on lactose indicator agar or milk agar medium. High ß-Gal activities were detected in crude extracts, together with moderate levels of ß-PGal (Table 1). In an attempt to localize the genes encoding these activities, the chromosomal and plasmid DNA of these strains was submitted to hybridization with a genetic probe containing the ß-PGal gene from *Lactococcus lactis* subsp. *lactis* MG 1299 (Mayo, B. *et al.* 1989, J. Dairy Res., in press), and with plasmid pUC19 that contains the ß-Gal gene from *E. coli*. No positive hybridization was observed in any case (data not shown).

No significant proteolytic activity was detected in any of the strains (Table 1). Values obtained were lower than 5 % of the activities determined in proteolytic strains of *L. lactis* subsp. *lactis* using the same method (Mayo, B. *et al.* 1989, J. Dairy Res., in press). Furthermore, washed suspensions of *L. plantarum* were unable to clot milk, except for strains LL441 and LL442 that produced coagulation after 5 d of incubation (Table 1). The proteolytic activity and milk clotting ability did not increase when the milk was supplemented with glucose, but the addition of a readily utilizable source of amino acids (peptone plus yeast extract) allowed coagulation of milk in two days (Table 2).

TABLE 1
LACTOSE FERMENTATION, PROTEOLYTIC ACTIVITY AND MILK CLOTTING ABILITY OF *L. PLANTARUM* STRAINS

Strain	Specific activity (mU/mg protein)		Proteolytic activity *	Milk clotting time (d)
	ß-Gal	ß-PGal		
INIA 169	703.1	53.2	0	>10
LL1	447.5	18.0	0	>10
LL2	541.7	33.7	0.006	>10
LL3	480.6	40.2	0	>10
LL7	885.7	44.7	0.008	>10
LL31	86.9	24.3	0.003	>10
LL232	744.2	40.0	0.006	>10
LL441	1,292.0	53.0	0.005	5
LL442	1,111.0	47.7	0	5

* Expressed as absorbance increase at 340 nm, as described in materials and methods.

Results on antimicrobial agent susceptibility are shown in Table 3. Except for lincomycin, the strains showed a homogeneous behaviour with the compounds tested. Disk susceptibility assays indicated low sensitivity to several agents, but the levels of resistance, as measured in MIC tests, were lower than those described for strains bearing specific resistance factors (4).

The presence of plasmid bands, ranging in size from 0.8 to 110 Mdal, was detected in all strains (Table 4). Despite the similarity in the results obtained with most of the properties studied, plasmid profiles differed between strains, except in the case of LL441 and LL442, which may represent a repeated isolation of a single strain present in the cheese.

When cultures of strain LL2 were plated after different plasmid curing treatments, and incubated in aerobiosis, colonies significantly smaller (0.1-0.5 mm in diameter instead of 1-2.5 mm of normal colonies) appeared at frequencies from 1 % to 2.5 %. These colonies did not cause a color change in the indicator media used for screening. However, under anaerobic conditions (hydrogen-carbon dioxide atmosphere), all colonies grew to a similar size and appeared to utilize lactose and citrate in the respective indicator media. Twelve of the small colonies observed in aerobiosis were isolated and their plasmid contents analyzed after cell lysis and purification in CsCl gradients. Plasmid losses were detected in eleven of these derivative strains (Table 5). When they were compared

TABLE 2
EFFECT OF SUPPLEMENTS ON PROTEOLYTIC ACTIVITY AND MILK CLOTTING ABILITY OF *L. PLANTARUM* STRAINS

Strain	Supplement		
	0.5 % Glucose		0.125 % peptone + 0.125 % Yeast extract
	Proteolysis *	Milk clotting (d)	Milk clotting (d)
LL2	0	>10	2
LL7	0.002	>10	2
LL442	0.004	5	2

* Expressed as absorbance increase at 340 nm, as described in materials and methods.

TABLE 3
ANTIMICROBIAL AGENT SUSCEPTIBILITY OF *L. PLANTARUM* STRAINS

Antimicrobial agent **	Disk tests *		MIC (μg/ml)	
	Resistant	Sensitive	Mode	Highest
Nalidixic acid (30)	9	0	32	64
Sulfadiacine (300)	9	0	100	500
Streptomycin (10)	9	0	64	64
Ampicillin (10)	0	9	2	2
Kanamycin (30)	9	0	32	32
Gentamycin (10)	9	0	32	32
Tetracycline (30)	0	9	ND	ND
Chloramphenicol (30)	0	9	4	4
Vancomycin (30)	0	9	ND	ND
Lincomycin (2)	5	4	ND	ND
Cephalotin (30)	9	0	32	32
Fosfomycin (50)	9	0	64	64
Penicillin G (10)	9	0	32	32
Rifampicin	ND	ND	4	4

* Number of resistant or sensitive strains are given. Interpretation of inhibition zones was done according to Lennette *et al.* (17).
** The numbers in brackets indicate the disk content in μg.
ND: Not determined.

TABLE 4
PLASMID PROFILES OF *L. PLANTARUM* STRAINS

Strain	Plasmids (Mdal)
INIA 169	30; 16.6; 12; 8; 6.6; 2; 1.4
LL1	34; 12; 8.6; 4.8; 2; 1.4; 0.9
LL2	110; 70; 60; 50; 44; 30; 18; 6; 1.2
LL3	30; 21; 18; 12; 4.6; 1.4; 0.9
LL7	50; 5.8; 4.6
LL31	50; 30; 18; 1.2; 0.8
LL232	50; 8; 4.8
LL441	30; 5.5; 3.7
LL442	30; 5.5; 3.7

with their parent strain (LL2) using the API 50 test, no change in the physiological properties examined was observed. Furthermore, the pattern of antimicrobial agent susceptibility, the ß-Gal and ß-PGal levels, and the production of diacetyl, remained unaltered in the cured strains.

Due to the high copy number and the stability observed in curing experiments, the 1.2 Mdal plasmid from strain LL2, designated pLL21, was selected for cloning into the *E. coli* plasmid pUC19. Both plasmids had a single *Eco* RI site and, after digestion with this enzyme, they were mixed, ligated «in vitro», and the ligation mixture used to transform competent cells of *E. coli* JM 83. After selection of the transformants, one of them contained a new plasmid with the expected size

TABLE 5
DERIVATIVE STRAINS OF *L. PLANTARUM* LL2 OBTAINED WITH CURING AGENTS

Strain	Screening medium *	Cured plasmids (Mdal)	New plasmids (Mdal)
LL21	A	44	—
LL22, LL23, LL24	A	50; 44	—
LL25	B	44	—
LL27, LL28, LL29, LL210	C	60; 50	26
LL211	C	110	—
LL212	C	60	—

* The screening media used were: A, lactose indicator agar; B, Elliker medium + X-gal; C, citrate indicator medium.

for a hybrid pUC19-pLL21 plasmid. This clone was purified and its plasmid DNA utilized for a new transformation of *E. coli* JM 83. The transformants contained the new plasmid that , after extraction and purification in CsCl gradients, was physically mapped with several restriction enzymes (Fig. 1). The 1.2 Mdal fragment inserted in pUC19 had restriction sites coinciding with pLL21, which confirmed the identity of the recombinant plasmid.

Discussion

All the *L. plantarum* strains studied here were efficient lactose fermenters and they appeared to use both the ß-Gal and ß-PGal pathways, a result previously found by Premi *et al.* (28) with one strain of this species. Unfortunately, the degree of homology between the genes coding for the galactosidase activities and the probes used for hybridization, was not enough to allow their localization. The probe used for the ß-PGal gene had been previously found to hybridize with DNA from different *Lactococcus lactis* strains that present this activity (Mayo, B. *et al.* 1989, J. Dairy Res., in

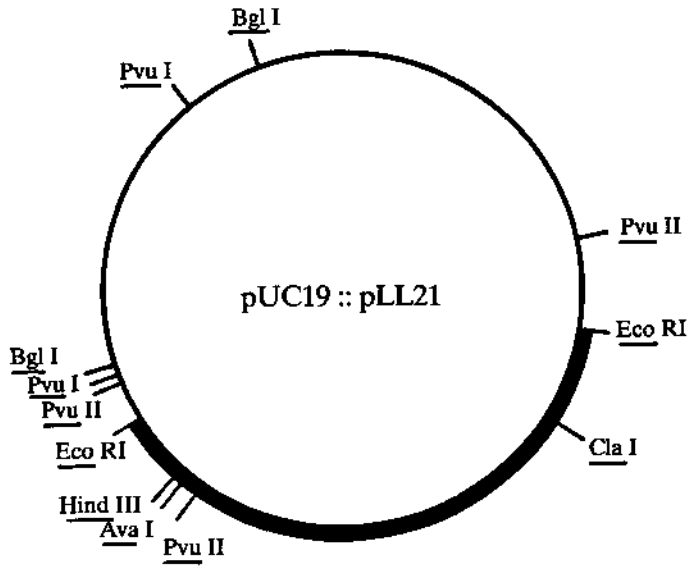

Fig. 1. Restriction endonuclease map of the hybrid plasmid comprising pUC19 (thin line) and pLL21 (thick line).

press). Therefore, a significant interspecific genetic diversity among lactic acid bacteria appears to exist, at least concerning this gene.

Since *L. plantarum* strains can catabolize lactose and acidify culture media with this sugar as the carbon source, their inability to coagulate milk seems to be due to some nutritional requirement not present in milk but provided by the supplement of peptone plus yeast extract. The existence of such a requirement might also explain the lack of significant proteolytic activity after inoculation in skim milk, simply due to the inability of the cells to grow in this medium. Alternatively, there may be a true deficiency in their proteolytic system, which would be compatible with all these results. Whatever the explanation is, *L. plantarum* is present at high numbers in Cabrales cheese (25, 26). Therefore, it seems that the metabolic activities of other members of the cheese microflora would meet *L. plantarum* requirements (amino acids or other nutrients), and this bacterium could make a contribution to cheese maturation through their lactose fermenting ability and the production of compounds involved in flavour and aroma.

Resistance to high levels of antimicrobial agents does not seem to be a common trait in lactic acid bacteria (27, 29, 30). However, the moderate levels of resistance found in *L. plantarum* against antibiotics such as cephalotin and penicillin G, may be useful for its selective isolation from mixed populations of lactic acid bacteria. Growth of both *Lactococcus* and *Leuconostoc* species was inhibited by concentrations of these antibiotics much lower than those needed to inhibit *L. plantarum* (Mayo, B. 1988, Ph. D. thesis. Universidad de Oviedo).

The presence of small cryptic plasmids in strains of *L. plantarum* has been reported by other authors (24, 32, 33). This was also observed in our isolates, but the use of the lysis method of Anderson and McKay (1) allowed the detection of plasmids with a wide range of molecular weights. In other lactic acid bacteria, mainly lactococci, many of their relevant properties are encoded by extrachromosomal DNA (23). However, our attempts to relate the presence of plasmids in *L. plantarum* with a particular characteristic have not been successful. The maintenance of such an amount of plasmid DNA must have a high metabolic cost, and therefore it is reasonable to suposse that these plasmids contain some advantageous information for growth and survival under natural conditions, but that may not be obvious in laboratory conditions. In any case, the poor growth of our cured strains under aerobic conditions suggests that some plasmids may have a direct or indirect role on oxygen tolerance.

The application of recombinant DNA technology to the majority of *Lactobacillus* species lags well behind the situation for lactococci, although recent efforts have been made to close this gap (6, 10, 13, 19, 33). In the case of *L. plantarum,* no efficient cloning vectors have been developed to the present date. The recombinant plasmid obtained in this work, providing that a suitable marker is introduced to allow selection in *L. plantarum,* constitutes a starting point for the construction of a useful shuttle vector between *E. coli* and *L. plantarum.*

Acknowledgments

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The effect of *Trichoderma viride* C-1 UV mutagenization on cellulases activity

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Summary

Trichoderma viride C-1 strain was irradiated with UV until the survival level of 0.03 % was obtained. Sixteen mutants were isolated on the basis of the visible clearance zone around the colonies on the media with cellulose and glycerol. Then they were cultivated on a rotary shaker in the liquid Saunders medium supplemented with microcrystalline cellulosé and comminuted sugar beet pulp. Exo-1,4-β-glucanase, endo-1,4-β-glucanase and β-glucosidase were assayed in the supernatants of postcultural liquids at different time intervals of culture. The same mutants were characterized by higher biosynthesis level of exoglucanase (1.2-5.0 times) endoglucanase (1.2-2.5 times) and β-glucosidase (1.5-1.7 times) when compared with the wild type strain.
Keywords: Mutants, Trichoderma, *cellulases.*

Resumen

La cepa C1 de *Trichoderma viride* fue irradiada con luz ultravioleta hasta que se obtuvo el nivel de supervivencia del 0,03.%. Se aislaron 16 mutantes que formaron un halo translúcido alrededor de las colonias en medios con celulosa y glicerol. Los mutantes fueron cultivados en un agitador orbital en medio líquido Saunders enriquecido con celulosa microcristalina y pulpa de remolacha azucarera pulverizada. Las actividades exo-1,4-β-glucanasa, endo-1,4-β-glucanasa y β-glucosidasa se determinaron en los sobrenadantes de los cultivos a diferentes intervalos de tiempo. Los mutantes se caracterizaron por un nivel más alto de biosíntesis de exoglucanasa (1,2-5,0 veces), endoglucanasa (1,2-2,5 veces) y β-glucosidasa (1,5-1,7 veces) cuando se compararon con la cepa salvaje.

Introduction

The use of enzyme preparations of high activity is one of methods for reducing the costs of industrial hydrolisis of cellulose substrates. For this reason, in recent years much attention has been drawn to mutagenization of microorganisms in order to obtain the strains of high performance (3, 4, 6, 10, 11, 13, 14, 18). Mutans with enhanced exo-1.4-β-glucanase activity contributing to degradation

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of native cellulose are of particular value. In the present study, a wild strain *Trichoderma viride* C-1 was mutagenized using UV irradiation and mutants with enhanced cellulase activity were selected.

Materials and methods

Microorganism

The wild strain *Trichoderma viride* C-1 from the Department of Biotechnology and Food Microbiology at the Agricultural Academy in Wroclaw was used for the UV induction of mutants.

Media

Five different media were prepared. The solid Sabouraud (5) medium was used for cultivation and preservation. The cells were UV irradiated on a mineral agar medium with a trace element solution II according to Theodorou (20) supplemented with 33.3 µg/ml of rose bengal. The above medium but supplemented with 1 % cellulose type Heveten 10 (DDR) instead of glucose was used for screening and selection. For the same purpose we used the above medium supplemented with 5 % glycerol and 1 % microcrystalline cellulose (Merck) (11).

The submerged cultures of the examined strains were carried out in the Saunders medium (15) containing 2 % microcrystalline cellulose (Merck), 2 % beet pulp 0.5 % yeast extract (Difco), 0.5 % wheat germs and 0.1 % glucose.

Induction and selection of mutans

Mutants were inducted by UV irradiation. Water suspension (with 0.1 % Tween 80) of 4.9×10^4 conidia free of mycelia per plate (100 plates) obtained from 7 day cultures on Sabouraud plates were irradiated with UV Hanau Quarzlitat lamp (7.1 erg/mm^2/sec.) from a distance of 20 cm for 90 seconds. The survival level was 0.03 %. The 1,400 clones which survived UV irradiation were isolated on a plate on Theodorou medium (20) supplemented with glucose. The clones were incubated at 28° C for 3 days and then replicated on the selecting media as above. The replica plates were incubated at 28° C 3 days and then at 50° C for 24 h in order to intensify the clearing zone around the colonies on the media with cellulose and glycerol.

Further screening based on excretion of extracellular hydrolases, was done by growing the selected mutants and the wild type strain at 28-30° C for 144 h in the Saunders liquid medium on a rotary shaker. The experiments in triplicate were carried out in 500 ml Erlenmeyer flasks with 100 ml Saunders medium inoculated with 5×10^7 conidia. The culture liquid was maintained at pH=5.0 by means of sterile 1N HCI or 1N NaOH. After 2 days of incubation, samples were taken from the flasks each 24 h in order to assay the enzymatic activity and protein.

Enzyme assay

Exo-1.4-β-glucanase activity was determined at 50° C, pH=4.8 using strips (1 x 6 cm) of Whatman No. 1 (8). Endo-1.4-β-glucanase activity was determined at 50° C, pH=4.8 using CMCNa as a substrate in 0.05M citrate buffer (8). One unit enzyme activity in these assays is defined as the amount of enzyme which produced one µmol of reducing sugar per minute per millilitre of culture

filtrate. Reducing sugar was estimated colorimetrically using dinitrosalicylic acid reagent (9). β-glucosidase activity was determined at 37° C, pH=3.2 using cellobiose (Merck) as a substrate (16). One unit of β-glucosidase activity is defined as the amount of enzyme which catalyses the formation of one μmol of glucose per minute, per millilitre of culture filtrate. The released glucose was estimated by the glucose-oxidase-peroxidase-reaction (Fermognost, Veb Laborchemie Apolda, DDR).

Proteolytic activity was determined according to Anson (2). Soluble proteins were determined in the culture according to Lowry (7).

Cell proteins were determined by subtraction of extracellular proteins (Lowry) from quantity of total culture proteins [Kjeldahl, N(protein) x 6.25]. The content of cell proteins was used as a rough index of mycelium growth.

Results and discussion

After the mutagenization with UV irradiation of a wild *T. viride* C-1 strain, 49 clones were isolated from the 1,400 survivals. Next, 16 clones were isolated from the above 49 clones, and these exhibited different zones of visible clearance around them on cellulose solid medium.

In the submerged culture, the examined mutants and the wild strain showed different β-glucanase activity (Fig. 1).

The greatest changes in the activity of the examined enzymes were observed in exo-glucanases, since 1.2-5 fold increase in their specific activity was obtained (0.2-0.79 U/mg) as compared with the wild strain (0.16 U/mg). On the other hand, the mutants M4-5 and M7-1 proved to be the best producers of exo-glucanases (0.79 and 0.68 U/mg) (Fig. 2).

In the case of endo-glucanases, the ability to their biosynthesis was twice higher in 50 % of the UV mutants, whereas M4-5, M4-6 and M7-1 mutants exhibited the highest activity (1.7, 1.78 and 1.8

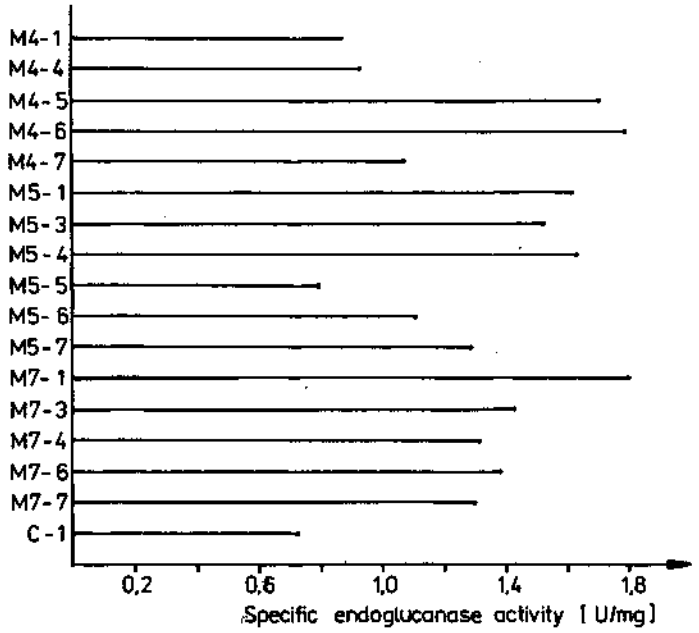

Fig. 1. Specific endo-glucanase activity of wild strain. *T. viride* C-1 and its UV mutans after 96 hours cultivation.

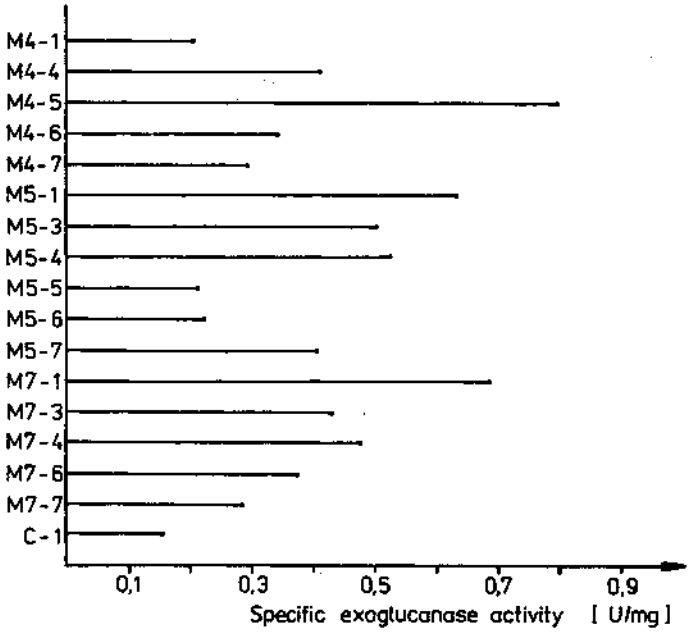

Fig. 2. Specific exo-glucanase activity of wild strain. *T. viride* C-1 and UV mutans after 96 hours cultivation.

U/mg, respectively) as compared with the wild strain (Fig. 1). The increased β-glucosidase biosynthesis was obtained only in the case of two mutants M4-1 and M7-1. Specific activities of β-glucosidases (0.11 U/mg and 0.095 U/mg) of these mutants were 1.5-1.8 fold higher than those of the wild strain. The other mutans showed their enzyme activity decreased by 6-90 % (Fig. 3).

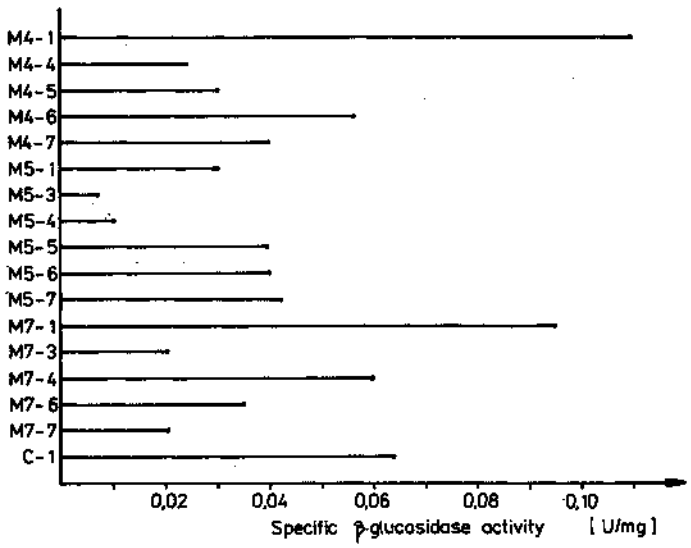

Fig. 3. Specific β-glucosidase activity of wild strain. *T. viride* C-1 and its UV mutans after 96 hours cultivation.

TABLE 1
EXTRACELLULAR HYDROLASE ACTIVITY OF MILD STRAIN *T. VIRIDE* C-1 AND ITS UV MUTANT M-7-1 IN SUBMERGED CULTURE AFTER 96 HOURS.

Strains	Exo-glucanase		Endo-glucanase		β-glucosidase		Proteinase		Protein
	U/ml	U/mg	U/ml	U/mg	U/ml	U/mg	U/ml	U/mg	mg/ml
C-1	0.38 ± 0.03	0.16	1.74 ± 0.16	0.72	0.155 ± 0.014	0.064	1.4 ± 0.09	0.58	2.4 ± 0.11
M7-1	2.71 ± 0.23	0.68	7.2 ± 0.46	1.8	0.38 ± 0.02	0.095	0.72 ± 0.03	0.18	4.0 ± 0.25

The results obtained in the study indicate that the UV mutants *T. viride* C-1 especially mutant M7-1 were similar in some cellulolytic activities of other kinds of fungi obtained by other authors (Table 1).

Zaldivar *et al.* (21) obtained a B_1 mutant *pseudoköningi* by NG mutagenization. It exhibited three times higher exoglucanase (from 0.14 to 0.44 U/ml) and endo-glucanase (from 5.3 U/ml to 15.9 U/ml) but 1.5 times higher β-glucosidase activity (from 0.13 U/ml to 0.2 U/ml) than the wilds type strain.

The mutant *T. reesei* after UV and NG mutagenization, obtained by Bland and Montenecourt (3) exhibited a 5-fold increase in exo-1,4-β-glucanase (4.65 U/ml), 2-fold increase in endo-1,4-β-glucanase (15 U/ml) and 4-fold increase in β-glucosidase activity (1.35 U/ml) in comparison with *T. reesei* QM 9414. Ostrikowa and Konowalow (12, 13) obtained an active *T. viride* 44 mutant as a result of three-stage mutagenesis carried out in the presence of nitroso-dimethylurea, ethylenimine and thermal shock at 100° C for 1 min. The mutant thus obtained exhibited four times as high exo-glucanase activity (22.2 mg/ml/h) as the wild strain (5.7 mg/ml/h).

Targonski (19) examined an UV mutant of *Fusarium* sp. FS-27 and found 1.7 fold increase in endo-glucanase activity (from 12.4 μmol/ml to 21.5 μmol/ml, visc method) and 1.6 fold increase in exo-glucanase (from 0.73 to 1.2 U/ml) and a decrease in β-glucosidase activity (from 0,25 μmol/ml to 0.12 μmol/ml) in comparison with the wild strain. The same mutant but in semicontinous culture showed 2.4 fold increase in endo-glucanase activity (1).

Two out of the four UV mutants of *T. viride* QM 9414 obtained by Farkas et al. (4), M6 and MHC 22, showed 1.2-1.4 higher exo-glucanase but MHC15 and MHC 22 mutants showed 2.3-3.2 higher β-glucosidase activity than the parent strain.

In the present study, we also draw our attention to the kinetics of cellulase synthesis of UV mutant M7-1 and of the wild type *T. viride* C-1 strain. Figs. 4 and 5 show that maximal production occurs after 96 h of cultivation, i. e., at the end of the stationary phase of growth. In the next hours of cultivation, cellulase activity gradually decreased and this coincided with the lowering curve of growth.

The decreased cellulase activity in post cultural liquid (Fig. 4 and 5) at the final phase of growth is difficult to explain. This decrease is affected by several factors, i. e. increasing pH, aeration of the culture or presence of proteolytic enzymes in the culture.

Farkas *et al.* (4) explained that decreased cellulolytic activity at the end of cultivation of *T. viride* in submerged cultures could be attributed to inactivation of cellulases by the increased pH of the cultures in the stationary phase of growth.

In our studies, the pH of the submerged cultures of *T. viride* C-1 and the mutans was controlled each 24 h and maintained at pH=5.0 but in the course of time, until the next control after 24 h, the pH increased to 6.0-7.0 which could also affect cellulase activity (Witkowska D., 1988 in preparation). The decreased cellulase activity at the final phase of growth of *A. terreus* has been explained by Szczodrak (17), as an effect of proteolytic enzymes whose activity increased regularly during their growth, as found in our experiments (Figs. 4 and 5).

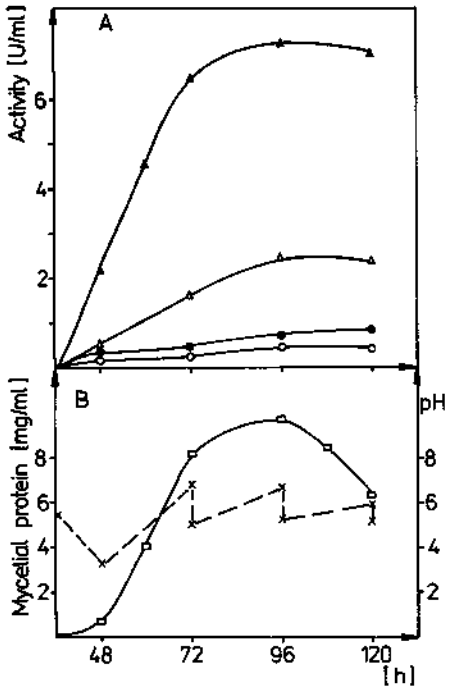

Fig. 4. Dynamics of cellulolytic enzymes and proteinase synthesis in submerged culture of UV mutant M7-1 *T. viride:*

4A: exo-glucanase (Δ), endo-glucanase (▲)
β-glucosidase (o), proteinase (•),
4B: mycelial protein (□), pH (x)

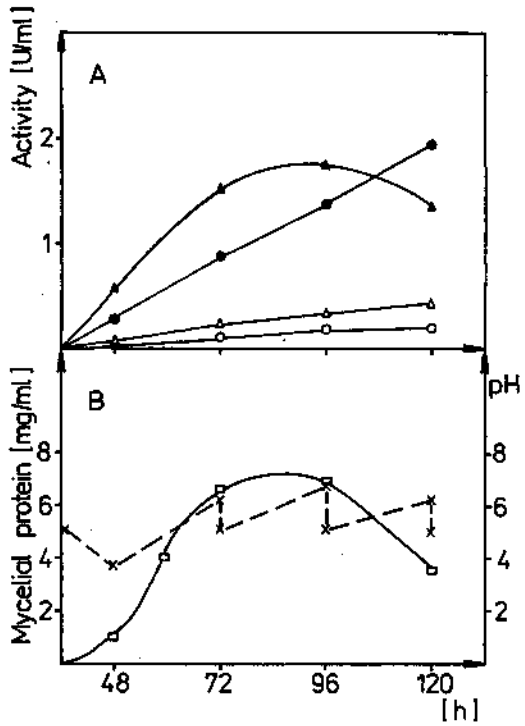

Fig. 5. Dynamics of cellulolytic enzymes and proteinase synthesis in submerged culture of wild strain *T. viride* C-1. Symbols as in Fig. 4.

Out current studies on the mutans described here are directed to increase extracellular hydrolase activities by several stages of mutation of the wild strain and to get detailed characteristics of other hydrolases produced by the resultant mutants.

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MICROBIOLOGIA SEM 5 (1989), 121-125 **NOTES**

Saprophytic fungi on hair and feathers from apparently healthy animals

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Summary

The occurrence of saprophytic fungi on hair and feathers samples taken from apparently healthy domestic animals (cows, pigs, rabbits, and chickens) has been studied. A total of 221 strains classified in 24 genera were isolated. The most frequent genera were *Aspergillus, Penicillium, Mucor, Absidia* and *Alternaria,* and some statistically significant differences of contamination were detected among the animal species.

Key words: Mycoflora, hair, feathers, healthy animals.

Resumen

Se ha realizado un estudio de los hongos presentes en muestras de pelos y plumas de animales aparentemente sanos (vacas, cerdos, conejos y gallinas). Se han aislado 221 cepas pertenecientes a 24 géneros. Los géneros aislados más frecuentemente fueron *Aspergillus, Penicillium, Mucor, Absidia* y *Alternaria,* y se obsevaron diferencias significativas al comparar la frecuencia de aparición de los distintos géneros y especies fúngicas en las especies animales estudiadas.

The hair and feathers of animals represent a suitable substrate for the colonization of keratinophilic fungi, some of which are pathogens, opportunistic pathogens or allergens. Studies focused on the knowledge of mycoflora of this substrate have gradually revealed that the findings of fungi in this kind of samples may reflect specific relationships between the fungi and their hosts.

It is not known whether most of the saprophytic fungi can produce infections in healthy animals. However, some of them become invasive in conditions of decreased resistance, thus being opportunistic in their pathogenicity. Cases of suspected fungal infections caused by normally saprophytic fungi have been described in animals during the last two decades (1). However, only a few surveys about the fungal flora of asymptomatic skin of animals have come out (1, 2, 6, 8, 11, 14). In this work we study the occurrence of saprophytic fungi on hair and feather samples taken from apparently healthy domestic animals.

Seventy breeding places in Cataluña, Spain, have been sampled (20 of cows, 20 of pigs, 15 of

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rabbits and 15 of chickens). It was taken hair or feather samples of one selected animal from each breeding place. Samples were placed on the surface of Sabouraud's dextrose agar with chloramphenicol (DIFCO) and incubated at 28° C. Three plates per animal were used. Cycloheximide was not used because its selective influence on the growth of contaminant moulds and yeasts. The taxonomic identification was performed according to the macro- and microscopic morphology of the suspected colonies. All the strains were cultivated on the appropriate culture medium to achieve typical growth and sporulation and identified according to the criteria established by the mycological handbooks published for each genera. The yeast colonies were identified using the API 20 C AUX system. The χ^2 test was used when possible to determine if there were significant differences of contamination among the animal species.

A total of 221 strains classified in 24 genera were isolated during the study. Figure 1 shows the percentage occurrence of the 10 most frequent genera arranged in accord with the descending frequency of occurrence. All the dematiaceous strains which did not sporulate under laboratory conditions within 30 days, are grouped together as «dematiaceous sterile mycelia».

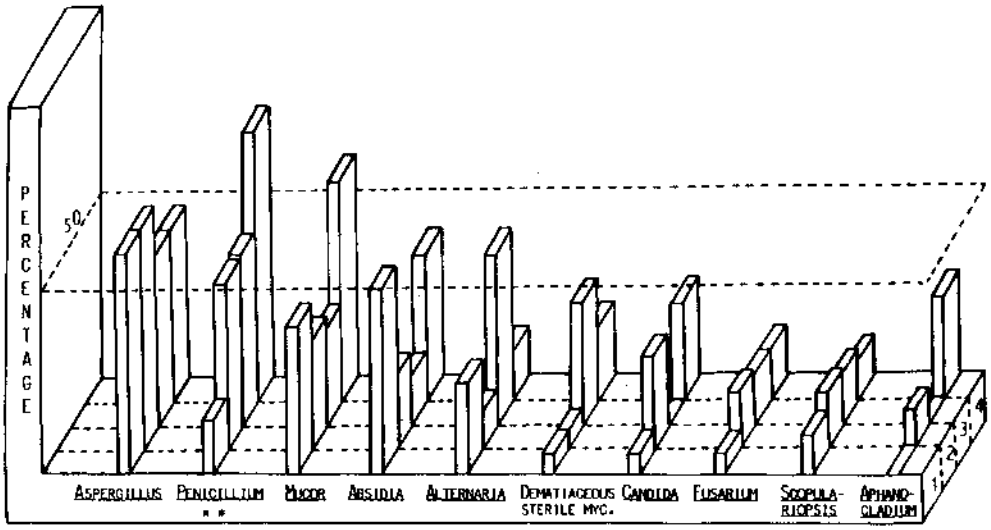

Fig. 1. Percentage occurrence of the 10 most frequent genera (including dematiaceous sterile mycelia) isolated from cow's hair (1), pig's hair (2), rabbit's hair (3) and chicken's feather (4).
**: Statistically significant differences (χ^2 test) $p < 0.01$.

Table 1 shows the various species of fungi isolated and the frequency of their occurrence among the animal species studied. In both cases, the significant differences among the animal species (χ^2 test) are pointed out.

The most frequent genera were *Aspergillus, Penicillium, Mucor, Absidia* and *Alternaria.* All these genera are ubiquous in nature and nearly all the isolated genera have been reported in similar studies made on asymptomatic animals. In a survey of the normal fungal flora of dogs (14), the authors reported that *Cladosporium* and *Alternaria* were the commonest genera isolated. Other studies made in asymptomatic dogs (2, 8) showed the isolation of some dermatophyte species and strains of genera *Scopulariopsis, Penicillium, Aspergillus* and *Alternaria* among others. Marsella *et al.* (11) studying the occurrence of fungi in animals of the Zoological Park of Rome reported among the ac-

TABLE 1
FREQUENCY OF FUNGAL SPECIES OCCURRENCE AMONG THE ANIMALS EXAMINED

Fungal species	Cows n = 20	Pigs n = 20	Rabbits n = 15	Chickens n = 15
Absidia corymbifera	10	4	2	6
Acremonium strictum	2	—	1	—
Acremonium terricola	—	—	1	—
Alternaria alternata **	5	1	7	1
Alternaria longipes	—	—	—	1
Alternaria tenuissima	—	1	—	—
Aphanocladium album	—	2	—	4
Artrinium phaeospermum	—	2	—	—
Arthroderma sp.	—	—	2	—
Aspergillus amstelodami	2	2	2	—
Aspergillus awamori	1	—	1	—
Aspergillus candidus	—	6	2	3
Aspergillus chevalieri	1	1	—	—
Aspergillus flavus	3	1	1	1
Aspergillus fumigatus	3	3	2	3
Aspergillus nidulans	—	—	1	—
Aspergillus terreus	1	—	—	—
Aspergillus terricola	1	—	—	1
Aspergillus tubingensis *	6	2	—	—
Aspergillus versicolor	2	—	—	—
Candida famata	1	4	—	3
Candida lusitaniae	—	1	—	1
Circinella umbellata	—	—	—	1
Cladosporium cladosporioides	1	—	—	—
Cladosporium herbarum	1	—	1	1
Cladosporium macrocarpum	—	—	1	—
Epicoccum purpurascens	—	—	1	—
Fusarium oxysporum	1	3	1	2
Fusarium equiseti	—	—	1	—
Geotrichum candidum	2	—	1	1
Gliocladium catenulatum	—	—	1	—
Gliocladium virens	—	—	1	—
Mucor circinelloides	1	—	—	—
Mucor hiemalis	1	—	1	—
Mucor plumbeus	2	1	2	2
Mucor racemosus	4	5	1	7
Paecilomyces variotii	—	—	—	2
Penicillium carneo-lutescens	—	—	—	1
Penicillium chrysogenum	1	2	3	4
Penicillium frequentans	—	2	1	—
Penicillium fellutanum	—	—	—	1
Penicillium jenseni	—	—	—	1
Penicillium martensii	—	—	1	3
Penicillium olivino-viride	—	1	—	—
Penicillium palitans	—	1	—	2
Penicillium variabile	2	2	—	1
Penicillium viridicatum	1	2	2	3

TABLE 1 (CONTINUED)

Fungal species	Cows n = 20	Pigs n = 20	Rabbits n = 15	Chickens n = 15
Phoma glomerata	1	—	—	—
Rhizopus oryzae	—	—	—	1
Rhizopus oligosporus	1	—	—	—
Rhizopus stolonifer	—	1	—	—
Scopulariopsis brevicaulis	—	—	1	1
Scopulariopsis candida	2	3	2	—
Scytalidium lignicola	1	—	—	—
Trichoderma viride	1	—	—	1
Trichothecium roseum	—	—	2	—
Ulocladium consortiale	1	1	—	—

* Statistically significant differences (χ^2 test) $p < 0.05$.
** Statistically significant differentes (χ^2 test) $p < 0.01$.

companying fungal flora, strains of the genera *Alternaria, Aspergillus, Acremonium, Cladosporium, Geotrichum, Penicillium* and *Scopulariopsis.* Hubalek *et al.* (6), in a survey of the fungi isolated on the hair of small wild mammals reported a high incidence of different species belonging to the genera *Penicillium, Aspergillus* and *Alternaria.* Aho (1) examined hair samples of dogs, cats, horses, cows, rats and other animals for the presence of saprophytic fungi. The commonest in order of frequency were members of the genera *Penicillium, Cladosporium, Aspergillus, Mucor, Aureobasidium, Alternaria, Scopulariopsis, Trichoderma* and *Trichothecium.* Statistically significant differences in contamination of animal skin were noted in some cases.

In our study, the genus *Penicillium* was isolated significantly more often (χ^2 test $p < 0.01$) from chicken samples than those of rabbits, pigs or cows. *P. chrysogenum* and *P. viridicatum* were the most frequent species of *Penicillium* isolated.

The commonest species of *Aspergillus* isolated were *A. fumigatus, A. candidus,* and *A. tubingensis.* Statistically significant differences (χ^2 test $p < 0.05$) were noted in *A. tubingensis,* more frequent in cow's hair.

Alternaria alternata, one of the most frequent species in the study was isolated significantly more often (χ^2 test $p < 0.01$) from rabbit samples than those of cows, pigs or chickens.

We have to point out the isolation of *Arthroderma* sp., perfect state of *Trichophyton mentagrophytes* in two rabbit's hair samples without apparent injury. The dermatophyte *T. mentagrophytes* is one of the most common causes of tinea in rabbits, and it has been isolated from healthy animals as wild rodents (10, 12, 13), laboratory animals (3, 4, 5, 9), bird feathers (7) and dogs (2), suggesting that in some cases, animals can act as healthy carriers. They do represent a potential risk of infection both for other animals living in the same animal house and for the people working with them.

The hair of animals serves as a collector of environmental fungi and thus it acquires a transient fungal flora. In this study the fungal flora isolated from the skin of the animals reflected in general the abundance of fungi in their environments because the samples were taken inside the farmhouses.

It is unknown how long the fungi can survive on the skin of animals and how some fungi can multiply on the coats of animals contaminated by soil and foodstuffs. The presence of opportunitic fungi on the coats of animals creates an opportunity for them under special circumstances to become invasive and thus to cause primary or secondary infections. Because of that, further studies are obviously required.

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MICROBIOLOGIA SEM 5 (1989), 127-128 **NOTES**

A rational proposal for plasmid nomenclature

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Summary

We propose a more rational system for nomenclature of wild plasmids of bacteria.With this proposal for nomenclature of bacterial plasmids, it is established in an unambiguous way: 1) if a plasmid is wild or derivative, and 2) in which species and bacterial strain it was found (in the case of wild plasmids).

Key words: Plasmid nomenclature.

Resumen

Nosotros proponemos un sistema más racional para la nomenclatura de los plásmidos salvajes de las bacterias. Con esta proposición para la nomenclatura de los plásmidos de las bacterias se establece sin ambigüedades: 1) si un plásmido es salvaje o derivado, y 2) en qué especie y cepa bacteriana fue encontrado (en el caso de los plásmidos salvajes).

Biological nomenclature has as a principal objective to name organisms/molecules in a very specific and unambiguous way, following internationally recognized rules. In bacterial nomenclature, following the binomial system, the names of bacterial species are necessary and must be stable and unambiguous (6). With this system, new bacterial species can be introduced in bacterial taxonomy in an unambiguous way. In molecular nomenclature, unambiguous names have been proposed for the several molecular species in a logic way. In the case of restriction endonucleases (5), these enzymes have the general name of restriction endonucleases, followed by a code of three letters (formed by the first letter, in capital letter, of the genus name, and the two first letters, in small letters, of the specific epithet of the bacterial species that presents a particular modification-restriction system), and the name of the bacterial strain and/or a serial number that identifies a particular bacterial strain. For example, the restriction endonuclease R. Eco RI means: «R» of restriction endonuclease, «E» of *Escherichia,* «co» of *coli,* and «RI» of first endonuclease codified by a resistance plasmid (plasmid RY13) in *E. coli.* With this system of restriction endonuclease nomenclature, it is not ambiguous to name the new endonucleases that are continually being isolated (1).

Bacterial plasmids, in their first period, were named: «F» (fertility factor), «Col» followed by

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an identification (for bacteriocinogenic plasmids of *E. coli*) and «R» followed by an identification number (for resistance plasmids). Due to the isolation of new plasmids, this system was unacceptable. In 1976, a system of nomenclature for bacterial plasmids was proposed (3). In this system it was recommended to use the letter «p» to designate a plasmid, followed by a prefix (two/three/four letters) to identify a particular investigator/laboratory and a serial number of that investigator/laboratory. With this system, plasmid pXY1234 means: plasmid number 1234 from the collection of the investigator/laboratory using the prefix/initials of identification XY. A great number of prefixes have been registered for plasmid designations to avoid undesirable duplications (2). This system of nomenclature for bacterial plasmids is completely unacceptable, due to: 1) it does not distinguish between wild plasmids and derivative plasmids (mutants, recombinants, etc.); 2) it does not offer information about the primary natural host of plasmids, and 3) the prefix offers neither information nor reability due to plasmids are being isolated from many bacterial species (throughout the world) and continually new derivative plasmids are being made [for example, pHH is used to name plasmids of Hammersmith Hospital (London) (3) and plasmids of *Halabacterium halobium* (4)]. Therefore, this system of nomenclature for bacterial plasmids tends towards a future collapse, because it does not permit the introduction of new plasmid names in an indefinite and unambiguous way.

Until very specific and unambiguous rules are developed, we propose here a more rational system for plasmid nomenclature. In this proposal, we put forward that the nomenclature for wild plasmids must be different from the nomenclature for derivative plasmids (mutants, recombinants, etc.). In the case of derivative plasmids, we propose the nomenclature already established in 1976 (3), until a better solution be found. In the case of wild plasmids of bacteria, we propose that they be named: «p» to designate a plasmid, followed by a code of three letters (formed by the first letter, in capital letter, of the genus name, and the two first letters, in small letters, of the specific epithet of the bacterial species where the plasmid was found) as in the system of restriction endonucleases nomenclature, followed by a hyphen and the name of bacterial strain (not a prefix and a serial number of the plasmid). The bacterial strain could be designated by a prefix of the investigator/laboratory followed by a serial number or a prefix of a reference collection of bacteria followed by a serial number. If the bacterial strain has more than one plasmid, the designation of the strain could be followed by «A», «B», «C», etc., in order to designate plasmids from larger to smaller molecular weight. With this new system, plasmid pEco-XY1234A means: first wild plasmid (in molecular weight) of the bacterial strain XY1234 (whose prefix/initials of identification is XY and whose serial number is 1234) of *Escherichia coli*. Principally, this proposal for nomenclature of bacterial plasmids establishes in an unambiguous way: 1) if a plasmid is wild or derivative (for example, pEco-XY1234A is a wild plasmid and pXY1234 is a derivative plasmid), and 2) in the case of wild plasmids, in which species and bacterial strain it was found (for example, pEco-XY1234A is the first wild plasmid of the strain XY1234 of *Escherichia coli*).

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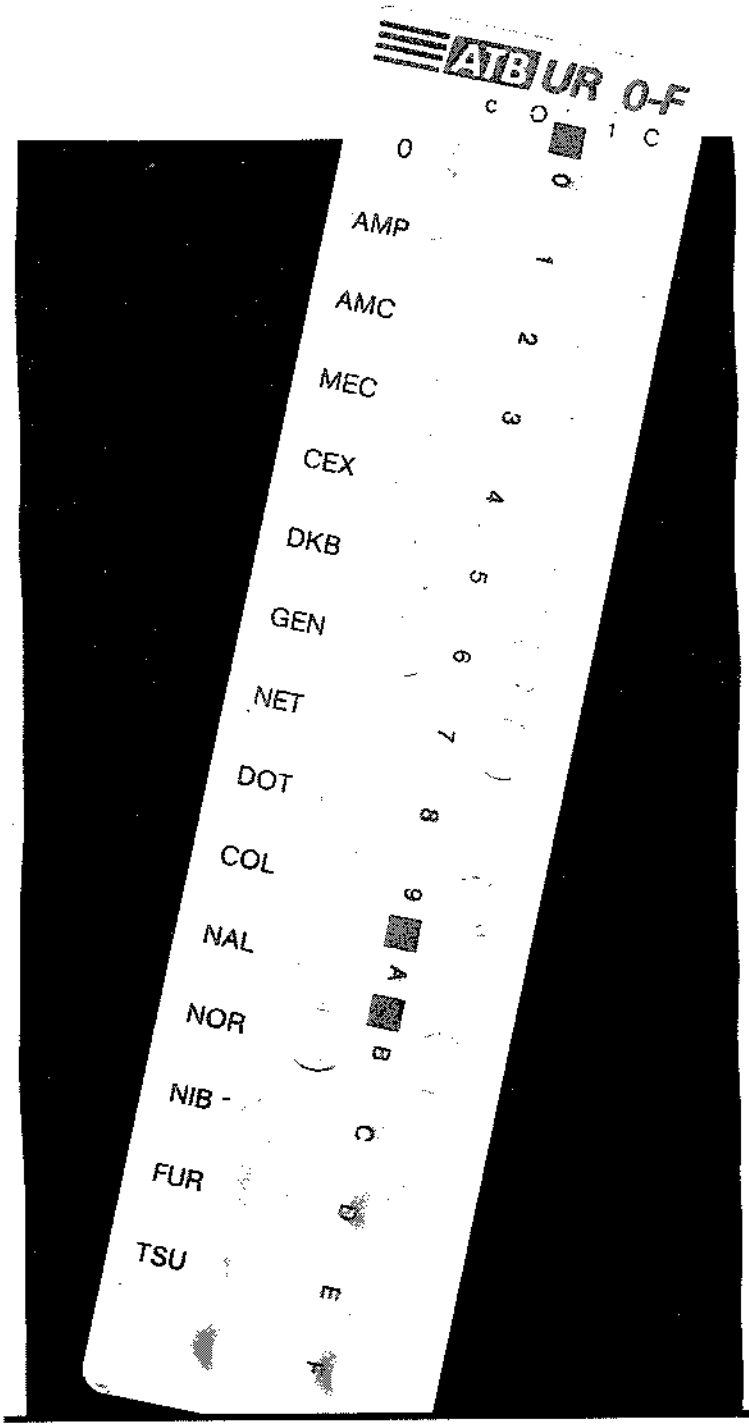

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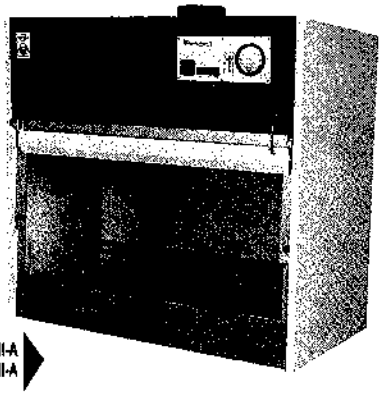

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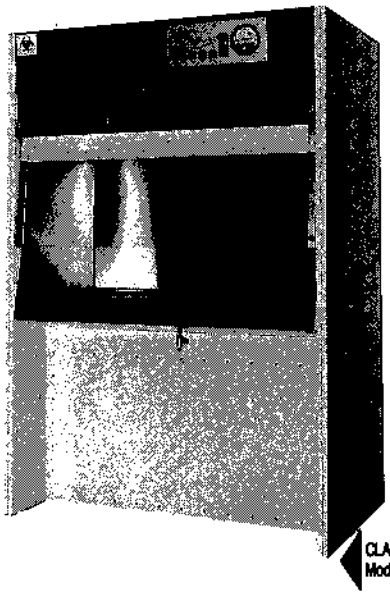

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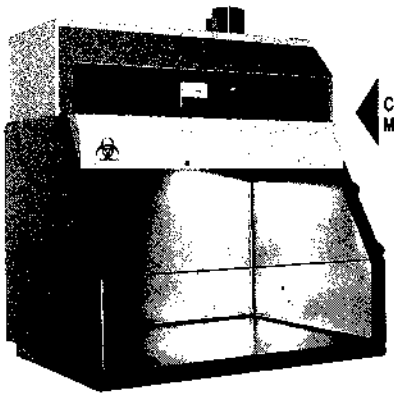

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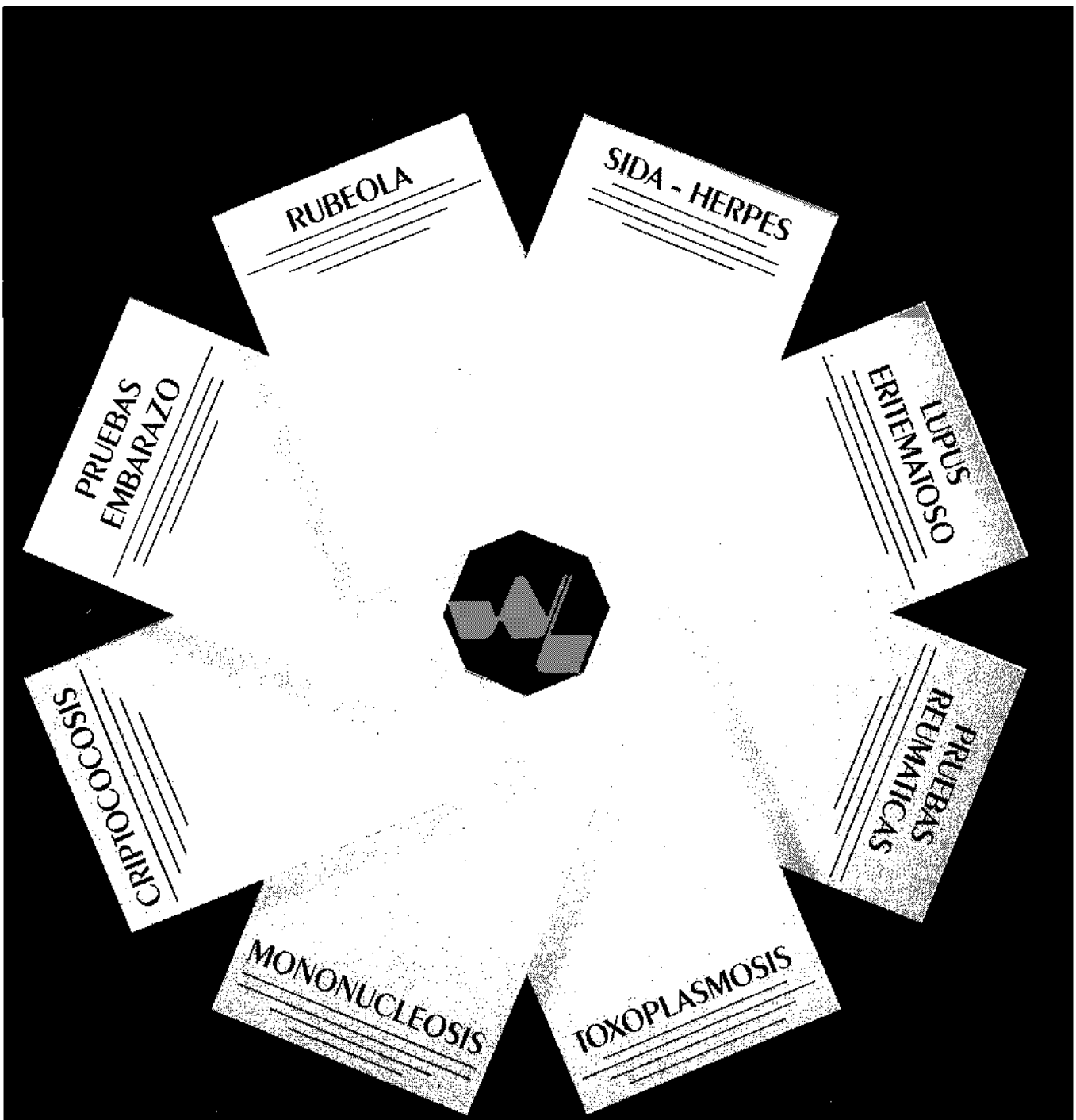

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