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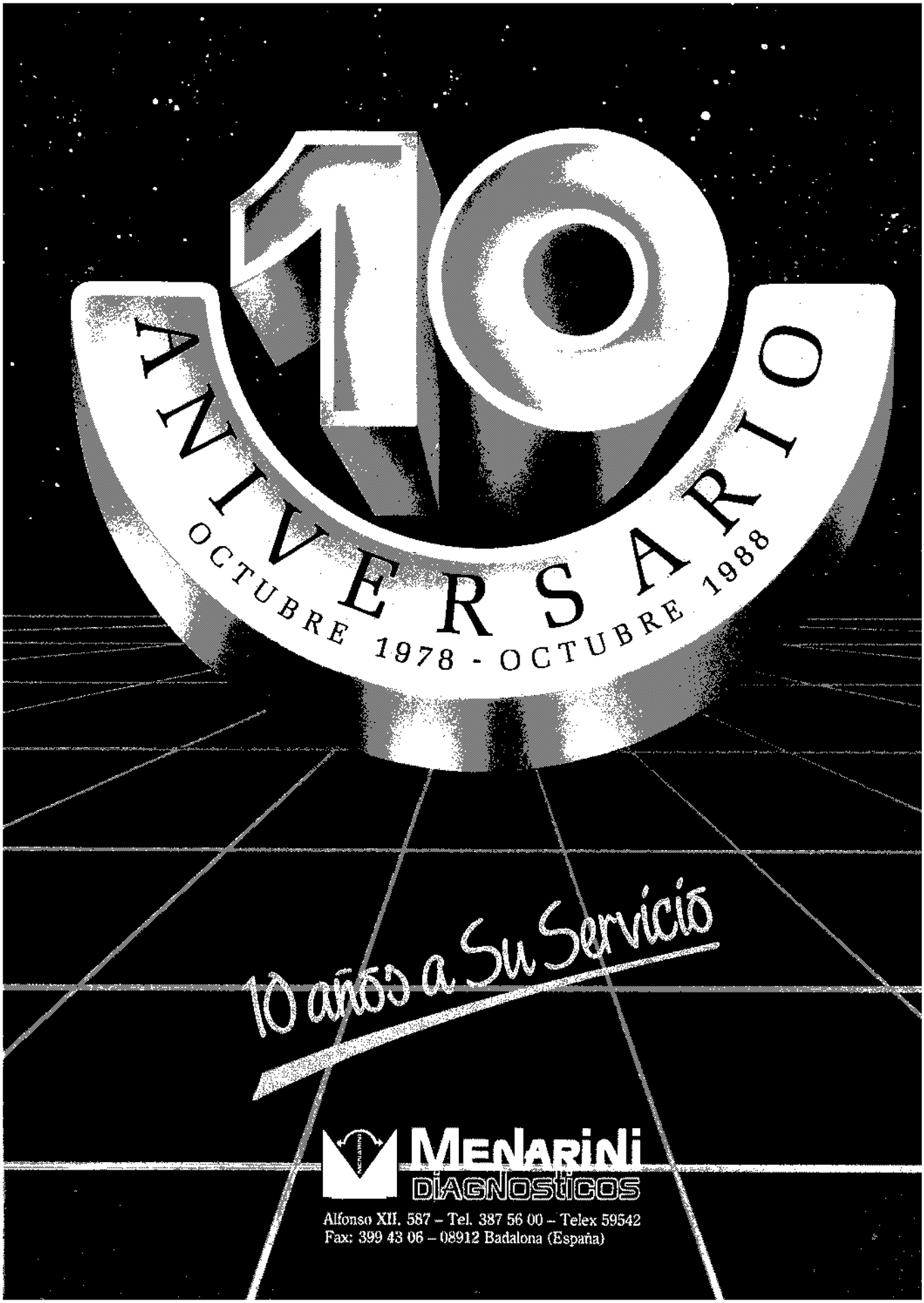
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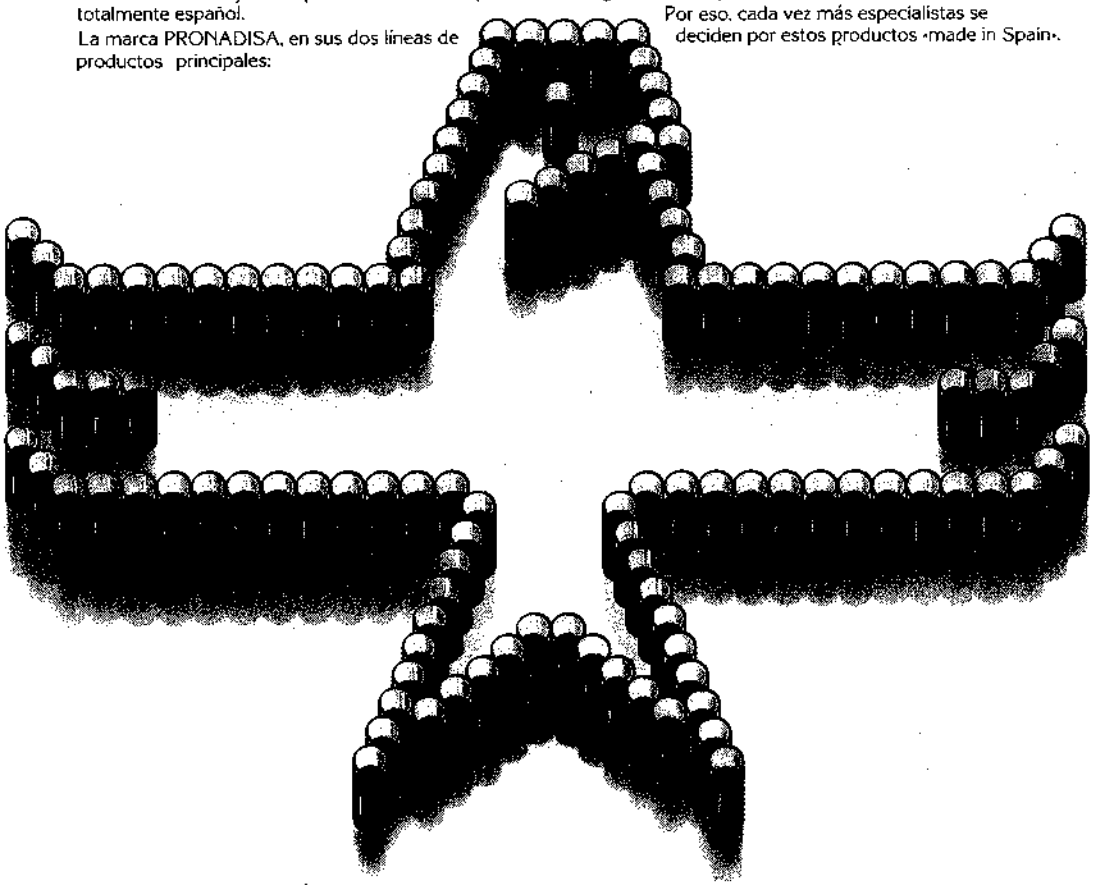

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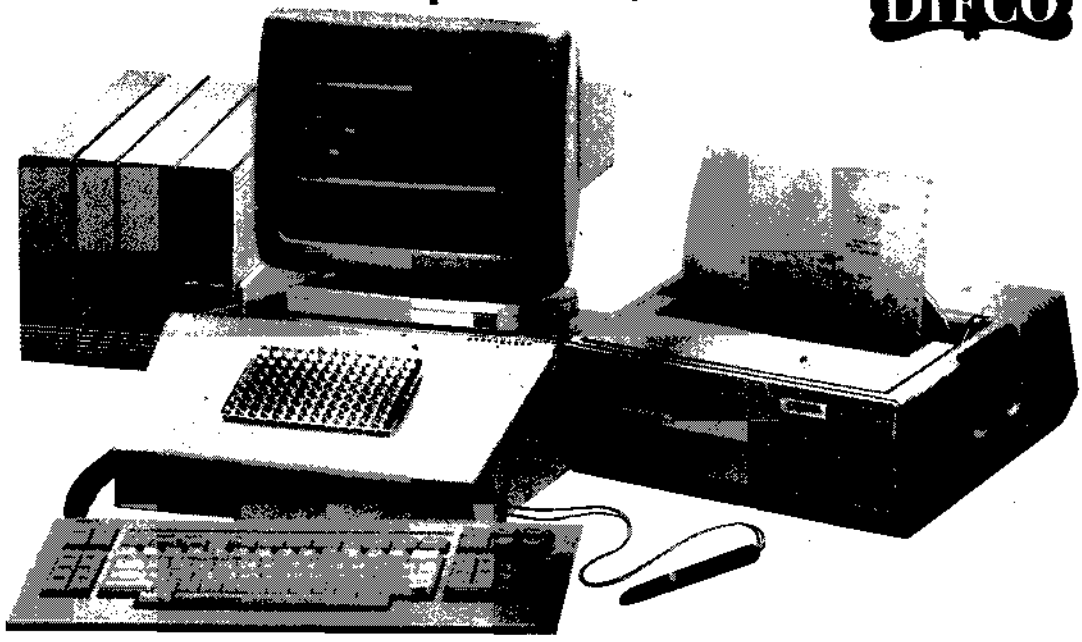

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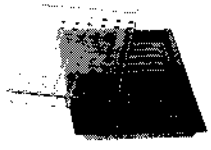

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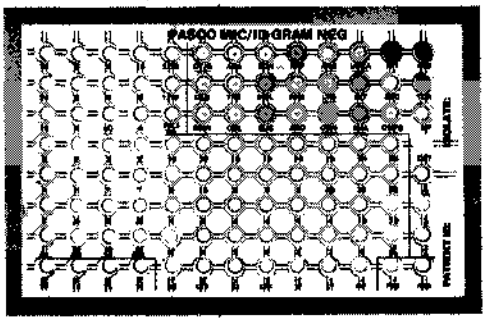

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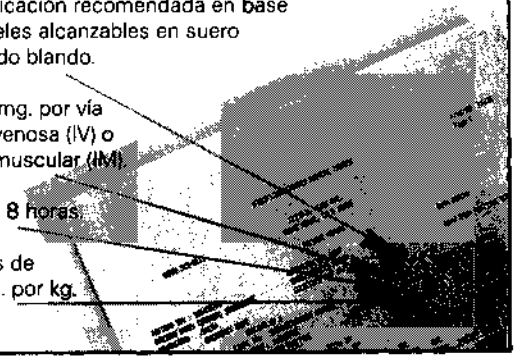

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MICROBIOLOGIA SEM 4 (1988), 73 - 85 **MINIREVIEW**

Protein secretion and compartmentalization in yeast

Enrique Herrero* and Rafael Sentandreu

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Summary

Genetic and biochemical techniques are being employed to study the transport and compartmentalization of proteins in *Saccharomyces cerevisiae*. A number of thermosensitive *sec* mutants have been isolated which are blocked at different stages of the secretory route (endoplasmic reticulum, Golgi complex, secretory vesicles, cell surface). They offer the possibility of analyzing protein processing along this pathway, by studying the molecular intermediates accumulated in the mutants at the nonpermissive temperature. Compartmentalization of the proteins is influenced by cellular functions, as well as by their own peptide sequence of the molecules to be targeted. The physical structure of the wall is also important for the incorporation of mannoproteins into it. Factors affecting expression and externalization of heterologous proteins in yeasts are also reviewed.

Key words: Yeasts, protein transport, glycosylation, cell wall.

Resumen

Se están empleando técnicas genéticas y bioquímicas para estudiar el transporte y la compartimentalización de proteínas en *Saccharomyces cerevisiae*. Se ha aislado un cierto número de mutantes *sec* termosensibles bloqueados en diversos estadios de la ruta secretora (retículo endoplásmico, complejo de Golgi, vesículas secretoras, superficie celular). Estos mutantes ofrecen la posibilidad de analizar el procesamiento de las proteínas a lo largo de la ruta, mediante el estudio de los intermediarios moleculares acumulados a la temperatura no permisiva. La compartimentalización de las proteínas viene influenciada por funciones celulares, además de por la propia secuencia polipeptídica de las moléculas transportadas. La estructura física de la pared también es importante para la incorporación de las manoproteínas en ésta. Asimismo se revisan los factores que afectan a la expresión y externalización de proteínas heterólogas en levaduras.

Introduction

Protein secretion in eucaryotic cells is a process which is structurally and physiologically much more complex than in bacteria, since it involves a diversity of membranous structures that transport the secreted molecules. The process has been studied mainly by electron microscopy and biochemi-

(*) Corresponding author.

cal techniques (23, 58, 65). The genetic approach to the problem is difficulted by the apparently large number of gene functions involved and the complexity of genetic manipulations in higher organisms. Yeasts, particularly *Saccharomyces cerevisiae,* offer a relatively easy and multidisciplinary (cytological, genetical, physiological and biochemical) approach to analyze problems such as protein transport, and by using this eucaryotic microorganism as a model, knowledge in this area of cell biology has accumulated throughout the last decade (see reviews in Ref. 76 and 77).

In this review, we will describe two aspects in particular: i) the secretory pathway of yeast proteins inside the cytoplasm, and ii) the assembly of secreted proteins at the final compartments. We distinguish between the secretory pathway (followed by extracytoplasmic, plasma membrane and vacuolar proteins) and the transport of cytoplasmically-synthesized proteins to the mitochondria and nucleus; these latter processes will not be considered here, and reviews on them are found in Ref. 18 and 16 respectively.

Synthesis and transport of proteins to the cell surface and vacuole

A series of proteins (mostly of mannoprotein nature) synthesized by yeast cells are found externally to the cytoplasmic membrane, being located either in the wall, in the periplasmic space or released to the surrounding medium. Distinction between the two first compartments is experimental: integral wall mannoproteins cosediment with the structural components (glucan and chitin, see below) that form the network of the wall, while periplasmic enzymes (such as invertase, acid phosphatase or α-galactosidase) are released after cells are converted into protoplasts or broken mechanically, but do not cosediment with the walls. Alternatively, other protein molecules (such as the sexual α-factor or the killer toxin) are released into the growth medium. All of these molecules seem to follow the same route from the site where they are synthesized [the endoplasmic reticulum (ER)] to the cell surface.

Genetics of protein secretion

Schekman and coworkers (76) have isolated different thermosensitive *(sec)* mutants that accumulate organelles and molecular intermediates of the secretory proteins at the restrictive temperature (37° C). In mutants of class A, divided in 23 complementation groups, secretion is blocked at one of the following steps: transport from the ER (i.e. *sec18* and *sec20*), assembly of secretory vesicles at the Golgi apparatus (i.e. *sec7)* and discharge of secretory vesicles (i.e. *sec1* and *sec5).* By analysis of the organelles accumulated in double mutants, the order of events of the yeast secretory pathway has been established (55), revealing a vectorial movement between organelles identical to that in higher cells: ER → Golgi apparatus → secretory vesicles → cell surface.

In addition, Schekman and coworkers have isolated other mutants (class B, i.e. *sec53* and *sec59)* blocked in the secretion process at steps previous to class A ones (25, 26). In these mutants, the precursors are accumulated, which in the case of periplasmic enzymes such as invertase, are inactive. With the support of biochemical studies (for example, analysis of protein internalization based on the sensitivity of the protein molecules in intact transport membranous vesicles to externally added proteases), it was established that the *sec53* mutant represents a step previous to *sec59* (25, 26). In both mutants, internalization of nascent proteins into the ER lumen seemed not to occur, although later experiments raised some doubt on this previous conclusion, at least for the *sec53* block (24). Recently, the isolation of a new mutant *(sec61)* has been reported (15), which defines a gene product needed at an earlier step than that defined by *sec53;* this mutation produces the accumulation of the precursors of the proteins with a cytoplasmic orientation.

TABLE 1
PROTEINS WHOSE SECRETION IS BLOCKED IN *SACCHAROMYCES CEREVISIAE SEC* MUTANTS

	Reference
Vacuolar enzymes	
Carboxypeptidase Y	86
Plasma membrane proteins	
Sulfate, galactose, arginine and proline permeases	93
Proton-translocating ATPase	36
Periplasmic enzymes	
Invertase	55
Acid phosphatase	55
α-Galactosidase	93
Asparaginase	93
Wall mannoproteins	
High molecular weight mannoprotein	56, 74
33 kDa mannoprotein	74
Sexual agglutination substance	90
Extracellular proteins	
α-Factor	40
Killer toxin	8
Exoglucanases I and II	33

Secretion of periplasmic enzymes and extracellular proteins is blocked in the class A and B *sec* mutants at the non-permissive temperature (see Table 1 for a list), as well as some plasma membrane proteins, indicating a common export route for mannoproteins (or proteins) of these different compartments. The blockage also occurs for integral cell wall mannoproteins (56, 74).

Using *sec* strains, it has been established that yeast vacuolar enzymes such as carboxypeptidase Y also follow the secretory pathway until the Golgi stage, while *sec* mutants that prevent transport of secreted proteins at the secretory vesicles stage do not affect delivery of vacuolar enzymes (86). This situation is analogous to the sorting of proteins in higher eucaryotic cells, that also occurs in the Golgi complex. Overproduction of carboxypeptidase Y (87) or mutations in a number of genes (named VPL, for vacuolar protein localization) required for the proper function of the sorting apparatus (68) leads to mislocalization of carboxypeptidase Y at the cell surface. This points to the existence of a saturable critical step that occurs as a late Golgi, or post-Golgi process.

Figure 1 shows scheme of the secretory pathway ant the gene functions involved at the different steps, as known today.

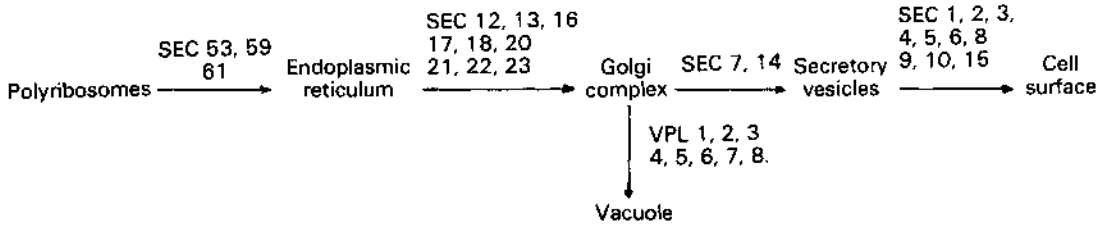

Fig. 1. Scheme of the secretory pathway in *Saccharomyces cerevisiae*. The genes whose products are known to act at some stage of the route are indicated.

The biochemical functions corresponding to the steps defined by the *sec* mutations are still unknown. Recently, the *sec4* gene (its product acting at a post-Golgi stage) has been cloned and the product has been found to share homology with viral *ras* proteins and to be essential for growth (71); best homology occurs with *ras* regions of GTP-binding and hydrolysis activity.

In higher cells, membrane vesicles that mediate transport to the surface seem to be coated by triskelion structures formed by a protein called clathrin (63). This protein has also been isolated from *S. cerevisiae* cells (49) and the corresponding gene has been cloned (62). Gene disruption experiments have shown that cells lacking functional clarthrin are viable, although they grow slower than normal cells and protein transport from the Golgi stage to the surface may be delayed (45, 61); thus, clathrin seems to play a non-essential activity in cell growth and protein secretion. On the other hand, actin may have a role in vesicle transport late in the yeast secretory pathway, although temperature-sensitive actin mutants do not show an absolute block in protein secretion (54). Finally, some of the gene products defined by the CDC (for cell division cycle) genes may also participate in this transport, as some mutants show an abnormal deposition of the wall mannoproteins (83).

Synthesis and processing of secreted proteins along the export pathway

Most of the molecules transported along the secretory pathway are of mannoprotein nature (77). Two types of carbohydrate chains exist in yeast mannoproteins, which differ in the type of linkage with the protein moiety (3, 44, 88). One type or carbohydrate chain is linked to the peptide through N-linkages to asparagine residues, with an inner core of two glucosamines and 12 to 15 mannose units in a branched structure; in mannoproteins such as invertase (92) or the high molecular weight structural component of the yeast cell wall (28, 94), this inner core is extended with a varying number of blocks of mannose units that form an outer region of branched structure that containts phosphodiester bonds in the side chains. In contrast, other carbohydrate chains consist of one to five mannose units linked to serine or threonine residues in the peptide through O-glycosidic linkages.

Synthesis of secretory mannoproteins is initiated on free ribosomes and subsequently the become associated to the ER thanks to the nascent N-terminal peptide (signal peptide) of the molecules. The latter has been shown to be present in the precursors of the exported mannoproteins such as invertase (11), α-factor (39), killer toxin (6) and vacuolar carboxypeptidase Y (96). The elongation of the peptide takes place at the same time as its translocation to the lumen of the ER (cotranslational translocation). Elimination of the signal peptide and initiation of glycosylation also occurs during translocation. These processes have been analyzed both *in vivo*, studying the precursors accumulated in early-blocked *sec* mutants (see above), and *in vitro* with mRNAs translated in homologous cell-free systems that have been developed recently (66, 99). *In vitro*, the precursor of α-factor, but not invertase, can be translocated post-translationally into the ER lumen (67, 99); however, internalization of completely synthesized peptide chains has yet to be demonstrated in yeast cells *in vivo*.

Initial N-glycosylation at the ER occurs by transfer of the group $GlcNAc_2$-Man_9-Glc_3 from a lipid intermediate (dolichyl-P-P-oligosaccharide) to an asparagine residue of the nascent peptide, followed by elimination of the three glucose residues and one mannose residue, and later addition of some mannose residues (with GDP-mannose as donor) to complete the core region (9). Elongation of the N-glycosidically-linked sugar chains takes place at the Golgi level, since *sec* mutants blocked in the transport from the ER lack the outer chain in the mannoprotein molecules (76). Synthesis of the outer chain occurs by a stepwise addition of mannose residues catalyzed by several mannosyltranferases, for which GDP-mannose is also the sugar donor (44, 88). One of the factors that affect elongation of the outer chain is the esteric accesibility of the transferases to the sustrate (92), which may explain the heterogeneity in the length of the outer chain even in the same mannoprotein molecule, and also the fact that some mannoprotein species do not contain outer chains. On the other hand,

initiation of O-glycosylation takes place between the ER and the Golgi organelle, and it is normally finished in this latter organelle (31, 101).

Besides elimination of the signal peptide and glycosylation, secretory mannoproteins may have other kinds of processing. α-Factor is synthesized as a N-glycosylated precursor (prepro-α-factor) from which the signal peptide is initially processed, and the suffers several endoproteolytic cleavages to give four mature tridecapeptides lacking sugar residues for each precursor molecule (40). Similar proteolytic processing of larger precursors occurs with acid phosphatase (78), killer toxin (6) and carboxypeptidase Y (86). Specific proteinases are involved in these proteolytic events along the secretory route (1, 2, 17).

The role of peptide sequence in transport

As indicated above, signal peptides in secretory proteins are involved in the initial interaction of the nascent polypeptide chain with the ER membrane. Amino acid sequences of procaryotic and eucaryotic signal peptides have common features: one or more basic amino acids are found near the amino terminus of the signal sequence, followed by a hydrophobic region and a less hydrophobic carboxy-terminal end (98). Mutations have been introduced in the signal sequences of secretory proteins to study the role of these sequences in transport and their structural requirements. Extensive deletions are needed in the invertase signal peptide (i.e. disruption of the positively charged amino end) to prevent export and glycosylation of the molecules (41, 64). Surprisingly, a deletion mutation that removes the entire carboxypeptidase Y signal sequence diminishes but does not eliminate the efficiency of transport and processing of the peptide to the vacuole, part of the molecules being post-translationally glycosylated (5).

Thus, the signal sequence may not always be strictly necesary to promote translocation through the ER membrane, perhaps because internal sequences may replace the function of the original N-terminal sequence (42), or because polypeptides lacking the signal sequence may still assume a conformation in the cytoplasm that allows translocation. In relation to this, Schauer *et al.* (75) have isolated a mutant with a single amino acid change in the mature invertase polypeptide that diminishes the rate of transport of the altered molecules to the surface; in this mutant, invertase is partially glycosylated, but signal peptide cleavage takes place normally. In other words, peptide sequences in the mature part of the molecule are also essential for adequate intracytoplasmic transport. Finally, recent results suggest that ATP hydrolysis may supply the energy needed in the protein translocation process (99).

The role of glycosylation in protein transport

There is a correlation between protein secretion and glycosylation that might indicate the need of sugar addition for effective secretion of the protein molecules. The problem has been addressed in yeast cells *in vivo* with the use of tunicamycin, an inhibitor of N-glycosylation. Although no inhibitors of O-glycosylation are known, apparently the only extracytoplasmic mannoprotein species containing this type of linkages in *S. cerevisiae* and *Candida albicans* are the high molecular weight components or the wall (94, 101, Elorza *et al.*, in press).

Non-glycosylated acid phosphatase (78) and precursors of α-factor (40) and killer toxin (8) have been reported to accumulate in internal membranous structures. Using partially inhibitory concentrations of tunicamycin and *mnn* mutants defective in the synthesis of N-linked carbohydrate outer chains (3). Mrša *et al.* (48) have observed that outer chain addition is not essential for secretion of active acid phosphatase, and that only four N-linked saccharide core regions (out of eight in the nor-

mal molecules) are required for enzyme transport to the periplasmic space. On the other hand, exo-1,3-D-glucanase (73) and the protein moiety of a 33 kDa structural mannoprotein component of the *S. cerevisiae* cell wall (60) are still secreted in the presence of tunicamycin. Non-glycosylated carboxypeptidase Y is also targeted to the vacuole (79). With respect to invertase, secretion of non-glycosylated molecules is blocked at 37° C, but not at 25° C (25).

In vitro, several yeast mannoproteins are more sensitive to proteases, heat or other denaturing conditions in their non-glycosylated form (4, 12, 13), which points to a role of glycosylation in the determination of the correct tertiary or quaternary structure of the molecules (57). Thus, carbohydrates would not be a direct signal for secretion, but would act (at least in some molecular species) providing an adequate conformation of the molecules to be efficiently recognized by the secretory machinery. In this context, the divergent behaviour of different mannoproteins *in vivo* in the presence of tunicamycin, as well as the differential effect of temperature in these conditions, would be easily explained in terms of different spatial conformations.

Protein compartmentalization

All mannoproteins (or proteins) listed in Table 1 follow the same general pattern of transport along the secretory route, although they are targeted to different compartments. A number of cellular functions (i.e. those defined by the SEC and the VPL genes) participate in the secretory process interacting directly or indirectly with determinants located in the molecules to be transported. Thus, cells must be able to discriminate among compartmentalization determinants depending on the particular target of the molecules. Once at their target compartment, the proteins must interact with other components to become firmly established in it; the particular spatial structure of the molecules must also have an important role at this stage.

Targeting of vacuolar enzymes

In yeast cells, the vacuole plays a role similar to the lysosomes in mammalian cells, that is, to store a number of degrading enzynes. Lysosomal glycoproteins contain mannose 6-phosphate groups at the carbohydrate moieties that act as signals to be recognized by receptors located at the lysosomal membrane (97). The situation is different in yeast cells, at least for carboxypeptidase Y, which is correctly internalized in the vacuole in the absence of the sugar moiety of the molecules (79). From the analysis of the final location of carboxypeptidase Y-invertase hybrid molecules, it seems that no more than 30 N-terminal amino acids act as a targeting signal for the vacuolar enzyme, since they are sufficient to address a 511 residues carboxy-terminal domain of invertase to the vacuole (38). This 30 amino acids region of the peptide may interact functionally with some of the products of the VPL genes.

Targeting of cytoplasmic membrane proteins

A number of yeast plasma membrane proteins, probably lacking carbohydrate moieties, are transported to their final destination through the secretory pathway (i.e. the permeases for sulfate, galactose, arginine or proline, and the proton-translocating ATPase; see Table 1) (36, 93). Compartmentalization of these proteins can be explained by hypothesizing the existence of an «anchoring signal» of hydrophobic nature in the polypeptide that would render the later unable to traverse completely the ER membrane while being translated (70); that is, the peptide would remain anchored at the

vesicle membrane with at least one domain at the luminal side and another one at the cytroplasmic side (in a more complex situation, there may be more than one anchoring domain). These proteins would move along the secretory route as integral components of the transport organelle membrane, and after fusion of the secretory vesicles with the plasma membrane, would become stably incorporated in the later (70).

The *S. cerevisiae* plasma membrane protein more extensively studied at a molecular level is the proton-translocating ATPase, which is transported to the plasma membrane by the already described secretory pathway (36). Its gene has been cloned and the amino acid sequence has been deduced (81). It contains several hydrophobic domains that may function as anchoring regions, as would be expected from the model described above (29, 81).

Incorporation of proteins to external compartments

Yeast extracytoplasmic proteins are transported inside the lumen of the membranous organelles involved in secretion (26, 74), in such a way that after fusion of the latter with the plasma membrane, the proteins are released into the periplasmic space. There are three possible targets of the molecules at this stage: the periplasmic space, the cell wall structure and the extracellular medium, and examples of the three situations are known Table 1).

The yeast cell wall is a rigid structure formed by a fibrillar network of glucan plus chitin, and an amorphous mannoprotein material that fills the structural network and is specially enriched at the more external layers of the wall (10, 80). The periplasmic space is supposed to be delimited by the plasma membrane and the cell wall (77), although periplasmic mannoprotein enzymes may be interacting loosely with the wall structure (80). Mannoproteins can be liberated in a native state from purified cell walls by glucan digestion with the gulcanase complex Zymolase or by endogenous glucanases. In this way, two types of wall mannoproteins are solubilized in *S. cerevisiae* (28, 60, 100): a polydisperse high molecular weight material (larger than 120 kDa) which is probably covalently linked to glucan, and a 33 kDa mannoprotein non-covalently linked to other wall components. A similar pattern of wall mannoproteins is observed in a number of yeast species (34). In *C. albicans,* the high molecular weight material is resolved in four and two molecular species respectively in blastospores and mycelial cells (21); a 34 kDa mannoprotein that crossreacts with antibodies against the *S. cerevisiae* 33 kDa species is also present in both morphological forms (21, 34).

Before being stably incorporated into the wall, mannoproteins destined to this compartment are detected transiently at the periplasmic space in pulse and chase experiments followed by fractionation of the extracytoplasmic compartments (50, 59). The reasons why these mannoproteins finally integrate into the rigid structure of the wall in contrast with invertase and other periplasmic enzymes are unknown at present. However, several observations may shed some light on the problem:

i) In whole cells treated with antibiotics that inhibit glucan synthesis such as aculeacin A and papulacandin B (19), wall mannoproteins are released in part into the growth medium (50, 95).

ii) Regenerating protoplasts of *S. cerevisiae* and *C. albicans* are unable to retain mannoproteins in the forming wall until a dense fibrillar network of glucan plus chitin has been formed on the protoplast surface (20, 51, 95); in *C. albicans,* incorporation of the high molecular weight mannoprotein occurs later than the 34 kDa species and it marks the time when protoplasts become osmotically stable, a situation that is not reached in *S. cerevisiae* protoplasts suspended in liquid medium.

From these results, a model has been proposed to explain the biogenesis of the yeast cell wall, a process that is localized at the bud region in normal cells and occurs over the whole surface in regenerating protoplasts (20, 80). Essentially, as they are synthesized by means of enzyme complexes located at the plasma membrane (10), glucan and chitin form a cristalline structure that is required for mannoproteins to be deposited on the surface; incorporation of the high molecular weight mannoprotein

would be the last event in wall maturation: it occurs with the formation of covalent linkages with glucan and parallels the transformation form alkali-soluble to alkali-insoluble glucan. Some mannoprotein material is also probably covalently linked to chitin (Elorza *et al.*, in press). In whole cells of *S. cerevisiae* and *C. albicans*, the non-glycosylated forms of the 33-34 kDa species and of the high molecular weight material that are transported to the cell surface become efficiently incorporated into the walls, so that molecules lacking the N-linked carbohydrate are not released into the growth medium (60, our unpublished results). Thus, no N-linked sugar chains are needed for these molecules to become integrated in the walls. Since the large molecular weight material of *S. cerevisiae* and *C. albicans* contains a significant amount of O-linked carbohydrate (94, 101, Elorza *et al.*, in press), the possibility exists that these oligosaccharides covalently link the mannoprotein with glucan.

The yeast cell wall can be contemplated as a barrier that avoids the release of soluble periplasmic mannproteins into the growth medium. Periplasmic invertase is in the form of octamers (14, 22), and other periplasmic enzymes such as acid phosphatase and α-galactosidase also form oligomers (22). These observations support the view that retention of yeast mannoproteins in the periplasmic space is facilitated by the formation of high molecular weight complexes.

On the other hand, liberation of α-factor into the growth medium may be caused by the small size of the mature molecule, which would be able to traverse the wall structure. Mature killer toxin is constitued by two subunits of 86 and 83 amino acids each, linked by three disulfide bonds (89); obviously, this size seems too high for free diffusion across the wall, and the same happens with two *S. cerevisiae* exoglucaneses also secreted into the medium (33, 72). However, the killer toxin and the exoglucanases appear transiently associated with tha wall structure, before their diffusion into the growth medium (33, 89). Thus, it is reasonable to believe that liberation of these large protein molecules to the extracellular space is due to an active process involving localized alteration of the wall structure.

In the near future, studies on the final location of hybrid peptides (such as those described for carboxypeptidase Y-invertase hybrids) may lead to a better understanding of the mechanisms underlaying the differential location of proteins at the extracytoplasmic compartments.

Secretion of heterologous proteins

The rapid progress in the molecular genetics of *S. cerevisiae* in recent years has made this organism a useful host for the expression of heterologous proteins. Compared with bacteria, yeasts have the advantage of being able to glycosylate the proteins in a way which is similar (but not identical) to higher eucaryotes; this may help to preserve the functionally active sites or the correct antigenic epitopes. However, for the system to be feasible at an industrial level, the heterologous product should be efficiently secreted into the growth medium, which would allow its rapid and simple isolation. Thus, a number of expression and secretion vectors have been constructed trying to optimize these parametres. Ref. 43 gives a list of higher eucaryotic proteins that are expressed in *S. cerevisiae* using such vectors. Here we will consider some of the problems that arise when trying to express and secrete heterologous proteins in yeast.

In some cases, yeast cells can recognize the heterologous signal peptide, so that the native foreign protein can be delivered into the medium without further genetic manipulation. For example, wheat (69), mouse (27) and human (52) α-amylases are expressed and secreted with their own signal peptides, although the recovery from the medium reaches at most 50 % of the total synthesized product. Human influenza virus hemaglutinin is also secreted (and concommitantly glycosylated) with its own signal peptide, and in this case it has been shown that the yeast signal peptidase recognizes and correctly processes the signal peptide (37). However, other examples are known [i.e. human interferon-γ (35)] where the efficiency of secretion with native signal sequences is low, and a fraction of

the molecules are processed improperly by the host signal peptidase. Although native human lysozyme can be expressed at a high level in *S. cerevisiae*, the product accumulates in the cytoplasm in an insoluble and biologically inactive form (32), as happens with many foreign proteins expressed in *Escherichia coli* which are not delivered to the external space.

The above problems reflect the high stringency of *S. cerevisiae* in the recognition of strange signal peptides by the internalization machinery of the cell at the ER level. Therefore, secretion vectors have been made by DNA recombinant techniques in which the gene sequence for the mature foreign protein is fused in frame to a yeast DNA coding for a signal sequence that replaces that of the protein to be expressed. Signal sequences of α-factor (7, 30), killer toxin (91) and invertase (53, 84) are employed. In the two former cases, hybrid proteins are formed that contain the N-terminal prepro region of the respective precursor of α-factor or killer toxin, fused to the heterologous protein; such hybrids are processed by the host proteolytic machinery along the secretory pathway, allowing the delivery of the foreign protein to the growth medium. In the case of the invertase signal peptide, this is directly linked to the sequence of the mature foreign protein, and the constructions are adequately processed by the host signal peptidase. All the constructions are put under the control of strong yeast promotors (i.e. from the genes for alcohol dehydrogenase, phosphoglycerate kinase, invertase or acid phosphatase). However, the levels of foreign protein reached in the growth medium are far from optimal. From their studies, Smith *et al.* (84) have concluded the existence of a rate-limiting step in the *S. cerevisiae* secretory pathway and have isolated supersecreting mutants in which the secretion levels of heterologous proteins are increased more than ten fold; using these mutants, they have obtained titers of more than 20 mg of activable calf prochymosine per litre of growth medium.

Even after attaining a high level of secretion, the heterologous product may remain trapped at the periplasmic space or at the cell wall. This may not affect negatively the solubility of the product, but adds a new step for its recovery and purification.

To optimize the conditions for expressing foreign genes, some more aspects have to be considered:

i) Upstream and downstream DNA sequences may influence in a subtle way the expression of the foreign gene (47, 85), for example affecting the activity of positive or negative regulators of transcription, the spatial structure of the mRNA, or the frequency of translation initiation.

ii) Yeast genes do not employ synonymous codons in a random way: there exists a codon usage bias that is different for highly and lowly expressed genes (82). This preference for certain codons correlates with factors such as tRNA abundance, and may affect expression of foreign genes with a different frequency of codon usage (46).

Thus, expression of the heterologous genes and secretion of the products in yeast cells is affected by a number of factors (not always well understood) that should be investigated in each particular case when it is intended to maximize the production.

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References

1. Achstetter, T. and Wolf, D. H. (1985). Hormone processing and membrane-bound proteinases in yeast. The EMBO J. **4**, 173-177.
2. Ammerer, G., Hunter, C. P., Rothman, J. H., Saari, G. C., Valls, L. A. and Stevens, T. H. (1986). PEP4 gene of *Saccha-*

romyces cerevisiae encodes proteinase A, a vacuolar enzyme required for processing of vacuolar precursors. Mol. Cell. Biol. **6,** 2490-2499.

3. Ballou, C. E. (1982). Yeast cell wall and cell surface. *In:* J. N. Strathern, E. W. Jones and J. R. Broach (eds.). The Molecular Biology of the Yeast Saccharomyces. Vol. **2,** pp 335-360. Cold Spring Hanbor Laboratory, Cold Spring Harbor, NY.
4. Barbaric, S., Mrša, V. and Mildner, P. (1984). Role of the carbohydrate part of yeast acid phosphatase. Arch. Biochem. Byophys. **234,** 567-575.
5. Blachly-Dyson, E. and Stevens, T. H. (1987). Yeast carboxypeptidase Y can be translocated and glycosylated without its amino-terminal signal sequence. J. Cell. Biol. **104,** 1183-1191.
6. Bostian, K. A., Elliot, Q., Bussey, H., Burn, V., Smith, A. and Tipper, D. J. (1984). Sequence of the perprotoxin dsRNA gene of type I killer yeast: multiple processing events produce a two-component toxin. Cell. **36,** 741-751.
7. Brake, A. J., Merryweather, J. P., Coit, D. G., Heberlein, U. A., Masiarz, F. R., Mullenback, G. T., Urdea, M. S., Valenzuela, P. and Barr, P. J. (1984). α-Factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. USA. **81,** 4642-4646.
8. Bussey, H., Saville, D., Green, D., Tipper, D. J. and Bostian, K. A. (1983). Secretion of *Saccharomyces cerevisiae* killer toxin: processing of the glycosylated precursor. Mol. Cell. Biol. **3,** 1362-1370.
9. Byrd, J. C., Tarentino, A. L., Maley, F., Atkinson, P. H. and Trimble, R. B. (1982). Glycoprotein synthesis in yeast. Identification of Man_8 $GlcNA_{c2}$ as an essential intermediate in oligosaccharide processing. J. Biol. Chem. **257,** 14657-164666.
10. Cabib, E., Roberts, R. and Bowers, B. (1982). Synthesis of the yeast cell wall and its regulation. Annu. Rev. Biochem. **51,** 763-793.
11. Carlson, M., Taussig, R., Kustu, S. and Botstein, D. (1983). The secreted form of invertase in *Saccharomyces cerevisiae* is synthesized from mRNA encoding a signal frequence. Mol. Cell. Biol. **3,** 439-447.
12. Chu, F. K. and Maley, F. (1982). Stabilization of the structure and activity of yeast carboxypeptidase Y by its high-amannose oligosaccharide chains. Arch. Biochem. Biophys. **214,** 134-139.
13. Chu, F. K., Takase, K., Guarino, D. and Maley, F. (1985). Diverse properties of external and internal forms of yeast invertase derived from the same gene. Biochemistry **24,** 6125-6132.
14. Chu, F. K., Watorek, W. and Maley, F. (1983). Factors affecting the oligomeric structure of yeast external invertase. Arch. Biochem. Biophys. **223,** 543-555.
15. Deshaies, R. J. and Schekman, R. (1987). A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. J. Cell Biol. **105,** 633-645.
16. Dingwall, C. and Laskey, R. A. (1986). Protein import into the cell nucleus. Annu. Rev. Cell Biol. **2,** 367-390.
17. Dmochowska, A., Dignard, D., Henning, D., Thomas, D. Y. and Bussey, H. (1987). Yeast KEX1 gene encodes a putative protease with a carboxypeptidase B-like function involved in killer toxin and α-factor precursor processing. Cell **50,** 573-584.
18. Douglas, M. G., McCammon, M. T. and Vassarotti, A. (1986). Targeting proteins into mitochondria. Microbiol. Rev. **50,** 166-178.
19. Durán, A., Varona, R., Pérez, P. and García-Acha, I. (1984). Biosynthesis of fungal wall ß-glucan and its inhibition by antifungal agents. *In:* C. Nombela (ed.). Microbial Cell Wall Synthesis and Autolysis, pp. 297-305. Elsevier Science Pub. B.V. Amsterdam.
20. Elorza, M. V., Murgui, A., Rico, H., Miragall, F. and Sentandreu, R. (1987). Formation of a new cell wall by protoplasts of *Candida albicans:* effect of papulacandin B, tunicamycin and nikkomycin. J. Gen. Microbiol. **133,** 2315-2325.
21. Elorza, M. V., Murgui, A. and Sentandreu, R. (1985). Dimorphism in *Candida albicans:* contribution of mannoproteins to the architecture of yeast and mycelial cell walls. J. Gen. Microbiol. **131,** 2209-2216.
22. Esmon, P. C., Esmon, B. E., Schauer, I. E., Taylor, A. and Schekman, R. (1987). Structure, assembly and secretion of octameric invertase. J. Biol. Chem. **262,** 4387-4394.
23. Farquhar, M. G. (1985). Progress in unraveling pathways of Golgi traffic. Annu. Rev. Cell Biol. **1,** 447-488.
24. Feldman, R. I., Bernstein, M. and Schekman, R. (1987). Product of SEC53 is required for folding and glycosylation of secretory proteins in the lumen of yeast endoplasmic reticulum. J. Biol. Chem. **262,** 9332-9339.
25. Ferro-Novick, S., Hansen, W., Schauer, I. and Schekman, R. (1984). Genes required for completion of import of proteins into the endoplasmic reticulum in yeast. J. Cell Biol. **98,** 44-53.
26. Ferro-Novick, S., Novick, P., Field, C. and Schekman, R. (1984). Yeast secretory mutants that block the formation of active cell surface enzymes. J. Cell Biol. **98,** 35-43.
27. Filho, S. A., Galembeck, E. V., Faria, J. B. and Frascino, A. C. S. (1986). Stable yeast transformants that secrete functional α-amylase encoded by cloned mouse pancreatic cDNA. Biotechnology. **4,** 311-315.
28. Frevert, J. and Ballou, C. E. (1985). *Saccharomyces cerevisiae* structural cell wall mannoprotein. Biochemistry. **24,** 753-759.
29. Ghislain, M., Schelesser, A. and Goffeau, A. (1987). Mutation of a conserved glycine residue modifies the vanadate sensitivity of the plasma membrane H^+-ATPase from *Schizosaccharomyces pombe.* J. Biol. Chem. **262,** 17549-17555.
30. Green, R., Schaber, M. D., Shieds, D. and Kramer, R. (1986). Secretion of somatostatin by *Saccharomyces cerevisiae.* Correct processing of an α-factor-somatostatin hybrid. J. Biol. Chem. **262,** 7558-7565.
31. Haselbeck, A. and Tanner, W. (1983). O-glycosylation in *Saccharomyces cerevisiae* is initiated at the endoplasmic reticulum. FEBS Lett. **158,** 335-338.

32. Hayakawa, T., Toibana, A., Marumoto, R., Nakahama, K., Kikuchi, M., Fujimoto, K. and Ikehara, M. (1987). Expression of human lysozyme in an insoluble form in yeast. Gene. **56,** 53-59.
33. Hernández, L. M., Ramírez, M., Olivero, I. and Larriba, G. (1986). Accumulation and secretion of exoglucanase activity in yeast secretory mutants. Arch. Microbiol. **146,** 221-226.
34. Herrero, E., Sanz, P. and Sentandreu, R. (1987). Cell wall proteins liberated by Zymolyase from several Ascomycetous and imperfect yeasts. J. Gen. Microbiol. **133,** 2895-2903.
35. Hitzeman, R. A., Leung, D. W., Perry, L. J., Kohr, W. J., Levine, H. L. and Goeddel, D. V. (1983). Secretion of human interferon by yeast. Science. **219,** 620-625.
36. Holcomb, C. L., Hansen, W. J., Etcheverry, T. and Schekman, R. (1988). Secretory vesicles externalize the major plasma membrane ATPase in yeast. J. Cell Biol. **106,** 641-648.
37. Jabbar, M. A. and Nayak, D. P. (1987). Signal processing, glycosylation and secretion of mutant hemagglutinins of a human influenza virus by *Sacharomyces cerevisiae*. Mol. Cell. Biol. **7,** 1476-1485.
38. Johnson, L. M., Bankaitis, V. A. and Emr, S. D. (1987). Distinct sequence determinants direct intracellular sorting and modification of a yeast vacuolar protease. Cell. **48,** 875-885.
39. Julius, D., Blair, L., Brake, A., Sprague, G. and Thorner, J. (1983). Yeast α-factor is processed from a larger precursor polypeptide: the essential role of a membrane-bound dipeptidyl aminopeptidase. Cell. **32,** 839-852.
40. Julius, D., Schekman, R. and Thorner, J. (1984). Glycosylation and processing of prepo-α-factor through the yeast secretory pathway. Cell. **36,** 309-318.
41. Kaiser, C. A. and Botsein, D. (1986). Secretion-defective mutations in the signal sequence of *Saccharomyces cerevisiae* invertase. Mol. Cell Biol. **6,** 2382-2391.
42. Kaiser, C. A., Preuss, D.; Grisafi, P. and Botstein, D. (1987). Many random sequences functionally replace the secretion signal sequence of yeast invertase. Science. **235,** 312-317.
43. Kingsman, S. M., Kingsman, A. J. and Mellor, J. (1987). The production of mammalian proteins in *Saccharomyces cerevisiae*. Trends Biotechnol. **5,** 53-56.
44. Kukuruzinska, M. A., Bergh, M. L. E. and Jackson, B. J. (1987). Protein glycosylation in yeast. Annu. Rev. Biochem. **56,** 915-944.
45. Lemmon, S. K. and Jones, E. W. (1987). Clathrin requirement for normal growth of yeast. Science. **238,** 504-509.
46. Maruyama, T., Gojobori, T., Aota, S. and Ikemura, T. (1986). Codon usage tabulated from the Gene Bank genetic sequence data. Nucl. Acids. Res. **14,** r151-r197.
47. Meilor, J., Dobson, M. J., Roberts, N. A., Kingsman, A. J. and Kingsman, S. M. (1985). Factors affecting heterologous gene expression in *Saccharomyces cerevisiae*. Gene. **33,** 215-226.
48. Mrša, V., Barbarić, S., Ries, B. and Mildner, P. (1987). Role of glycosylation in secretion of yeast acid phosphatase. FEBS Lett. **217,** 174-179.
49. Mueller, S. and Branton, D. (1984). Identification of coated vesicles in *Saccharomyces cerevisiae*. J. Cell. Biol. **98,** 341-346.
50. Murgui, A., Elorza, M. V. and Sentandreu, R. (1985). Effect of papulacandin B and calcofluor white on the incorporation of mannoproteins in the wall of *Candida albicans* blastospores. Biochim. Biophys. Acta. **841,** 215-222.
51. Murgui, A., Elorza, M. V. and Sentandreu, R. (1986). Tunicamycin and papulacandin B inhibit incorporation of specific mannoproteins into the walls of *Candida albicans* regenerating protoplasts. Biochim. Biophys. Acta. **884,** 550-558.
52. Nakamura, Y., Sato, T., Emi, M., Miyanohara, A., Nishide, T. and Matsubara, K. (1986). Expression of human salivary α-amylase gene in *Saccharomyces cerevisiae* and its secretion using the mammalian signal sequence. Gene. **50,** 239-245.
53. Nishizawa, M., Ozawa, F. and Hishinuma, F. (1987). Construction of an expression and secretion vector for the yeast *Saccharomyces cerevisiae*. Agric. Biol. Chem. **51,** 515-521.
54. Novick, P. and Botstein, D. (1985). Phenotypic analysis of temperature-sensitive yeast actin mutants. Cell. **40,** 405-416.
55. Novick, P., Ferro, S. and Schekman, R. (1981). Order of events of the yeast secretory pathway. Cell. **25,** 461-469.
56. Novick, P. and Schekman, R. (1983). Export of major cell surface proteins is blocked in yeast secretory mutants. J. Cell Biol. **96,** 541-547.
57. Olden, K., Bernard, B. A., Humphries, M. J., Yeo, K. T., White, S. L., Newton, S. A., Bauer, H. C. and Parent, J. B. (1985). Function of glycoprotein glycans. Trends Biochem. Sci. **10,** 78-82.
58. Palade, G. E. (1975). Intracellular aspects of the process of protein secretion. Science. **189,** 347-358.
59. Pastor, F. I. J., Herrero, E. and Sentandreu, R. (1982). Metabolism of *Saccharomyces cerevisiae* envelope mannoproteins. Arch. Microbiol. **132,** 144-148.
60. Pastor, F. I. J., Valentín, E., Herrero, E. and Sentandreu, R. (1984). Structure of the *Saccharomyces cerevisiae* cell wall: mannoproteins released by Zymolyase and their contribution to wall architecture. Biochim. Biophys. Acta. **802,** 292-300.
61. Payne, G. S., Hasson, T. B., Hasson, M. S. and Schekman, R. (1987). Genetic and biochemical characterization of clathrin-deficient *Saccharomyces cerevisiae*. Mol. Cell Biol. **7,** 3888-3898.
62. Payne, G. S. and Schekman, R. (1985). A test of clathrin function in protein secretion and cell growth. Science. **230,** 1009-1014.
63. Pearse, B.M.F. and Bretscher, M. S. (1981). Membrane recycling by coated vesicles. Annu. Rev. Biochem. **50,** 85-101.
64. Perlman, D., Raney, P. and Halvorson, H. O. (1986). Mutations affecting the signal sequence alter synthesis and secretion of yeast invertase. Proc. Natl. Acad. Sci. USA. **83,** 5033-5037.

65. Pfeffer, S. R. and Rothman, J. E. (1987). Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Annu. Rev. Biochem. **56**, 829-852.
66. Rothblatt, J. A. and Meyer, D. I. (1986). Secretion in yeast: reconstitution of the translocation and glycosilation of α-factor and invertase in a homologous cell-free system. Cell. **44**, 619-628.
67. Rothblatt, J. A. and Meyer, D. I. (1986). Secretion in yeast: translocation and glycosylation of prepro-α-factor *in vitro* can occur via an ATP-dependent post-translational mechanism. The EMBO J. **5**, 1031-1036.
68. Rothman, J. H. and Stevens, T. H. (1986). Protein sorting in yeast: mutants defective in vacuolar biogenesis mislocalize vacuolar proteins into the late secretory pathway. Cell. **47**, 1041-1051.
69. Rothstein, S. J., Lahners, K. N., Lazarus, C. M., Baulcombe, D. D. and Gatenby, A. A. (1978). Synthesis and secretion of wheat α-amylase in *Saccharomyces cerevisiae*. Gene. **55**, 353-356.
70. Sabatini, D. D., Kreibach, G. Morimoto, T. and Adesnik, M. (1982). Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. **92**, 1-22.
71. Salminen, A. and Novick, P. J. (1987). A *ras*-like protein is required for a post-Golgi event in yeast secretion. Cell. **49**, 527-538.
72. Sánchez, A., Villanueva, J. R. and Villa, T. G. (1982). *Saccharomyces cerevisiae* secretes two exo-ß-glucanases. FEBS Lett. **138**, 209-212.
73. Sánchez, A., Villanueva, J. R. and Villa, T. G. (1982). Effect of tunicamycin on exo-1,3-β-D-glucanase synthesis and secretion by cells and protoplasts of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **128**, 3051-3060.
74. Sanz, P., Herrero, E. and Sentandreu, R. (1987). Secretory pattern of a major integral mannoprotein of the yeast cell wall. Biochim. Biophys. Acta. **924**, 193-203.
75. Schauer, I., Emr, S., Gross, C. and Schekman, R. (1985). Invertase signal and mature sequence subsitutions that delay intercompartmental transport of active enzyme. J. Cell Biol. **100**, 1664-1675.
76. Schekman, R. (1985). Protein localization and membrane traffic in yeast. Annu. Rev. Cell Biol. **1**, 115-143.
77. Schekman, R. and Novick, P. (1982). The secretory process and cell surface assembly. *In:* J. Strathern, E. Jones and J. Broach (eds.). The Molecular Biology of the Yeast Saccharomyces. Vol. **2**, pp. 361-393. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
78. Schönholzer, F., Schweingruber, A. M., Trachsel, H. and Schweingruber, M. E. (1985). Intracellular maturation and secretion of acid phosphatase of *Saccharomyces cerevisiae*. Eur. J. Biochem. **147**, 273-279.
79. Schawaiger, H., Hasilik, A., von Figura, K., Wiemken, A. and Tanner, W. (1982). Carbohydrate-free carboxypeptidase Y is transfered into the lysosome-like yeast vacuole. Biochem. Biophys. Res. Commun. **104**, 950-956.
80. Sentandreu, R., Herrero, E., Martínez, J. P. and Larriba, G. (1984). Biogenesis of the yeast cell wall. Subcell. Biochem. **10**, 193-235.
81. Serrano, R., Kielland-Brandt, M. C. and Fink, G. R. (1986). Yeast plasma membrane ATPase is essential for growth and has homology with $(Na^+ + K^+)$, K^+-and Ca^{2+}-ATPases. Nature. **319**, 689-693.
82. Sharp, P. M., Tuohy, T. M. F. and Mosurski, K. R. (1986). Codon usage in yeast: cluster analysis clearly differentiates highly and lowly expressed genes. Nucl. Acids Res. **14**, 5125-5143.
83. Sloat, B., Adams, A. and Pringle, J. R. (1981). Roles of the CDC24 gene product in cellular morphogenesis during the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. **89**, 395-405.
84. Smith, R. A., Duncan, M. J. and Moir, D. T. (1985). Heterologous protein secretion from yeast. Science. **229**, 1219-1224.
85. Stanway, C., Kingsman, A. J. and Kingsman, S. M. (1987). The control of transcription in *Saccharomyces cerevisiae*. BioEssays. **7**, 62-67.
86. Stevens, T., Esmon, B. and Schekman, R. (1982). Early stages of the yeast secretory pathway are required for transport of carboxypeptidase Y to the vacuole. Cell. **30**, 439-448.
87. Stevens, T. H., Rothman, J. H., Payne, G. S. and Schekman, R. (1986). Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. J. Cell Biol. **102**, 1551-1557.
88. Tanner, W. and Lehle, L. (1987). Protein glycosylation in yeast. Biochim. Biophys. Acta. **906**, 81-89.
89. Tipper, D. J. and Bostian, K. A. (1984). Double-stranded ribonucleid acid killer systems in yeasts. Microbiol. Rev. **48**, 125-156.
90. Tohoyama, H. and Yanagishima, N. (1986). The sexual agglutination substance is secreted through the yeast secretory pathway in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **201**, 446-449.
91. Tokunaga, M., Wada, N. and Hishinuma, F. (1987). A novel yeast secretion vector utilizing secretion signal of killer toxin encoded on the yeast linear DNA plasmid PGKL1. Biochem. Biphys. Res. Commun. **144**, 613-619.
92. Trimble, R. B., Maley, F. and Chu, F. K. (1983). Glycoprotein biosynthesis in yeast. Protein conformation affects processing of high mannose oligosaccharides on carboxypeptidase Y and invertase. J. Biol. Chem. **258**, 2562-2567.
93. Tschopp, J., Esmon, P. C. and Schekman, R. (1984). Defective plasma membrane assembly in yeast secretory mutants. J. Bacteriol. **160**, 966-970.
94. Valentín, E., Herrero, E., Rico, H., Miragall, F. and Sentandreu, R. (1987). Cell wall mannoproteins during the population growth phases in *Saccharomyces cerevisiae*. Arch. Microbiol. **148**, 88-94.
95. Valentín, E., Herrero, E. and Sentandreu, R. (1986). Incorporation of mannoproteins into the walls of aculeacin A-treated yeast cells. Arch. Microbiol. **146**, 214-220.

96. Valls, L. A., Hunter, C. P., Rothman, J. H. and Stevens, T. H. (1987). Protein sorting in yeast vacuolar carboxypeptidase Y resides in the propeptide. Cell. **48**, 887-897.
97. von Figura, K. and Hasilik, A. (1986). Lysosomal enzymes and their receptors. Annu. Rev. Biochem. **55**, 167-193.
98. von Hejne, G. (1985). Signal sequences: the limits of variation. J. Mol. Biol. **184**, 99-105.
99. Waters, M. G. and Blobel, G. (1986). Secretory protein translocation in a yeast cell-free system can occur posttranslationally and requires ATP hydrolysis. J. Cell Biol. **102**, 1543-1550.
100. Zlotnik, H., Fernández, M. P., Bowers, B. and Cabib, E. (1984). *Saccharomyces cerevisiae* mannoproteins from an external cell wall layer that determines wall porosity. J. Bacteriol. **159**, 1018-1026.
101. Zueco, J., Mormeneo, S. and Sentandreu, R. (1986). Temporal aspects of the O-glycosylation of the *Saccharomyces cerevisiae* mannoproteins. Biochim. Biophys. Acta. **884**, 93-100.

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Inducción de celulasas y xilanasas en *Aureobasidium pullulans*

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Summary

The specificity or induction of wood-degrading enzymes using series of mono, di or polissacharides as carbon source for the yeasts *Geotrichum candidum* and *Trychosporon penicillatum* and the yeast-like organism *Aureobadidium pullulans* were studied. The strain *A. pullulans* was the only one that when xylan or «steam exploded wood» were used as carbon source all the enzymes tested were found. This strain was unable to grow on carboxymethyl or Avicel cellulose. D-xylose was the nutritional inducer of ß-xylosidase and ß-xylanase but D-glucoronic acid induced CMCase activity and ß-glucosidase was produced with every carbon source. The 1,4 ß-xylobiose was not an inducer of ß-xylanase but with the structural 1,2-ß-and 1,3-ß-xylobiose isomers high levels of this enzyme were obtained.

Key words: Cellulases, xylanases, yeast, fungi, Aureobasidium pullulans.

Resumen

Se ha estudiado la inducción de los enzimas celulolíticos y hemicelulolíticos por diferentes mono, di o polisacáridos utilizados como fuente de carbono en el desarrollo de las especies *Geotrichum candidum, Trichosporon penicillatum* y *Aureobasidium pullulans. A. pullulans* fue la especie que presentó un mayor desarrollo celular y la única que al utilizar las cadenas de xilanos o el «steam exploded wood» como fuente de carbono excretó completo el complejo enzimático estudiado. Sin embargo, no pudo desarrollarse sobre carboximetilcelulosa (CMC) o sobre celulosa Avicel. El inductor nutricional de la ß-xilosidasa y de la ß-xilanasa fue la D-xilosa y el ácido D-glucurónico indujo la actividad CMCasa. La ß-glucosidasa fue producida con todas las fuentes de carbono utilizadas, aunque en bajos niveles. La 1,4-ß-xilobiosa no fue inductor de la ß-xilanasa, pero sus isómetros estructurales 1,2 y 1,3-ß-xilobiosa produjeron altos niveles de enzima.

Introducción

La producción de celulasas en microorganismos ha sido descrita sobre todo en hongos como *Trichoderma viride, Myrothecium verrucaria, Aspergillus niger* (7, 14, 15, 18, 27) *Penicillium finiculosum* (22) y *Fusarium solani* (28) entre otros. Se conocen muy pocos microorganismos celulolíticos que sinteticen y excreten al medio de cultivo el complejo enzimático completo capaz de degradar la celulosa nativa altamente ordenada de la fibra de algodón. Así, por ejemplo, la especie *Trichoderma reseii* excreta el complejo enzimático completo de celulasas que contiene endo y exoglucanasas ade-

(*) A quien debe dirigirse la correspondencia.

más de ß-glucosidas (celobiasa), las cuales actúan sinergéticamente para degradar la celulosa (incluso la cristalina) en azúcares solubles (8). También en hongos y bacterias han sido aisladas y caracterizadas numerosas enzimas ß-xilanasas y ß-xilosidasas capaces de hidrolizar las cadenas de los xilanos (4, 5).

Sin embargo, los datos referentes a la producción de celulasas y xilanasas en levaduras son bastante limitados (1, 2, 6, 22, 24, 26) a pesar de que el desarrollo de levaduras sobre celulosas y hemicelulosas de residuos agrícolas es de un gran interés para la obtención de proteínas para alimentación animal.

En este trabajo se ha estudiado la producción de celulasas, ß-xilanasas y ß-xilosidasas por las especies *Geotrichum candidum, Trichosporon penicillatum* y *Aureobasidium pullulans,* seleccionadas entre las especies utilizadas en el aprovechamiento de residuos celulósicos en anteriores trabajos (19, 20). El estudio con *A. pullulans* se realizó a partir de medios sintéticos conteniendo mono, di o polisacáridos como fuente de carbono o bien sobre maderas tratadas por explosión al vapor («exploded wood»), con vistas a la utilización de este sistema de hidrólisis para el aprovechamiento de residuos agrícolas en alimentación animal.

Materiales y métodos

Microorganismos

En este trabajo se utilizaron las especies de levaduras *Geotrichum candidum* (cepa 1471) y *Trichosporon penicillatum (Geotrichum penicillatum)* (cepa 1735), y la especie *Aureobasidium pullulans* (cepa 1740) que presenta características similares a las de levaduras, todas ellas procedentes de la colección del Instituto de Fermentaciones Industriales (CSIC).

Métodos de cultivo

El cultivo de las levaduras se llevó a cabo en un agitador a 30° C y a pH 5 utilizando frascos Erlenmeyer con 500 ml de medio de cultivo.

Los medios utilizados para la selección de microorganismos, en placa Petri, fueron los empleados por Bravery (3). Todos estos medios contenían 1 g/l de KH_2PO_4; 0,5 g/l de KCl; 0,2 g/l de Mg $SO_4 7H_2O$; 0,1 g/l de Ca Cl_2 y 20 g/l de agar agar, con las adiciones que figuran en la tabla 1 y las fuentes de carbono que se detallan en cada caso.

El medio utilizado para la obtención de inóculos y en los ensayos de inducción contenía: 1 g/l de KH_2PO_4, 1,5 g/l de $(NH_4)_2SO_4$; 0,8 g/l de urea; 0,5 g/l de KCl; 0,5 g/l de $MgSO_4 \cdot 7H_2O$; 0,1 g/l de $CaCl_2$; 1 ml/l de una solución de vitamina B_1 (0,1 mg/l), 1 ml/l de solución biotina (0,025 mg/l)

TABLA 1
CONSTITUYENTES DE LOS MEDIOS UTILIZADOS POR BRAVERY (3)

Medios de cultivo	I	III	IV	V	VI	VII
	g/l					
DL-asparagina	0,5	0,5	0,25	0	0	0
Extracto de levadura	0,5	0	0	0,5	0,25	0
$(NH_4)_2 SO_4$	0,5	0,5	0,521	0,543	0,543	0,543
Clorhidrato de tiamina	0	0,001	0,001	0	0	0,001

y 1 ml/l de una solución de metales ($M_nCl_2 \cdot H_2O$; $ZnSO_4 \cdot 7H_2O$; $CuSO_4 \cdot 5H_2O$ y $Cl_3Fe \cdot 6H_2O$, 1,8 mg/l) y la fuente de carbono que se detalla en cada caso.

A los tiempos de incubación que se indican en cada caso, las células se centrifugaron (3.500 xg) 15' a 4° C y en los sobrenadantes se analizaron las actividades enzimáticas.

Determinación de actividades enzimáticas

Las actividades CMCasa y ß-xilanasa se midieron, a 45° C, siguiendo la liberación de los azúcares reductores a partir de una solución de carboximetil celulosa al 1 % (7H35 x F Hércules) o de una suspensión (2,5 g/l) de xilanos (Larch Wood de Sigma) (10). Los azúcares liberados se determinaron por el método del ácido dinitrosalicílico (DNS) (16). Una unidad de CMCasa o ß-xilanasa se define como la cantidad de enzima capaz de liberar 1 μmol de azúcares reductores (calculado como glucosa o xilosa) por minuto.

La ß-glucosidasa y la ß-xilosidasa se ensayaron por el método descrito por Ghose *et al.* que utiliza p-nitrofenil ß-D-glucopiranosido o xilopiranosido, respectivamente (10). Una unidad de ß-glucosidasa o de ß-xilosidasa se define como la cantidad de enzima capaz de liberar 1 μmol de p-nitrofenol por minuto.

Determinación de proteínas

En los casos que se indican la determinación de proteínas se realizó según el método Lowry (13), después de someter las muestras a un proceso de ultrafiltración (células Amicon con membranas de PM 10) para eliminar los artefactos debidos a la fuente de carbono o a las sales minerales que producían interferencias.

Determinación del peso seco

El crecimiento celular se siguió por la determinación del peso seco. Las células centrifugadas se lavaron 2 veces y se secaron en estufa a 105° C hasta peso constante.

Hidrólisis por «steam explosion»

El tratamiento de hidrólisis por el sistema de explosión al vapor y los análisis de las muestras así obtenidas se realizaron según los métodos descritos por Barnet (1984, Tesis doctoral, Universidad de Grenoble, Francia).

Ensayos de inducción

Las células crecidas sobre 1 % de glucosa y recolectadas al final de la fase logarítmica se separaron del medio de cultivo por centrifugación (4° C, 3.500 g durante 15') y se lavaron 2 veces con solución de KCl (9 g/l).

En cada caso las muestras con su correspondiente fuente de carbono se inocularon con 2 ml de una suspensión celular equivalente en materia seca a 1-2 mg/ml.

Productos utilizados

La 1,4-ß-xilotriosa y la 1,2 y 1,3-ß-xilobiosa fueron proporcionadas por el doctor Heyraud y el

doctor Utille del CERMAV respectivamente (9). La 4-tioxilobiosa y la 4-tiocelobiosa procedían de trabajos de síntesis realizados en el CERMAV (4, 21). Las muestras de maderas hidrolizadas (álamo, *Populus tremula)* por el sistema de explosión al vapor fueron proporcionadas por los doctores A. Excoffier y M. Vignon también del CERMAV.

Todos los otros compuestos utilizados procedían de casas comerciales: papel filtro Whatman, celulosa Avicel, Carboximetilcelulosa, 7H35 x f Hércules, Xilanos Larch Wood de Sigma, ß-metil-D-xilosido Sigma. Los restantes productos procedían de la casa Merck.

La pureza de estos compuestos fue comprobada por HPLC con columna μBondapak-NH_2 y con aceto-nitrilo-agua como solvente.

Resultados

Utilización de diferentes fuentes de carbono por Trichosporon penicillatum *(cepa 1735),* Geotrichum candidum *(cepa 1471)* y Aureobasidium pullulans *(cepa 1740).*

Utilizando las especies de levaduras *T. penicillatum* (cepa 1735), *G. candidum* (cepa 1471) y *A. pullulans* (cepa 1740) seleccionadas en trabajos previos (19, 20) para el aprovechamiento de residuos celulósicos, se estudió la utilización de diferentes polisacáridos como fuente de carbono por dichas especies.

En primer lugar se llevaron a cabo cultivos en placa Petri de *T. penicillatum, G. candidum* y *A. pullulans* sobre los medios de cultivo ya citados (medios I al VII) (3) y utilizando una mezcla de xilanos y papel de filtro como fuente de carbono.

Los resultados demostraron que *A. pullulans* (cepa 1740) fue el único que se desarrolló sobre dicha fuente de carbono con todos los medios utilizados. Al no haber diferencias apreciables en el desarrollo obtenido sobre los diferentes medios de cultivo, en los ensayos siguientes se utilizaron tan sólo el medio I (que contenía asparagina y extracto de levadura) y el medio VII (que contenía Vit. B1).

Con objeto de comprobar si estas especies de levaduras podrían utilizar diferentes di o polisacáridos (cadenas de xilanos o celulosa) y, por tanto, presumiblemente, poseer el complejo enzimático celulolítico o hemicelulolítico, se cultivaron dichas especies sobre diferentes fuentes de carbono en placas Petri. Los resultados obtenidos se presentan en la tabla 2.

Como podemos observar, *A. pullulans* (cepa 1740) presentó un mejor desarrollo celular que *T. penicillatum* (cepa 1735) y *C. candidum* (cepa 1471) al utilizar los xilanos, la α-celulosa, la celobiosa y el almidón como fuentes de carbono, aunque *T. penicillatum* presentó el mismo desarrollo al utilizar el papel de filtro. Por otra parte, *G. candidum* y *T. penicillatum* presentaron un mejor desarrollo al utilizar el ácido poligalacturónico.

Efecto de la fuente de carbono en la producción de enzimas celulolíticos y hemicelulolíticos

En primer lugar se realizaron unos ensayos previos para determinar la actividad de enzimas celulolíticos y hemicelulolíticos que presentaban las especies estudiadas al crecer sobre diferentes polisacáridos como fuente de carbono. En estos ensayos previos únicamente la especie *Aureobasidium pullulans* (cepa 1740) presentó dicho complejo enzimático, por lo que se eligió esta cepa para continuar este trabajo sobre producción e inducción de celulasas y xilanasas.

Por tanto, utilizando únicamente *A. pullulans* se estudió el tiempo necesario para inducir la actividad enzimática sobre diferentes monosacáridos y polisacáridos, utilizados como fuente de carbono, durante 1, 4, 6, 9, y 11 días de cultivo. Los resultados mostraron que no había ninguna variación enzimática después de 24 horas de cultivo. Por ejemplo, utilizando xilano como fuente de car-

TABLA 2
CRECIMIENTO DE *TRICHOSPORON PENICILLATUM, GEOTRICHUM CANDIDUM* Y *AUREOBASIDIUM PULLULANS* SOBRE DIFERENTES FUENTES DE CARBONO

Fuente de carbono	Tiempo de cultivo (días)	*Trichosporon penicillatum* (1735)		*Geotrichum candidum* (1471)		*Aureobasidium pullulans* (1740)	
		Medio de cultivo					
		I	VII	I	VII	I	VII
Xilanos	3						
	7	—	—	—	—	+++	+++
	14	—	—	—	—	+++	+++
Papel filtro	3	++	+	+	—	++	+
	7	++	+	+	—	++	+
	14	++	+	+	—	++	+
α-celulosa	3	+	—	+	—	++	+
	7	+	—	+	—	++	—
	14	+	—	+	—	+	—
Celobiosa	3	++	+	++	+	++	++
	7	++	+	++	+	+++	+++
	14	++	+	++	+	+++	+++
Almidón	3	++	+	++	—	++	++
	7	++	+	++	+	+++	+++
	14	++	+	++	—	+++	++
Acido poligalacturónico	3		+++		+++		+
	4		+++		+++		++
	14		++		++		++
Glucosa	3		+++		+++		+++
	4		+++		+++		+++
	14		+++		+++		+++

Crecimiento en placas de Petri, a 25° C de temperatura:
Crecimiento abundante +++, moderado ++, débil + y nulo —.
Medios utilizados por Bravery (3) que difieren en el contenido de extracto de levadura, asparagina y vitamina B_1.

bono ya en la primera muestra (24 h) se alcanzaron valores aceptables de actividad ezimática total (80 mU/ml de CMCasas, 145 mU/ml de ß-xilanasas y 155 mU/ml para ß-xilosidasas).

También se estudió la producción de enzimas celulolíticos y hemicelulolíticos a partir de diferentes polisacáridos utilizados como fuente de carbono. Como puede verse (Tabla 3), excepto en el caso de ß-glucosidasas, no se observó ninguna actividad enzimática ni crecimiento celular cuando se utilizó la carboximetilcelulosa (MCMI) y la α-cleulosa como fuente de carbono. Sin embargo, al utilizar los xilanos o el «steam exploded wood» se encontraron altos niveles de actividad enzimática y buen crecimiento celular en todos los casos (2,4 mg peso seco/ml).

La Tabla 4 muestra las actividades enzimáticas de CMCasas ß-xilanasas, ß-xilosidasas y ß-glucosidasas sobre diferentes monosacáridos a las 24 horas de cultivo. Como puede observarse tan

TABLA 3
ACTIVIDADES ENZIMATICAS EN *AEROBASIDIUM PULLULANS* CRECIDO SOBRE DIFERENTES POLISACARIDOS

Fuente de carbono (5 g/l)	ß-glucosidasa	ß-xilosidasa	ß-xilanasa	CMCasa
	mU/mg peso seco celular			
α-celulosa*	4	0	0	0
Carboximetil* celulosa	6	0	0	0
Xilanos	11	88	70	36
«Exploded wood»	25	60	67	37

Inóculo procedente de un cultivo sobre 1 % de glucosa.
Muestras obtenidas a las 24 horas de cultivo.
* Valores medios de 5 experimentos.

sólo la xilosa indujo la síntesis de ß-xilanasas (56 y 40 mU/mg de peso seco). Por otra parte, el ácido D-glucurónico fue el único monosacárido que indujo la producción de CMCasas (70 mU/mg peso seco) y con el ácido D-galacturónico se encontró el máximo nivel de ß-glucosidasas (34 mU/mg peso seco). En cambio, al utilizar como fuente de carbono D-fructuosa, D-arabinosa y D-glucosa la actividad enzimática fue apenas apreciable (0 a 13 mU/mg peso seco).

Producción de enzimas en presencia de diferentes mono, di o trisacáridos

Con vistas a determinar el verdadero inductor de los enzimas celulolíticos y hemicelulolíticos en *Aureobasidium pullulans* se utilizaron diferentes mono, di o trisacáridos a una concentración muy baja (4mM). En estos ensayos, debido a esta cantidad tan pequeña de fuente de carbono, los resultados se expresaron en mU/ml, ya que no existía desarrollo celular.

Los resultados obtenidos (ver Tabla 5) mostraron que ninguno de estos compuestos inducía las

TABLA 4
ACTIVIDADES ENZIMATICAS EN *AUREOBASIDIUM PULLULANS* CRECIDO SOBRE DIFERENTES MONOSACARIDOS

Fuente de carbono (5 g/l)	ß-glucosidasa	ß-xilosidasa	ß-xilanasa	CMCasa
	mU/mg peso seco molecular			
Ninguna	17	0	0	0
D-xilosa	7	56	40	0
D-fructosa	13	1	0	0
D-arabinosa	10	3	0	0
D-glucosa	1	0	0	0
D-ác. glucurónico	9	3	0	70
D-ác. galacturónico	34	4	0	0

Inóculo procedente de un cultivo sobre 1 % de glucosa.
Muestras obtenidas a las 24 horas de cultivo.

TABLA 5
ACTIVIDADES ENZIMATICAS EN *AUREOBASIDIUM PULLULANS* CRECIDO SOBRE DIFERENTES MONO, DI O TRISACARIDOS

Fuente de carbono (4 mM)	ß-glucosidasa	ß-xilosidasa	ß-xilanasa	CMCasas
	mU/ml			
Ninguna	17	0	0	0
Celobiosa	19	0	0	0
4-tiocelobiosa	14	0	0	0
Soforosa	15	0	0	0
ß-metil-D-xilosido	12	0	0	0
4-tioxilobiosa	6	0	0	0
1,2-ß-xilobiosa	12	46	120	0
1,3-ß-xilobiosa	13	35	113	0
1,4-ß-xilobiosa	25	50	0	0
1,4-ß-xilotriosa	26	66	0	0
D-xilosa	16	52	0	0

Inóculo procedente de un cultivo sobre 1 % de glucosa.
Muestras obtenidas a las 24 horas de cultivo.

CMCasas. Cuando se utilizaron los derivados de glucosa o compuestos conocidos como no metabolizables, como el ß-metil-D-xilósido o la 4-tioxilobiosa no se encontró ninguna actividad enzimática a nivel apreciable, excepto para la ß-glucosidasa. La inducción de la ß-xilanasa se produjo sólo con la 1,2-ß-xilobiosa y la 1,3-ß-xilobiosa; sin embargo, la ß-xilosidasa presentó una inducción con todos los di o trisacáridos que contienen xilosa o con la xilosa misma, excepto la 4-tioxilobiosa y el ß-metil-D-xilósido. La xilosa a una concentración de 4mM no indujo la producción de ß-xilanasas.

Por último, los compuestos inductores fueron también ensayados a concentraciones aún menores (1 mM). A esta concentración sólo los niveles de actividad de ß-xilanasas fueron similares a los obtenidos a concentraciones 4 mM en el caso de inducción con 1,3-ß-xilobiosa.

Discusión

Los resultados obtenidos con las especies ensayadas *Trichosporon penicillatum, Geotrichum candidum* y *Aureobisidium pullulans*, cultivadas en placas Petri sobre diferentes di o polisacáridos como fuente de carbono, indicaban (Tabla 2) que *A. pullulans* podría ser la única especie de las ensayadas que presentase completo el complejo enzimático de celulasas y hemicelulasas. Esta idea estaría basada en el desarrollo positivo que presentaba sobre xilanos, papel de filtro, α-celulosa y celobiosa, mientras que *T. penicillatum* y *G. candidum* no presentaban ningún crecimiento al utilizar xilanos como fuente de carbono, lo cual indicaría la ausencia de actividad ß-xilanasas. Sin embargo, estas dos especies presentaban un mejor desarrollo sobre el ácido D-poligalacturónico, resultado que podría interpretarse como indicativo de la existencia de pectinasas.

Como era de esperar por los resultados obtenidos en placas Petri, al desarrollar dichas especies en cultivo líquido con aireación para determinar en unos ensayos previos las actividades de enzimas celulolíticas y hemicelulolíticas, pudo comprobarse que *A. pullulans* era la única especie de las ensayadas que desarrolló el complejo enzimático completo de CMCasas, ß-xilanasas, ß-xilosidasas y ß-glucosidasas.

Sin embargo, no se obtuvo ninguna actividad enzimática al utilizar *G. candidum* y *T. penicillatum*, lo cual difiere de los resultados encontrados por otros autores que mostraron la producción de celulasas y xilanasas en el género *Trichosporon* (1, 24) y la biosíntesis de celulasas en la especie *G. candidum* (22, 26). Esto viene a confirmar, una vez más, el hecho repetidamente comprobado que diferentes cepas de un mismo género o incluso de una misma especie presentan niveles y/o contenidos enzimáticos notablemente diferentes.

Al tratar de determinar el tiempo necesario para inducir las actividades enzimáticas estudiadas se encontró que el valor máximo se alcanzó a las 24 horas, manteniéndose este nivel durante los 11 días del cultivo que duró el ensayo. Este resultado puso de manifiesto que dichos enzimas eran producidos en un período de tiempo más corto que los reseñados anteriormente por otros autores (24, 25, 29).

Los resultados obtenidos con *A. pullulans* coinciden con los de otros autores donde demuestran (1, 6, 25) que *A. pullulans* no presentaba desarrollo sobre carboximetilcelulosa (CMC) o celulosa cristalina. Sin embargo, cuando las cadenas de xilanos o el «Steam exploded wood» se utilizaron como fuentes de carbono se encontró una buena actividad de CMCasas junto con actividades de ß-glucosidasas, ß-xilosidasas y ß-xilanasas (Tabla 3).

Como ya es conocido que los polisacáridos no pueden penetrar en las células y, por tanto, inducir la biosíntesis de dichos enzimas, para determinar los verdaderos inductores se ensayaron la mayoría de los monosacáridos que se encuentran en las cadenas de los polisacáridos. Los resultados ya citados (Tabla 4) mostraron que la actividad ß-glucosidasa era inducida a mayor o menor nivel por varios monosacáridos al ser utilizados como fuente de carbono, alcanzándose el valor máximo de actividad en el caso de utilización del ácido D-galacturónico, y que el ácido D-glucurónico (o bien alguno de sus metabolitos) inducían la actividad de CMCasas. Estos resultados no pueden ser comparados con los de otros autores, ya que no se han encontrado en la bibliografía antecedentes en que se hayan utilizado los ácidos D-glucurónico y D-galacturónico como fuentes de carbono en cultivos de microorganismos. La xilosa (5 g/l) apareció como el inductor a la vez de ß-xilosidasas y ß-xilanasas, resultado que coincide con los obtenidos con otros microorganismos (25, 29). El hecho de que la inducción de la ß-xilanasa sólo se haya encontrado con la concentración alta de xilosa podría deberse a un problema de transporte. Por otra parte, utilizando la 1,4 ß-xilobiosa y la 1,4 ß-xilotriosa (a una concentración de 4 mM), que son productos naturales de la hidrólisis de los xilanos, no se encontró actividad de ß-xilanasas. Sin embargo, al utilizar como fuente de carbono, a esa misma concentración, 1,2 y 1,3-ß-xilobiosas (que son productos de síntesis) se obtuvieron los máximos niveles de ß-xilanasas. Este resultado coincide con los obtenidos por Biely y Petrakova (2) y Hármová *et al.* (11) en *Cryptococcus albidus* y *Trichosporon cutaneum*, respectivamente.

En todos los casos al utilizar los disacáridos que contenían glucosa fueron ineficaces como inductores de enzimas celulolíticios y hemicelulolíticos, resultados que difieren de los encontrados por Desrochers *et al.* (7) con *Schizophyllum commune* y Defaye *et al.* (4) con *Trichoderma lignorum*. Según los datos que aparecen en la Tabla 5 la 1,2 y 1,3-ß-xilobiosas o bien algún producto de su transformación fueron los inductores de la ß-xilosidasa y de la ß-xilanasa. Este resultado podría interpretarse como el reflejo de la inducción de una ß-xilosido-permeasa (2, 12) que permitiera la entrada en la célula de estos compuestos o bien que dichos compuestos presenten una mejor afinidad hacia los receptores específicos de la superficie de las células.

En conclusión podemos decir que la especie *A. pullulans* es capaz de sintetizar el complejo enzimático de ß-glucosidasa, ß-xilosidasa, ß-xilanasa y CMCasa, según la fuente de carbono que se utilice y que, por tanto, podría desarrollarse sobre residuos lignocelulósicos pretratrados para aumentar su contenido en proteína y revalorizar así estos productos de bajo precio para la alimentación de rumiantes. Actualmente en nuestros laboratorios se está llevando a cabo un proceso de producción de proteínas a partir de desechos lignocelulósicos en cultivo semisólido utilizando dicha especie.

Agradecimientos

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Bibliografía

1. Biely, P., Krátký, Z., Kocková-Kratochvílová, A. and Bauer, S. (1978). Xylan-degrading activity in yeasts growth on xylose, xylan and hemicelluloses. Folia Microbiol. **23**, 366-371.
2. Biely, P. and Petraková, E. (1984). Novel inducers of the xylan-degrading enzyme system of *Cryptococcus albidus*. J. Bacteriol. **160**, 408-412.
3. Bravery, A. F. (1968). Microbiological breadkdown of cellulose in the presence of alternative carbon sources. J. Sci. Fd. Agric. **19**, 133-135.
4. Defaye, J., Driguez, H., John, M., Schmidt, J. and Ohleyer, E. (1985). Induction of D-xylan degrading enzymes in *Trichoderma lignorum* by nonmetabolizable inducers. A synthesis of 4-thioxylobiose. Carbohydr. Res. **139**, 123-132.
5. Dekker, R. T. H. and Richards, G. N. (1976). Hemicellulases, their occurrence, purification, properties and mode of action. Adv. Carbohydr. Chem. Biochem. **32**, 277-352.
6. Dennis, C. (1972). Break-down of cellulose by yeast especies. J. Gen. Microbiol. **71**, 409-411.
7. Desrochers, M., Jurased, L. and Paice, M. G. (1981). Production of cellulase, ß-glucosidase and xylanase by *Schizophyllum commune* grown on a cellulose-peptone medium. Dev. Ind. Microbiol. **22**, 675-684.
8. Dewey, D., Ryu, Y. and Mandels, M. (1980). Cellulases: biosynthesis and applications. Enzyme Microb. Technol. **2**, 91-102.,
9. Excoffier, G., Gagnaire, D. and Utille, J. P. (1984). Synthesis of reducing disaccharides of D-xylopyranose. Carbohydr. Res. **128**, 217-226.
10. Ghose, T. K., Montenecourt, B. S. and Eveleigh, D. E. (1980). «Measure of cellulose activity». International Union of Pure and Applied Chemistry. Comission on Biotechnology.
11. Hrmová, M., Biely, P., Vršanská, M. and Petráková, E. (1984). Induction of Cellulose and Xylan-degrading Enzyme Complex in the Yeast *Trhichosporon cutaneum*. Arch. Microbiol **138**, 371-376.
12. Kráktý, Z. and Biely, P. (1980). Inducible β-xyloside permease as a constituent of the xylan-degrading enzyme system in the yeast *Cryptoccocus albidus*. Europ. J. Biochem. **112**, 367-373.
13. Lowry, O. H., Rosebrourgh, N. J., Farr, A. L. and Randall, R. J. (1951). Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. **193**, 265-275.
14. Mandels, M. and Weber, J. (1969). The production of cellulases. *In:* R. F. Gould (ed.). «Cellulases and their Applications». Adv. in Chem. series n.º **95**, pp. 391-413. Washington: American Chemical Society.
15. Mandels, M., Weber, J. and Pazipek, R. (1971). Enhanced Cellulase Production by a Mutant of *Trichoderma viride*. Appl. Microbiol. **21**, 152-154.
16. Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. Anal. Chem. **31**, 426-428.
17. Pathak, A. N. and Ghose, T. K. (1973). Cellulases-1: Sources Techn-ology. Proc. Biochem. **8**, 35-38.
18. Pathak, A. N. and Ghose, T. K. (1973). Cellulases-2: Applications. Proc. Biochem. **8**, 20-21.
19. Pou, J., Figarella, X., Fernández, M. J. y Garrido, J. (1985). Obtención de proteínas a partir de bagacillo de caña. I. Estudio de hidrólisis de bagacillo y selección de especies de levaduras. Microbiol. Españ. **38** (3-4), 81-88.
20. Pou, J., Fernández, M. J. y Garrido, J. (1985). Obtención de proteínas a partir de bagacillo de caña. II. Estudio de la multiplicación de *Trichosporon penicillatum* en cultivo semisólido en bandejas. Microbiol. Españ. **38** (3-4), 89-95.
21. Rho, D., Desrochers, M., Jurasek, L., Driguez, H. and Defaye, J. (1982). Induction of cellulase in *Schizophyllum commune*: Thiocellobiose as a new inducer. J. Bacteriol. **149**, 47-53.
22. Rodionova, N. A., Rumyantseva, N. G., Tiunova, N. A., Martinovich, L. I. and Bakhtadze, L. N. (1977). β-glucosidase from de fungus *Geotricum candidum*. A. N. Bakh. Institut of Biochemistry MOSCOW. All —Union Scientific— Research Institute of Biotechnology MOSCOW, translated from Biokhimiya, **42**, 43-50.
23. Selby, L. (1986). Proc. Ist Intern. Biodeterioration of Materials. Symposium Southampton, Amsterdam, pp. 62.
24. Stevens, B. S. and Payne, S. (1977). Cellulase and xylanase production by yeast of the genus *Trichosporon*. J. of Gen. Microbiol. **100**, 381-393.
25. Stüttgen, E. and Sahm, H. (1982). Purification and properties of endo-1,4-ß-xylanase from *Trichosporon cutaneum*. European J. Appl. Microbiol. Biotechnol. **15**, 93-99.

26. Tionova, N. A., Zaikina, I. V., Uchasthova, L. N. Kobzeva, N. Y. and Zuvastina, N. A. (1982). Glucanases of *Geotrichum candidum*. Institute of Biochemistry. Academy of Sciences of the USSR. Translated from Mikrobiologiya. **51**, 510-514.
27. Toyoma, N. (1969). Applications of cellulases in Japan. *In:* R. F. Gould (ed.). «Cellulases and their applications». Adv. in Chem. series n.° **95**, pp. 359-390. Washington: American Chemical Society.
28. Wood, T. M. and Phillips, D. R. (1969). Nature. **222**, 986.
29. Yasui, T., Nguyen, B. T. and Nakanishi, K. (1984). Inducers for xylanase production by *Cryptococcus flavus*. J. Ferment. Technol. **62**, 353-359.

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Cellular location of asparginase activity in *Saccharomyces cerevisiae* and regulation of this activity by nitrogen compounds

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Summary

Three asparaginase activities have been detected in *Saccharomyces cerevisiae*. One is found outside the permeability barrier; a second one is found inside and is soluble in the cell, and the third one is localized in a system of membrane particles. Synthesis of the membrane and external asparaginases require «de novo» synthesis of RNA and protein. The synthesis of exocellular asparaginase is inhibited by several nitrogen compounds (catabolite repression). This inhibition might take place at the transcriptional level. Moreover, this isoenzyme is reversibly inactivated by its natural substrates (catabolite inhibition by substrates).

The half life of external asparaginase mRNA was calculated by two independent methods and values of 7.5 and 9.5 min were found.

Key words: Asparaginase, subcellular distribution, repression e inhibition, S. cerevisiae.

Resumen

Tres actividades asparaginásicas han sido detectadas en *Saccharomyces cerevisiae*. Una se encuentra localizada fuera de la barrera de permeabilidad (membrana plasmática); una segunda se encuentra dentro, y es soluble en el citosol, y la tercera actividad asparaginásica se encuentra localizada en el sistema de membranas. La síntesis de la asparaginasa de membrana y de la externa requieren la síntesis de «novo» de RNA y de proteínas.

Por otro lado, la síntesis de la asparaginasa externa es inhibida por diferentes compuestos nitrogenados (represión catabólica), y esta inhibición, al parecer, tiene lugar a nivel de transcripción. Además, este isoenzima es reversiblemente inactivado por sus sustratos naturales (inhibición catabólica por sustratos).

En este trabajo también se ha calculado el tiempo de vida media del mRNA que codifica a la asparaginasa externa por dos métodos diferentes, y los valores encontrados son de 7,5 y 9,5 minutos.

Introduction

There are many examples of enzymes that are located outside the microorganism plasma membrane, but retained inside by the cell wall and thus accessible by added substrates. One such enzyme is asparaginase (E.C.3.5.1.1.). Interest in this enzyme in bacteria increased considerably some time ago due to its potential therapeutic application (18).

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In *S. cerevisiae* the enzyme has been described in two forms: as a constitutive endocellular enzyme (10) and as an exoenzyme retained by the cell wall (1), the synthesis of which is regulated by nitrogen compounds (3, 12). These isoenzymes show different biochemical properties (2).

Nitrogen compounds that participate in the regulation of this exoenzyme might act on different steps of its biosynthesis (catabolite repression), but also upon the active enzyme itself (catabolite inactivation) (8, 17). Usually, the catabolite inactivation implicates the degradation of the enzyme (6, 7).

In the present work the regulation of the exocellular enzyme has been studied and a third asparaginase form has been found bound to a membrane preparation. Catabolite repression of the exoenzyme is brought about by several nitrogen compounds and possibly takes place at the transcriptional level. In addition, both D- and L-asparagine produce catabolite repression and catabolite inhibition of the external enzyme. But in this case, the catabolite inhibition is reversible after removal of the effectors.

Materials and methods

Organisms and culture conditions

The organism used was *S. cerevisiae* X 2180^{-136}ts obtained in our Department by crossing *S. cerevisiae* X 2180 1B and *S. cerevisiae*$^{-136}$ts (a gift from L. M. Hartwell, University of Washington, Seattle). Our mutant synthesizes asparaginase isoenzymes and RNA synthesis is blocked at the non-permissive temperature of 37° C.

Cells were grown in modified Vogel's medium (19): glucose 10 g, Na-citrate 3 g, $NaPO_4H_2$ 2 g, citric acid 0.25 g, $ZnSO_4$ 0.25 g, $CuSO_4$ 0.0125 g, $MnSO_4$ 0.0025 g, boric acid 0.025 g, histidine 0.011 g, methionine 0.002 g, biotine 0.02 mg, Na-pantothenate 0.4 mg, inositol 2 mg, and ammonium sulfate 1.32 g per litre of distilled water. Flasks containing 100 ml of medium were inoculated with 2 mg of cells (dry weight) and incubated with shaking at 23° C to early exponential phase.

The strains were maintained on slants of YM-1 medium (9) or Vogel medium solidified with 2 % agar.

Derepression of asparaginase synthesis was carried out as reported by Dunlop and Roon (1975), using a nitrogen-free medium containing 20 mM potassium phosphate buffer, pH 7.0 and 3 % glucose.

Protoplasts were incubated in the corresponding medium in the presence of 1 M sorbitol as osmotic stabilizer.

Protoplast preparation

Cells (100 mg dry weight) were treated with 10 ml of 5 mM dithiothreitol, 5 mM EDTA in 100 mM Tris-HCl buffer pH 8.0 for 20 min at 23° C. After washing, the cells were resuspended in 20 ml of 1 M sorbitol containing 20 mg of Zymolyase 5,000 (Kirin Breweries, Gumma, Japan) and incubated at 23° C for 30 min. After about 30 min quantitative transformation of cells in protoplasts was observed with a phase contrast microscope.

Cell permeabilization

Cells suspended in 0.5 ml of 20 mM phosphate buffer pH 6.8 were treated with 10 μl of toluene and washed free of organic solvent after 10 min shaking on a Vortex mixer.

Determination of asparaginase activity

Hydrolysis of asparagine was measured by determining the ammonia released with L-glutamate dehydrogenase (E.C.1.4.1.3.) (11) or by the method of Nessler (15). Alternatively a spectrophotometric assay for L-aspartate using L-glutamic oxalacetic transaminase (E.C.2.6.1.1.) and L-malic dehydrogenase (E.C.1.1.1.37) was employed (1).

One unit of asparaginase was the amount of enzyme which hydrolysed one micromole of substrate per minute per milligram of cells at 21° C (1).

Results

Subcellular distribution of asparaginase activity

Cellular localization of asparaginase was determined in cells grown in the presence and absence of a specified nitrogen source.

Derepressed cells showed enzyme formation kinetics typical of this type of enzyme (Fig. 1A). The external asparaginase remained associated with the cells but was released into the medium when cells were converted into protoplasts (Fig. 1B).

Exocellular activity was not detected in repressed cells but after treatment with small amounts of teluene (see Methods) activity was detected (intracellular enzyme) (Fig. 1A). When protoplasts were incubated without nitrogen source in the medium, the amount of secreted enzyme was proportional to the incubation time but the intracellular activity reached a plateau after a short period of time (data not shown).

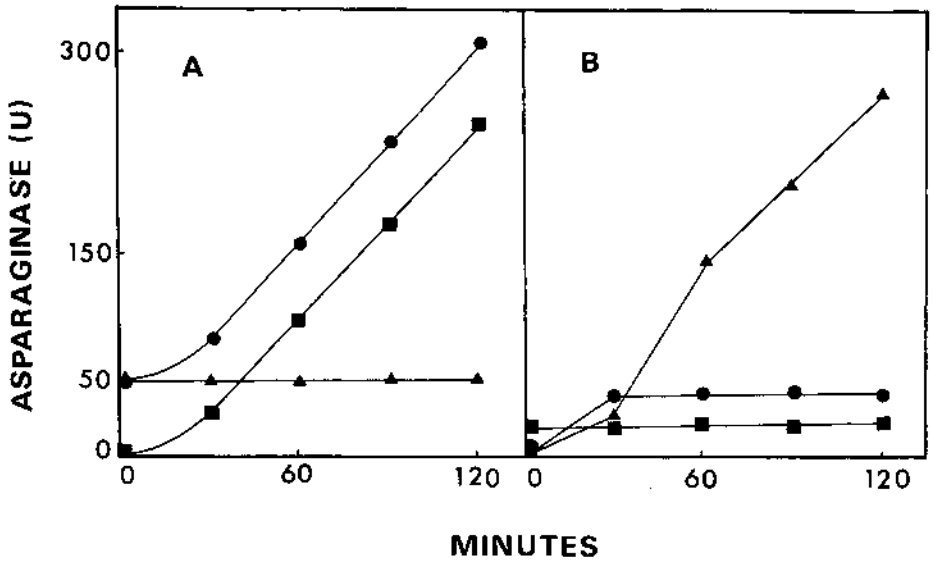

Fig. 1. Subcellular distribution of asparaginase activity. (A) Exponential phase cells grown in the presence of ammonia were incubated in derepression medium (0.5 mg/ml, dry weight) at 23° C. At the indicated times, samples were taken and the activity was determined in whole (■, extracellular asparaginase) and permeabilized washed cells (●, extracellular plus intracellular asparaginase). The difference between the activity found in permeabilized cells and in whole cells was taken as the intracellular asparaginase activity (▲). (B) Protoplasts obtained from exponentially growing repressed cells were incubated in a derepression medium supplemented with 1 M sorbitol. Samples were taken at the indicated times and the secreted enzyme (▲) was determined in the supernatant obtained after centrifugation at 5000 × g for 10 min. The protoplasts were lysed by osmotic shock and the resulting suspension centrifuged at 50,000 × g for 30 min and the enzyme activity was determined in the pellet (●, membrane-associated asparaginase activity) and supernatant fluid (■, cytosol-soluble asparaginase activity).

In repressed cells the enzyme activity was detected only as the soluble form in the cytoplasm, but in derepressed cells activity was also present in a particulate fraction obtained after centrifugation of the lysed protoplasts. This membrane-bound activity increased for a short period ot time (approx. 30 min.) and then remained constant. The soluble activity found under both repressed and derepressed conditions was always constant (Fig. 1B).

The possible relationship between the three isoenzymes was studied in experiments in which protein synthesis was inhibited with cycloheximide in derepressed cells. The activity of the membrane-bound as well as the exocellular and endocellular enzymes remained unchanged. If derepression was carried out in the presence of the drug neither exocellular nor membrane-bound activity was detected.

Effect of ammonia and other nitrogen sources on secretion of the exocellular asparaginase

The kinetics of enzyme formation by growing cells at different ammonia concentrations is shown in figure 2A. In the presence of 0.75 mM ammonia, only 50 % of the total activity detected without ammonia was found but at higher concentrations 5 mM, the enzyme synthesis was completely blocked.

The effect on the external asparaginase synthesis by other substances such as urea and different amino acids is shown in figure 2B. Asparagine was almost as good a repressor as ammonia. Histidine, lysine and alanine were less effective and urea showed almost no effect at the concentration used.

Effect of L- and D- asparagine on enzyme activity

Synthesis of several enzymes is subject to catabolite repression (8), but the molecular mecha-

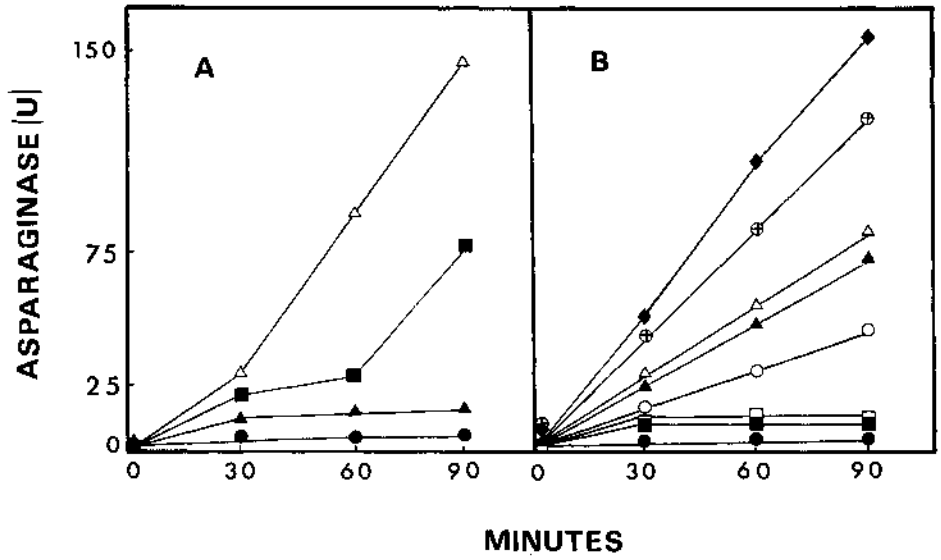

Fig. 2. Effect of ammonia (A) and other nitrogen compounds (B) on external asparaginase secretion. Cells growing exponentially in Vogel medium were resuspended in the derepression medium (0.5 mg/ml dry weight) where different amounts of ammonia were added. Samples for enzyme determination were taken at the times indicated. (A) Without ammonium or 0.15 mM (△), 0.75 mM (■), 1.5 mM (▲), 5 mM (●) of ammonium. (B) The cells were resuspended in derepression medium containing (at 2 mM concentration) urea (⊕), alanine (△), lysine (▲), histidine (○), D-asparagine (□), L-asparagine (■) and ammonium (●). The activity secreted by cells incubated in derepression medium was taken as a control (♦).

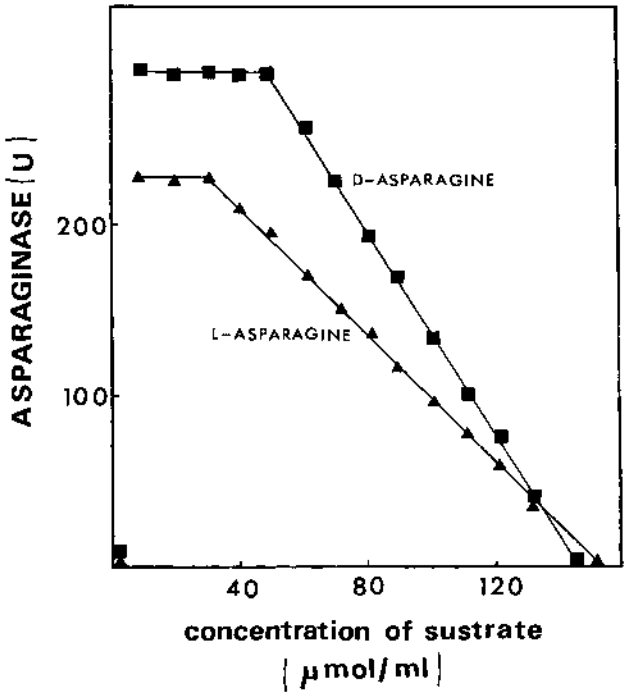

Fig. 3. Effect of L- and D-asparagine on asparaginase activity. Cells growing exponentially in Vogel medium were resuspended in derepression medium (0.5 mg/ml, dry weight) and incubated at 23° C during 120 min. The cell suspension was fractionated in aliquots and different concentrations of (▲) L- and (●) D-asparagine were added and the enzyme activity was determined.

nisms involved are not fully understood. Apparently, the presence of a source, such as glucose for energy or ammonia for nitrogen, produces the repression of enzyme synthesis. But neither glucose nor ammonia interfere with the enzyme activity directly.

In the case of asparaginase, D- and L-asparagine inhibit not only the synthesis of asparaginase but also the activity of the enzyme already formed. This inhibition of enzyme activity was found to be proportional to substrate concentration up to a concentration at which the inhibition of the activity was complete (Fig. 3). When the effectors were removed by washing the cells, the inhibition of asparaginase activity was found to be reversible. Other substances that had previously been shown to inhibit enzyme synthesis were also assayed but none of them inhibited enzyme activity (data not shown), suggesting that this inhibition of the asparaginase activity brought about by D- and L-asparagine is a highly specific process.

Effect of inhibition of RNA and protein synthesis on asparaginase secretion

In order to comprehend more fully the mechanisms of regulation of asparaginase synthesis, a study of the derepression process of the enzyme was carried out under conditions of inhibition of RNA and/or protein synthesis (fig. 4). No activity was detected when derepression of asparaginase was carried out either at 37° C or in the presence of cycloheximide (100 μg/ml). If derepression was carried out at 23° C, the enzyme was present. If the cells were then incubated at the nonpermissible temperature (37° C) the increase in activity was stopped in about 10-20 min.

Neither the transfer of the cells to the non-permissive temperature nor the addition of cycloheximide modified the level of the endocellular enzyme.

The decreased rate of enzyme synthesis was the same either if the cells were transferred to 37° C or if the derepression medium was supplemented with ammonia or asparagine.

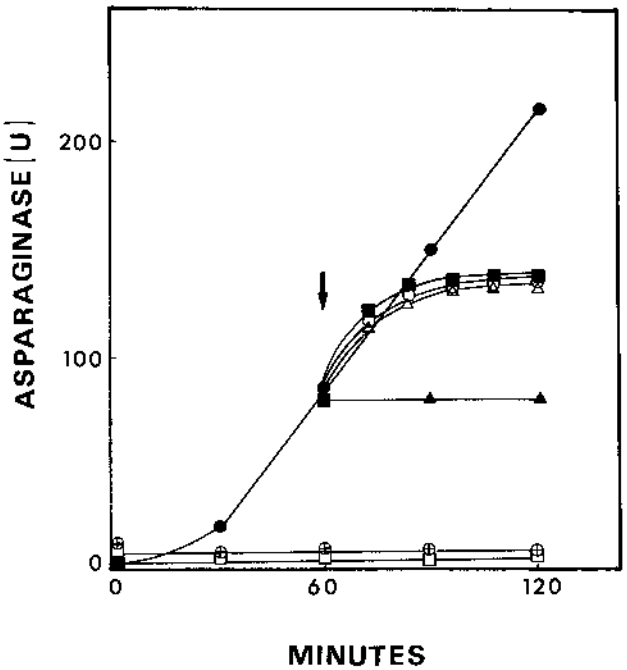

Fig. 4. Effect of protein and RNA synthesis inhibition on asparaginase secretion. Exponentially growing repressed cells were collected by centrifugation, resuspended in derepression medium (0.5 mg/ml, dry weight) and incubated at 23° C (●) and 37° C (⊕), and at 23° C in the presence of 100 μg/ml of cycloheximide (□). From the cell suspension incubated at 23° C without cycloheximide, aliquots weree separated and incubated again but now at 37° C (○), at 37° C plus 20 mM of ammonium (△) or with 2 mM of L-asparagine (■) and at 23° C with cycloheximide (100 μg/ml) (▲). Samples were taken at the times indicated and the asparaginase activity was determined.

Turnover of the RNA messenger of the exocellular asparaginase

Inhibition of RNA synthesis takes place when *S. cerevisiae* 2180^{-136}ts is transferred to the non-permissive temperature although translation of the already formed mRNA continues normally. Elevation of the incubation temperature to 37° C affords an easy way to determine the half life of specific mRNA (4). The drop in the increment during the time when the cells were incubated at the non-permissive temperature was expressed as percentage «decay» and the initial activity taken at 37° C was arbitrarily given a value of 100 %. The half life of the derepressed asparaginase mRNA was found to be 7.5 min (fig. 5).

A similar experiment was carried out by the addition of ammonium sulfate at 23° C. Now the half life of asparaginase mRNA was found to be 9.5 min.

Discussion

In *S. cerevisiae*, asparaginase activity is found in three different locations. One isoenzyme is found in the cytoplasm, another is membrane-bound, whereas the third activity is localized outside

the permeability barrier as this activity is released when the cells are converted into protoplasts. The enzyme is most probably located in the periplasmic space between the plasma membrane and the cell wall, as has been reported in the case of invertase (16), acid phosphatase (5), and other exoenzymes. The soluble enzyme in the cytoplasm and the external one have already been described by Dunlop *et al.* (1978). The membrane-bound enzyme, not previously described, might be the precursor of the periplasmic one since they appear only under derepression conditions; moreover, the kinetics of enzyme formation suggested a precursor-product relationship between them, as it ha been described in other cases (4, 16). The membrane-bound asparaginase was synthesized earlier than the exocellular enzyme and a plateu was reached after a few minutes (Fig. 1B). But, in order to confirm the relationship between the membrane-bound and periplasmic enzymes electrophoretic mobility, kinetic parameters, substrate specificity, pH optimum, etc., should be studied.

The total activity found in the cytoplasm remained constant under both repression and derepression conditions and also when protein or RNA synthesis was inhibited suggesting a very slow turnover.

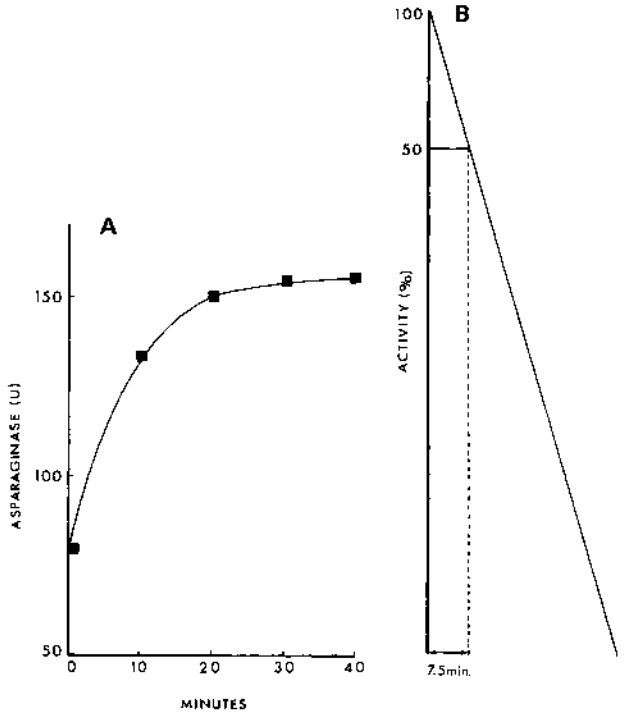

Fig. 5. Effect of RNA inhibition on exocellular asparaginase formation (A) and decay analysis of its mRNA (B). (A) The cells from a culture growing under derepression conditions at 23° C were transferred to 37° C and samples were taken at times indicated. (B) The ratios of the amounts of asparaginase for each portion (A) are plotted on a logarithmic scale as a function of time.

The synthesis of the extracellular and the particle-bound enzymes is regulated by catabolite repression caused by the presence of ammonia and other nitrogen-containing compunds (Fig. 2). In addition, their synthesis required «de novo» formation of mRNA because activity did not appear if derepression took place at the non-permissive temperature (Fig. 4).

S. cerevisiae 2180^{-136}ts shows inhibition of RNA synthesis when incubated at the non-permissive temperature (37° C) though translation of the already synthesized RNA continues (4). During cell growth there is no accumulation of mRNA and if both derepression and addition of cyclo-

heximide is carried out at the same time, no enzyme is detected (Fig. 4). This antibiotic inhibits protein formation but does not interfere with secretion (14).

The extent of inhibition of the synthesis of the extracellular enzyme by some nitrogen-containing substances (Fig. 2B) differs quantitatively from previously reported results (1, 12) suggesting a different level of sensitivity in the mechanism which regulates asparaginase synthesis by different yeast strains.

Regulation of asparaginase synthesis by ammonia and L- and D-asparagine might take place at the transcriptional level, because addition of these substances, once the cells have been transferred to a non-permissive temperature, does not modify the kinetics of enzyme formation (Fig. 4). If these substances acted at any other level, a decrease in the kinetics of accumulation would have been detected.

The fact that L- and D-asparagine do not only repress the synthesis of asparaginase but also inhibit its catalytic activity is of interest. A similar effect has been found in enzymes whose synthesis is regulated by the glucose effect (catabolite repression) and this phenomenon has been named catabolite inactivation (8). But in the case of the enzymes regulated by glucose the inhibition is irreversible due to the proteolytic degradation of the enzyme. In the case of asparaginase it was found to be reversible. The physiological significance of this effect is obscure due to the fact that high concentrations of the effectors are needed. Perhaps in this case the name «catabolite inhibition» is not the more appropriate.

Finally, the inhibition of RNA synthesis at the non-permissive temperature as well as by ammonium in *S. cerevisiae* X2180^{-136}ts afforded two easy ways of determining the half life of exocellular asparaginase mRNA. The values obtained, 7.5 min and 9.5 min, respectively, are similar, but the difference might be due to the time needed for ammonium to reach sufficient concentrations in the cell while the effect of the temperature may be an instantaneous one. The half-life of asparaginase mRNA corresponds to a relatively short-lived messenger as compared with other mRNA species of external (4) and intracellular (13) proteins.

References

1. Dunlop, P. C., and Roon, R. J. (1975). L-asparaginase of *Saccharomyces cerevisiae*: extracellular enzyme. J. Bacteriol. **125**, 999-1004.
2. Dunlop, P. C., Meyer G. M., Ban, D., and Roon, R. J. (1978). Characterization of two forms of asparaginase in *Saccharomyces cerevisiae*. J. Biol. Chem. **253**, 1297-1304.
3. Dunlop, P. C., Meyer, G. M., and Roon, R.J. (1980). Nitrogen catabolite repression of asparaginase II in *Saccharomyces cerevisiae*. J. Bacteriol. **143**, 422-426.
4. Elorza, M. V., Lostau, C. M., Villanueva, J. R., and Sentandreu, R. (1977). Invertase messenger ribonucleic acid in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. **475**, 638-651.
5. Elorza, M. V., Rodriguez, L., Villanueva, J. R., and Sentandreu, R. (1978). Regulation of acid phosphatase synthesis in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. **521**, 342-351.
6. Gancedo, J. M., and Gancedo, C. (1979). Inactivation of gluconeogenic enzymes in glycolytic mutants of *Saccharomyces cerevisiae*. Eur. J. Biochim. **101**, 455-460.
7. Haarasilta, S., and Oura, E. (1975). On the activity and regulation of anaplerotic and gluconeogetic enzymes during the growth process of Baker's yeast. Eur. J. Biochem. **52**, 1-7.
8. Holzer, H. (1976). Catabolite inactivation in yeast. Trends. Biochem. Sci. **1**, 178-181.
9. Hutchinson, H. T., Hartwell, L. H., and MacLaughlin, C. S. (1969). Temperature-sensitive yeast mutant defective in ribonucleic acid production. J. Bacteriol. **99**, 807-814.
10. Jones, G. E., and Mortimer, R. K. (1973). Properties of yeast L-asparaginase. Biochem. Genet. **9**, 131-145.
11. Kaltwasser, H., and Schlegel, H. G. (1966). NADH-dependent coupled enzyme assay for urease and other ammonia-producing systems. Anal. Biochem. **16**, 132-138.
12. Kang, L., Keeler, M. L., Dunlop, P.C., and Roon, R. J. (1982). Nitrogen catabolite repression in a glutamate auxotroph of *Saccharomyces cerevisiae*. J. Bacteriol. **151**, 29-35.

13. Koch, H., and Friesen, H. D. (1979). Individual messenger RNA half lives in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **170,** 129-135.
14. Moreno, F., Ochoa, A. G., Gascón, S., and Villanueva, J. R. (1975). Molecular forms of yeast invertase. Eur. J. Biochem. **50,** 571-579.
15. Roberts, J., Burson, G., and Hill, J. M. (1968). New procedures for purification of L-asparaginase with high yield from *Escherichia coli*. J. Bacteriol. **95,** 17-21.
16. Rodríguez, L., Ruiz, T., Villanueva, J. R., and Sentandreu, R. (1978). Yeast invertase: subcellular distribution and possible relationship between the isoenzymes. Curr. Microbiol. **1,** 41-44.
17. Switzer, R. L. (1977). The inactivation of microbial enzymes «in vivo». Ann. Rev. Microbiol. **31,** 135-137.
18. Vadlamudi, S., Padarathsing, H., Waravdekav, V. S., and Goldin, A. (1970). Factors influencing the therapeutic activity of L-asparaginase (NSC 109229) in leukemic (L 51784) mice. Cancer Res. **30,** 1467-1472.
19. Vogel, H. J. (1956). A convenient growth medium for *Neurospora* (medium N). Microbiol. Genet. Bull. **13,** 42-43.

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Epidemiology of *Yersiniae* in Barcelona (Spain)

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Summary

Between Aplil 1980 to December 1984 we undertook a study on the epidemiology of pathogenic *Yersinia*. Stool specimens from 5.199 patients with acute gastroenteritis were studied and *Y. enterocolitica* biotype 4 serotype 3 was isolated in 42 cases (0,8 %). The serological response was studied in 21 of these patients. Faecal specimens from 784 non-laboratory animals were also studied. The only pathogenic isolates from these animals were seven strains of *Y. enterocolitica* biotype 4 serotype 3 from six pigs and one dog and one strain of *Y. pseudotuberculosis* from a pig. The search of pathogenic *Yersinia* in 424 samples form row foods allowed the isolation of eight strains of *Y. enterocolitica* serotype 3 and one strain of *Y. pseudotuberculosis* from pig's tongue.

Key words: Yersinia, *Enteropathogeus, Epidemiology.*

Resumen

Entre abril de 1980 y diciembre de 1984 efectuamos un estudio sobre la epidemiología de las especies patógenas de *Yersinia.*

Se estudiaron muestras fecales de 5.199 pacientes con enteritis aguda aislándose *Y. enterocolítica* biotipo 4, serotipo 3, en 42 casos (0,8 %).

En 21 de estos pacientes se estudió la respuesta serológica.

También se efectuaron estudios de muestras fecales de 784 animales. Los únicos patógenos del género *Yersinia* aislados de esos animales fueron siete cepas de *Y. enterocolítica* biotipo 4, serotipo 3, de 6 cerdos y 1 perro y una cepa de *Y. pseudotuberculosis* de 1 cerdo.

La investigación de yersinias patógenas en 424 muestras de alimentos permitió el aislamiento de ocho cepas de *Y. enterocolítica* serotipo 3 y una de *Y. pseudotuberculosis* a partir de lenguas de cerdo.

Introduction

It is presently accepted there are species of yersiniae pathogenic to man, *Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis,* and four non-pathogenic species, *Y. intermedia, Y. kristensenii, Y.*

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fredericksenii and *Y. aldovae,* all widely distributed (3, 4). The taxonomic position of *Y. ruckeri* and *Y. philomiragia* is not so clear (13, 28).

Infections in humans due to *Y. enterocolitica* (18) and *Y. pseudotuberculosis* may produce diverse diseases, the most frequent being gastroenteritis, mesenteric adenitis and sepsis as well as immunologically mediated syndromes such as erythema nodosum and reactive arthritis (5).

Between April 1983 and December 1984 we carried out a microbiologic, clinical and epidemiologic study of the yersiniae in our environment.

Materials and methods

a) *Bacteriologic study*

1. Human specimens. Stool samples from 5,199 patients with gastroenteritis, 62 with reactive arthritis, 14 with Crohn's disease, 55 relatives in contact with affected patients and 218 healthy controls were studied.
2. Animal specimens. Stool samples from 784 animals were studied, including 170 birds (50 hens and 120 pigeons), 394 mammals (74 cows, 110 pigs, 100 rabbits, 110 dogs) and 220 wild rodents (from 7 different species).
3. Food. Following homogenization, 299 samples from various foods were processed, including 66 samples from vegetables, 208 from meat (24 from chicken, 19 from beef, 159 from pork, of which 125 were tongue, and 6 from lamb) and 25 from fish.

Isolation techniques

Human stools were processed by conventional procedures. They were plated out on to MacConkey agar (MacC), salmonella-shigella agar (SS) and cefsulodin-irgasan-novobiocin agar (CIN). For enrichment 1 ml from a dense suspension of fecal specimens in saline was inoculated on triptose broth and incubated at 4-6° C for 21 days. Then it was centrifugated for 15 minutes at 3.000 rpm and 2 ml of KOH at 0.5 % was added to 1 ml of the sediment during 2 minutes then a loop was plated on to a MacC. plate.

Animal fecal samples were studied only by the cold enrichment technique above described.

The food samples studied were homogenated and triptose broth was added for fluidification as necessary. The homogenates were processed by the enrichment technique described for human stool samples (12).

Metabolic Identification

The species and biotype identification of the isolated strains was made by standard procedures (14).

Serotype and Lysotype

Except for the 03 serotype strains which we type with our purpose antiserum, other serotypes and lysotypes were determined by Dr. H. H. Mollaret (I. Pasteur. Paris).

Pathogenic characters

Calcium dependency, autoagglutination and pyrazinamidase activity were studied in 45 *Y. enterocolitica* human strains from 14 distinct serotypes, using previously described techniques (16, 18, 19).

Serologic study

The methods used to study immune response in patients with enteritis were agglutination, complement fixation and ELISA in 22 patients infected with the 03 serotype and agglutination and complement fixation in 20 patients with other serotypes.

One hundred patients with reactive arthritis and 14 with Crohn's disease were also studied by agglutination.

Serotypes 03, 09, and 05,27 antigens were prepared with IP134, IP8450 and HSP 271 strains respectively. The other antigens were prepared with the patients homologous strain. The tests were done following previously described protocols (2, 15, 29).

TABLE 1
Y. ENTEROCOLITICA SEROTYPES ISOLATED FROM PATIENTS WITH GASTROENTERITIS

	Serotype	Lysotype	N. strains
Biotype 1			
	3	X_z	1
	5	X_z	16
	6	X_z	11
	6, 30	X_z	1
	7, 8	(4-X_o, 1-X_z)	5
	7, 8, 13	X_z	1
	7, 8, 13, 19	X_o	3
	7, 13, 19	X_o	2
	10, 34	X_o	2
	10, 34, K_1	X_o	1
	10, 37, K_1	X_o	1
	13	X_o	3
	14	(1-X_o, 1-X_z)	2
	25, 35	X_o	5
	25, 35, 28	X_o	3
	28	X_o	1
	30	X_z	9
	34	X_z	1
	36	X_o	2
	39, 41	X_z	1
	39, 41, 42 (41, 43)	(1-X_o, 1-X_z)	2
	Non typables		2
	Non typables		2
Biotype 3			
	7, 8	X_o	1
	16, 34	X_z	3
	Non typables	—	1
Biotype 4			
	3	VIII	42

TABLE 2
NON PATHOGENIC SPECIES OF *YERSINIA* ISOLATED FROM PATIENTS WITH GASTROENTERITIS

	Serotype	Lysotype	N. strains
Y. frederiksenii			
	3	X_z	1
	4, 10, 14, 16	X_o	1
	35	X_z	1
	44	X_z	1
	52 (52, 53)	X_o	1
	Non typables	($5\text{-}X_o$, $1\text{-}X_z$)	6
Y. kristensenii			
	11	X_z	1
	12	X_z	1
Y. intermedia			
	3, 37	X_z (1)	1
	18	X_o (2)	1

(1) Biotype 1. (2) Biotype 4.

Results

a) *Symptomatic Patients*

Gastroenteritis. From the 5,199 patients studied 139 *Yersinia* strains (2.6 %) were isolated, 124 corresponding to *Y. enterocolitica* species, 11 to *Y. fredericksenii*, 2 to *Y. kristensenii*, and 2 to *Y. intermedia* (Tables 1 and 2). The only pathogenic bioserotype isolated from man in this study was the biotype 4 serotype 3 of *Y. enterocolitica* of which 42 strains were isolated.

The three virulence associated characters studied in 21 strains belonging to serogroup 03 were all positive in 19. The pathogenicity tests of the other two strains of this serogroup were negative in one and the other did not autoagglutinate nor was calcium dependent but was pyrazinamidase negative.

These pathogenicity tests performed in 24 strains of human origin belonging to other serogroups (05; 06; 07,8; 07,13,19; 010,34; 014; 025,30; 025,35; 025,35,28; 028; 030; 039,41) were all negative.

Table 3 shows the media and method by which 136 of those strains were isolated. All non-pathogenic strains were isolated by cold enrichment and only 3 % by direct sampling, while 76,9 % of the pathogenic strains were isolated by direct plating and 89,7 % by enrichment.

The serologic response studied in the 22 patients with *Y. enterocolitica* 03 infection showed agglutination titers $\geqslant$ 1/128 in 86 % of these patients, ELISA titers $\geqslant$ 1/400 in 76 % and complement fixation $\geqslant$ 1/16 in 58 %.

The 20 patients with non-pathogenic serotypes did not show any serological response when studied by agglutination or complement fixation. In the other 62 patients with non-pathogenic serotypes no serological study was done.

Reactive Arthritis: Stool culture was done in 62 of the 101 patients with reactive arthritis. *Y. enterocolitica* serotype 03 was isolated in 3 cases, serotype 05 in two, and serotypes 06 and 030 each in one patient. Antibodies against *Y. enterocolitica* 03 were present in 8 patients which included the

TABLE 3
ISOLATION OF *YERSINIA* IN DIFFERENT MEDIA

STRAINS		DIRECT SAMPLING			COLD ENRICHMENT
Species Biotype	(N)	MacC	SS	CIN	TB 4-6.ª KOH MacC
Y. enterocolitica		N %	N %	N %	N %
Biotype 4	39	14 (35.8)	26 (66.6)	30 (76.9)	35 (89.7)
Biotype 3	5	0	0	0	5 (100)
Biotype 1	77	2 (2.6)	0	1 (1.3)	77 (100)
Y. frederiksenii	11	0	0	1 (10)	11 (100)
Y. kristensenii	2	0	0	0	2 (100)
Y. intermedia	2	0	0	0	2 (100)
TOTAL	136	16	26	32	132

N: Number of strains isolated.
MacC: Mac Conckey agar.
SS: Salmonella-shigella agar.
CIN: Cefsulodin-irgasan-novobiocin agar.
TB: Triptose broth.

3 patients with positive stool cultures for this serotype. Agglutination to the antigens 05,27 and 09 was negative in all cases.

Crohn's Disease: All cultures and agglutination tests were negative in these 14 patients.

b) *Relatives*

An epidemiologic study of the closest relatives of 22 patients infected by *Y. enterocolitica* 03 was done. In only 3 relatives of 2 patients (a brother who also presented gastroenteritis in one case and the asymptomatic mother and brother in the second individual) *Y. enterocolitica* 03 was isolated. All tests in relatives of patients infected by other serotypes were negative.

c) *Healthy Controls*

No strains of *Yersinia* were isolated in any of the 218 asymptomatic subjects studied.

d) *Animals*

Table 4 shows the results of isolations in animals. Fifty-six strains of *Yersinia* were isolated from the 170 birds and 394 mammals. The only pathogenic strains detected corresponded to *Y. enterocolitica* serotype 03 isolated in 6 pigs (5.5 % of pigs studied) and in 1 dog (0.9 %). One strain of *Y. pseudotuberculosis* was also isolated in the pigs (1 %). Table 5 shows the 88 strains of *Yersinia* isolated in the 220 wild rodents none of which were pathogenic species or bioserotypes.

TABLE 4
ISOLATION OF *YERSINIA* IN ANIMALS

ANIMALS	STRAINS ISOLATED				
	N. A.	Positives %	N. S.	Species	Biotype
BIRDS					
Hens	50	1 (2)	1	*Y. enterocolitica*	1
Pigeons	120	2 (1.6)	2	*Y. enterocolitica*	1
MAMMALS					
Cows	74	30 (40.5)	4	*Y. enterocolitica*	1
			3	*Y. enterocolitica*	3
			7	*Y. frederiksenii*	
			2	*Y. kristensenii*	
			18	*Y. intermedia*	
Pigs	110	13 (10.9)	1	*Y. pseudotuberculosis*	
			8	*Y. enterocolitica**	4
			5	*Y. enterocolitica*	1
Rabbits	100	1 (1)	1	*Y. intermedia*	
			1	*Y. enterocolitica*	1
Dogs	110	3 (2.7)	1	*Y. enterocolitica**	4
			1	*Y. intermedia*	1
			1	*Y. frederiksenii*	

N. A.: Number of animals studied.
N. S.: Number of strains isolated.
* Pathogenic serotypes.

e) *Food*

Table 6 shows the results of *Yersinia* isolated in the foods studied. The percentage of strains isolated oscillated between 36 % in 25 samples of fish and 10 % in 19 samples of beef. The only pathogenic strains detected were 8 strains of *Y. enterocolitica* 03 isolated in pork-tongue (6.5 % of tongue studied) and a strain of *Y. pseudotuberculosis* again in tongue (0.8 %).

Discussion

The objective of the study was to determine the incidence and distribution of the pathogenic bioserotypes of *Yersinia enterocolitica* in our environment.

The only pathogenic serotype of *Y. enterocolitica* detected was 03, isolated in 42 patients with enteritis (0.8 %). All corresponded to biotype 4 lisotype VIII and all except two were positive to pathogenicity tests. These results are comparable to those described by Calvo *et al.* (7) who revised 551 strains isolated in different regions of Spain and found 300 belonging to serotype 03, 2 to 09 and the rest to non-pathogenic bioserotypes. Pérez Trallero *et al.* (22) reported that 102 of the 103 strains isolated in Guipúzcoa, Spain, corresponded to the 03 serotype and the remaining strain to 0,7, 8, 13, 19. In other studies in Spain (8, 11, 20, 26) the only pathogenic serotype found was also 03 (Romero M. E., 1982, Ph D thesis Universidad de Navarra).

In our experience (23) *Y. enterocolotica* 03 was isolated less frequently (0.8 %) than *Salmonella enterica* (9 %) *Campylobacter jejuni* (8,4 %) and *Shigella* spp (5.5 %).

TABLE 5
ISOLATION OF *YERSINIA* IN WILD RODENTS

WILD RODENTS	STRAINS ISOLATED				
	N. A.	Positives %	N. S.	Species	Biotype
Apodemus sylvaticus	57	19 (33 %)	23	15: *Y. enterocolitica* 8: *Y. kristensenii*	1
Rattus norvergicus	52	34 (65.3 %)	54	14: *Y. frederiksenii* 34: *Y. enterocolitica* 2: *Y. kristensenii* 4: *Y. intermedia*	1
Arvicola terrestris	85	2 (2.3 %)	2	1: *Y. enterocolitica* 1: *Y. frederiksenii*	1
Arvicola sapidus	8	2 (25 %)	2	2: *Y. intermedia*	
Mus musculus	10	3 (30 %)	3	2: *Y. enterocolitica* 1: *Y. frederiksenii*	1
Mus spretus	6	1 (16.5 %)	1	1: *Y. enterocolitica*	1
Crocidura russulae	2	2 (100 %)	3	2: *Y. enterocolitica* 1: *Y. kristensenii*	1

N. A.: Number of animals studied.
N. S.: Number of strains isolated.

The frequency of *Y. enterocolitica* in stools in our study differs slightly from that of the other authors cited (8, 11, 20, 22, 26) and may be due to differences in the population studied and/or different methodology.

The proportion of serotype 09 strains detected in France in 1985 (21) and especially in Northen Europe (1, 10) is considerably higher than in Spain.

The remaining 97 strains of *Yersinia* isolated in 5,199 patients with enteritis belonged to the non-pathogenic bioserotypes of *Y. enterocolitica* and the species *Y. fredericksenii, Y. kristensenii* and *Y. intermedia.* This figure is very high even when compared to other studies in Spain which used an enrichment technique (8, 20). This is probably due to our methodology which combines cold enrichment with KOH treatment.

All 97 non-pathogenic strains of *Yersinia* were isolated by enrichment and only 3 by direct plating. These results coincide with those of Van Noyen *et al.* (29) regarding the efficacy of this cold enrichment isolating non-pathogenic serotypes of *Y. enterocolotica* from the digestive tract.

Of the 39 pathogenic strains in which we studied the means of isolation 35 (89.7 %) were by cold enrichment, 30 (76.9 %) by direct plating in CIN media, 26 (66.6 %) in SS and 14 (35.8 %) in MacC media.

Reactive arthritis was observed in one of the 22 patients with enteritis due to *Y. enterocolitica* 03 who were followed-up for over 3 months. Although the number of patients is limited, the percentage correlates with that observed by Soriano *et al.* in Spain (26).

Y. enterocolitica 03 was isolated in 3 of the 58 patients (5.2 %) who first presented reactive arthritis. Significative titres to *Y. enterocolitica* 03 by direct agglutination were detected in 8 (8.2 %) of the 97 patients studied.

The fact that no antibodies to the 09 and 05,27 antigens of *Y. enterocolitica* were detected in any of the 97 patients reflects the rarity of these serotypes in our country.

The lack of correlation between. *Y. enterocolitica* and Crohn's disease based on the microbiologic and serologic data also confirms the bibliografic findings (9, 31).

As in other studies regarding animal reservoirs (6, 17, 25), both pigs and dogs constitute a source of *Y. enterocolitica* 03 and this serotype was detected in 5.5 % of pig stools and in 0.9 % of dog stools. One strain of *Y. pseudotuberculosis* was also isolated in pig stools. Although we had isolated this species in humans in previous years, it was not detected in man in this study.

Regarding the foods studied, pathogenic strains were only detected in pork tongue, as previously stated (24, 27). In the 125 samples studied we isolated 8 corresponding to *Y. enterocolitica* 03 and 1 to *Y. pseudotuberculosis.* No pathogenic strains of *Yersinia* were detected in the other foods studied.

The only pathogenic *Yersinia* strains isolated in man in our study were *Y. enterocolitica* 03, and pigs and dogs were the only animal reservoir detected.

TABLE 6
ISOLATION OF *YERSINIA* IN FOOD SAMPLES

FOOD			STRAINS ISOLATED		
Samples	N. F.	Positives %	N. S.	Species	Biotype
VEGETABLES	66	11 (16.6)	1	*Y. enterocolitica*	1
			2	*Y. enterocolitica*	3
			7	*Y. intermedia*	
			1	*Y. kristensenii*	
MEAT					
Chicken	24	4(16.6)	3	*Y. intermedia*	
			1	*Y. kristensenii*	
Beef	19	2 (10)	1	*Y. enterocolitica*	
			2	*Y. intermedia*	
Pork					
tongues	125	42 (33.6)	1	*Y. pseudotuberculosis*	
			8	*Y. enterocolitica*	4*
			23	*Y. enterocolitica*	1
			13	*Y. intermedia*	
other	34	5 (14.7)	5	*Y. enterocolitica*	1
Lamb	6	2 (33.3)	1	*Y. enterocolitica*	1
			1	*Y. intermedia*	
FISH	25	9 (36)	8	*Y. intermedia*	
			1	*Y. frederiksenii*	
			1	*Y. kistensenii*	

N. F.: Number of samples studied.
N. S.: Number of strains isolated.

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References

1. Ahvonen, P. (1973). *Yersinia enterocolitica* infections in Finland. *In:* Winblad S. (ed.) *Yersinia, Pasteurella* and *Francisella*. pp. 133-134. S. Karger AG. Basel.
2. Alton, G. G., Jones, L. M. y Pietz, P. E. (1976). Las técnicas de laboratorio en la brucelosis. pp. 99-109. Organización Mundial de la Salud. Ginebra.
3. *Bercovier, H. and Mollaret, H. H. (1984). Genus XIV. Yersinia* Van Loghem 1944, 15^{AL}. *In:* Krieg, N. R., Molt, J. G. (eds.). Bergey's Manual of Systematic Bacteriology. Volume I, pp. 498-506. Williams and Wilkins. Baltimore.
4. Bercovier, H., Steigerwalt, A. G., Guiyoule, A., Huntley-Carter, G. and Brenner, D. (1984). *Yersinia aldovae* (formerly *Y. enterocolitica* like group X2): a new species of *Enterobacteriaceae* isolated from aquatic ecosystems. Int. J. Syst. Bacteriol. **34**, 166-172.
5. Boyce, J. M. (1985). *Yersinia* species. *In:* Mandell, G. D. Douglas, R. G., Bennett, J. E. (eds.). Principles and practice of infectious disease. 2nd ed. pp. 1296-1301. John Wiley and sons. New York.
6. Brewer, R. A. and Corbel, M. J. (1983). Characterization of *Yersinia enterocolitica* strains isolated from cattle, sheep and pigs in the United Kingdom. J. Hyg. Camb. **90**, 425-433.
7. Calvo, C., Ramos-Cormenzana, A., Guiyoule, A., y Mollaret, H. H. (1985). Incidencia de *Yersinia enterocolitica, Y. frederiksenii, Y. kristensenii* y *Yersinia intermedia* en España. Enf. Infec. y Microbiol. Clin. **2**, 105-109.
8. Castillo García, F. J., Lafarga Gil, M. A., Gil Torres, J. M., Clavel Parrilla, A., y Gómez Lus (1985). Contribución al estudio de la yersiniosis en la provincia de Zaragoza. Infectologika **VI**, 7-21.
9. Cerf, M., Mollaret, H. H. et See, A. (1982). Peut-on définitivement dissocier maladie de Crohn et infections à Yersinia? Med. Mal. Infect. **12**, 698-703.
10. De Groote, G., Vandepitte, J., and Wauters, G. (1982). Surveillance of human *Yersinia enterocolitica* infections in Belgium: 1963-1978. J. Infect. **4**, 189-197.
11. Dorronsoro, I., Viader, M. C. y Díaz, R. (1981). Contribución al estudio de la yersiniosis en Navarra. Rev. Clin. Esp. **162**, 39-42.
12. Ewing, W. H. (1986). Edwards and Ewing's Identification of *Enterobacteriaceae* Fourth Edition. Elsevier. New York.
13. Ewing, W. H., Ross, A. J., Brenner, D. J. and Fanning, G. R. (1978). *Yersinia ruckeri* sp. nov., the redmouth (RM) bacterium. Int. J. Syst. Bacteriol. **28**, 37-44.
14. Farmer III, J. J., Davis, B. R., Hickman-Brenner, F. W., McWhorter, A., Huntley-Carter, G. P., Asbury, M. A., Riddle, C., Wathen-Grady, H. G., Elias, C., Fanning, G. R., Steigerwalt, A. G., O'Hara, C. M., Morris, G. K., Smith, P. B. and Brenner, D. J. (1985). Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. J. Clin. Microbiol. **21**, 46-76.
15. Fowler, J. M. and Brubaker, R. R. (1986). Immunology of *Yersiniae. In:* Rose, N. R., Friedman, H., Fahey, J. L. (eds.). Manual of Clinical Laboratory Immunology. Third edition. pp. 371-374. American Society for Microbiology. Washington DC.
16. Higuchi, K. and Smith, J. (1961). Studies on the nutrition and physiology of *Pasteurella pestis* VI. A differential plating mediun for the estimation of the mutation rate to avirulence. J. Bacteriol. **81**, 605-608.
17. Hurvell, B. (1981). Zoonotic *Yersinia enterocolitica* infection. Host range, clinical manifestations and transmision between animals and men. *In:* Bottone, E. J. (ed.). *Yersinia enterocolitica*, pp. 145-160. CRC Press. Boca Raton.
18. Kandolo, K. and Wauters, G. (1985). Pyrazinamidase activity in *Yersinia enterocolitica* and related organisms. J. Clin. Microbiol. **21**, 980-982.
19. Laird, W. J. and Cavanaugh, D. C. (1980). Correlation of autoagglutination and virulence of *Yersiniae*. J. Clin. Microbiol. **11**, 430-432.
20. López Brea, M., Collado, L., De Rafael, L. y Baquero, M. (1984). *Yersinia enterocolitica* en coprocultivo: cinco años de experiencia. Rev. Sanid. Hig. Pública (Madr.). **58**, 481-487.
21. Mollaret, H. H. (1986). Activités du Centre des *Yersinia, Pasteurella* et *Francisella* en 1985. Ass. Anciens Eléves Ins. Pasteur. **109**, 21-28.
22. Pérez Trallero, E., Cillo-Eguiluz, G., López Lopategui, M. C. y Urbieta Egana, M. (1986). Gastroenteritis por *Yersinia enterocolitica* en Guipúzcoa. Estudio de 103 casos. Enf. Infec. y Microbiol. Clin. **4**, 262-266.
23. Prats, G., Mirelis, B., Portús, M., Rabella, N., Pericas, R., Ausina, V. and Coll, P. (1986). Gastroenteritis etiology in a University Hospital during 1983. Eur. J. Epidemiol. **2**, 118-123.
24. Schiemann, D. A. and Fleming, C. A. (1981). *Yersinia enterocolitica* isolated from the throats of swine in eastern and western Canada. Can. J. Microbiol. **27**, 1326-1333.
25. Shayegani, M., De Force, I., McGlynn, D. M. and Root, T. (1981). Characteristics of *Yersinia enterocolitica* and related species isolated from human, animal and environmental sources. J. Clin. Microbiol. **14**, 304-312.
26. Soriano, F., Ponte, M. C., Rivas, M. C., Oliva, M. y Gaspar, M. C. (1981). Infección humana por *Yersinia enterocolitica* y *Y. pseudotuberculosis* en España. Análisis de 80 casos recientes. Rev. Clin. Esp. **161**, 215-222.
27. Tauxe, R. V., Vandepitte, J., Wauters, G., Martin, S. M., Goossens, V., De Mol, P., Van Noyen, R. and Thiers, G. (1987). *Yersinia enterocolitica* infections and pork: the missing link. Lancet. **1**, 1129-1132.
28. Ursing, J., Steigerwolt, A. G., and Brenner, D. J. (1980). Lack of genetic relatedness between *Yersinia philomiragia* (the «Philomiragia» bacterium) and *Yersinia* species. Curr. Microbiol. **4**, 231-233.

29. Van Noyen, R., Vandepitte, J. and Wauters, G. (1980). Nonvalue of cold enrichment of stools for isolation of *Yersinia enterocolitica* serotypes 3 and 9 from patients. J. Clin. Microbiol. **11**, 127-131.
30. Voller, A. and Bidwell, D. (1986). Enzyme-linked immunosorbent assay. *In:* Rose, N. R., Friedman, H., Fahley, J. L. (eds.). Manual of Clinical Laboratory Immunology. Third edition. pp. 99-109. American Society for Microbiology. Washington DC.
31. Wasfish, J. S. and Sachar, D. B. (1981). *Yersinia enteritis* and Crohn's disease. *In:* Bottone, E. J. (ed.). *Y. Enterocolitica*. pp. 105-112. CRC Press, Bocal Raton.

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Hibridación de levaduras vínicas homotálicas con cepas heterotálicas

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Summary

Most of the yeast strains of industrial interest are homothallic, which makes genetic analysis as well as improvement of their desirable properties extremely difficult. In this work, all the wine yeasts analyzed, except for two «flor» yeasts which were able to generate heterothallic meiotic products, were homozygous for the *HO* allele of homothallism. The genetic analysis of one of the «flor» yeast spores, which was heterothallic, indicated that the strain carried an *HO* allele of homothallism and that its heterothallic behaviour was due to a genetic constitution *HML MAT HMR HO*. This *HO* allele of the «flor» yeast behaved differently from other *HO* alleles present in the wine yeasts. It gave rise to a very retarded homothallism, with formation of selfzygotes which presented an abnormal morphology. These zygotes generated selfdiploids whose ability to sporulate was very inefficient. The lag in the expression of the homothallism allowed the isolation of hybrids between meiotic products of the «flor» yeasts and heterothallic laboratory strains with a frequency of 10^{-1}, as compared to that of the meiotic products of other wine yeasts, which was of 10^{-4} to 10^{-6}. This result indicates that, among other factors which could influence the frequency of hybrid formation, the lag in the expression of homothallism could be the most important one. The use of heterothallic laboratory strains of mating type as opposed to a strain of *a* mating type, as well as the frequency of viable nucleus in the hybrid zygote after conjugation, also seemed to influence the success of hybrid formation between laboratory strains and the wine homothallic strains used.

Key words: Homothallism, heterothallism. Saccharomyces, *hybridation, genetic improvement.*

Resumen

El homotalismo que presentan la mayoría de las levaduras de uso industrial dificulta su mejora y el análisis genético de sus propiedades. Todas las cepas vínicas analizadas en este trabajo eran portadoras en homocigosis del alelo *HO* de homotalismo, excepto dos cepas de «flor» que generaron esporas heterotálicas. El análisis genético de una de estas esporas, de comportamiento heterotálico, reveló que la cepa es portadora de un alelo *HO* de homotalismo y que su comportamiento heterotálico se debe a una constitución genética *HMLα* MAT*α* HMR*α* *HO*. Este alelo *HO* presente en la levadura de «flor», a diferencia que el presente en las demás levaduras vínicas, dio lugar a un homotalis-

(*) A quien debe dirigirse la correspondencia.

mo muy retardado, con autocigotos de morfología anómala, que generaron autodiploides de ineficiente capacidad de esporulación. Este retraso en la expresión permitió obtener una frecuencia de hibridación con cepas heterotálicas de laboratorio de 10^{-1} frente a frecuencias de 10^{-4} a 10^{-6} obtenidas en las demás cepas vínicas, lo que sugiere que entre los distintos factores que pueden influir en la frecuencia de hibridación, el retraso en la expresión del homotalismo juega un papel decisivo. El empleo de una cepa heterotálica *MATα* respecto a una *MATa* y la frecuencia con que se formaron núcleos viables en el cigoto híbrido tras la conjugación influyeron también en el éxito de hibridación con las cepas homotálicas empleadas.

Introducción

El homotalismo es un carácter bien conocido en levaduras desde el punto de vista genético. El gen *HO* responsable del carácter presenta dos alelos, *HO* y *ho*, y sólo el dominante, *HO*, permite un comportamiento homotálico en el ciclo de vida, en el que una espora aislada germina y se divide mitóticamente, pero tras una o pocas generaciones se produce un cambio de sexo en una de las células. Como consecuencia de este cambio se produce posteriormente una conjugación entre ésta y alguno de sus descendientes mitóticos, originando así clones autodiploides isogénicos. Estos clones son homocigotos para todos los genes excepto para el locus sexual, en el que es heterocigoto *MATa/MATα*, lo que les permite volver a esporular (5). *HO* es esencial para que se produzca el cambio de sexo, pero también se requiere que en el gen *HML* o *HMR* exista una copia del alelo sexual distinta del que esté presente en el gen *MAT* (9). El alelo recesivo *ho* genera un comportamiento heterotálico en el que no existe cambio de sexo, con independencia de los alelos presentes en *HML* y *HMR*, y la levadura haploide portadora del alelo *ho* mantiene de forma estable el sexo determinado por el gen *MAT* (*a* o *α*) (5). En células diploides *a/α* el gen *HO* no actúa porque está sujeto a represión (10).

La mayoría de las levaduras de uso industrial son homotálicas, lo que dificulta la mejora y análisis genético de sus propiedades (4) porque sus productos meióticos, aun cuando se aislen espora a espora, generan células de distintos sexos que conjugan entre ellas con alta eficiencia. Con frecuencia se recurre a la obtención de mutantes heterotálicos (2) o a la búsqueda de esporas heterotálicas espontáneas (4), puesto que la pérdida de función del alelo *HO* genera una forma recesiva no funcional equivalente al alelo silvestre *ho*, heterotálico. Sin embargo, con cierta frecuencia una espora homotálica puede llegar a conjugar con una cepa heterotálica sin necesidad de buscar mutantes heterotálicos (7). En este trabajo se analiza esta situación en la que, mediante unas condiciones de selección apropiadas, se cuantifica la eficiencia de conjugación de cepas homotálica-heterotálica y se analizan algunos factores que influyen en la eficiencia de esta hibridación cruzada.

Materiales y métodos

Organismos. Las cepas utilizadas en este trabajo, y que han sido descritas con anterioridad (7, 8), han sido las siguientes: las cepas vínicas ACA4, IFI256, IFI82, FJF206, FJF414 (estas dos últimas son levaduras de «flor») y FSP414-6 (producto meiótico de FJF414, auxótrofa para metionina), así como las cepas de laboratorio D517-4B, D517-4BC (mutante resistente a cicloheximida procedente de D517-4B), MMY1, ES6 y K5-5A. Sus genotipos y origen aparecen descritos en la Tabla 1.

Medios y condiciones de cultivo. Se emplearon los medios (% p/v) YPD (2 % glucosa, 1 % bactopeptona, 0,5 % extracto de levadura) como medio nutritivo; YNB (0,67 % Difco-yeast nitrogen base with ammonium sulphate, 2 % glucosa) como medio mínimo fermentable/respirable; YNBG (0,67 % Difco-yeast nitrogen base with ammonium sulphate, 3 % glicerol) como medio de respiración suplementado con 50 μg/ml de ciclohexamida, 60 μg/ml de canavanina, 20 μg/ml de histidina y/o 30 μg/ml de adenina, según se indique; finalmente, SPO (0,1 % Difco-yeast extract, 1 % acetato potásico,

TABLA I
RELACION DE CEPAS UTILIZADAS EN ESTE TRABAJO

Denominación	Especie	Genotipo	Origen[1]
ACA4	*Sacch. fermentati*	*MATαMATa HO/HO*	A. Casas
IFI256	*Sacch. cerevisiae*	*MATα/MATa HO/HO*	V. Arroyo
IFI82	*Sacch. cerevisiae*	*MATα/MATa HO/HO suc/suc*	V. Arroyo
FJF206	*Sacch. rosei*	*MATα/MATa HO/ho MET/met*	J. Conde
FJF414	*Sacch. pretoriensis*	*MATα/MATa HO/ho MET/met*	J. Conde
FSP414-6	*Sacch. pretoriensis*	*MATα HO met*	Ref. (7)
D517-4B	*Sacch. cerevisiae*	*MATa ade2 lys9*	J. Conde
D517-4BC	*Sacch. cerevisiae*	*MATa ade2 lys9 cyh^R*	Ref. (7)
MMY1	*Sacch. cerevisiae*	*MATα ura3-Δ52 cyh^R*	R. Bailey
ES6	*Sacch. cerevisiae*	*MATa leu2-1 eth^R*	J. Polaina
K5-5A	*Sacch. cerevisiae*	*MATα his4-Δ15 ade2-1 can1 kar1-1*	J. Conde

[1] El origen de las cepas ha sido el siguiente: A. Casas. Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla; V. Arroyo. Instituto de Fermentaciones Industriales, Madrid; J. Conde. La Cruz del Campo, S. A., Sevilla; R. Bailey. Solar Energy Research Instituye, Golden, Colorado, EE. UU.; J. Polaina. Departamento de Genética, Facultad de Biología, Universidad de Sevilla.

0,05 % glucosa) como medio de esporulación. Todos los medios empleados han sido medios sólidos que se solidificaron con la adición de 2 % de agar.

Las cepas se cultivaron de forma rutinaria en cajas de Petri a 30° C, excepto para inducir la esporulación, en que se incubaron a 22° C.

Procedimientos genéticos

Esporulación. Colonias obtenidas en YPD a 30° C se pasaron con una espátula estéril a medio SPO, incubándose a 22° C durante 6-8 días hasta observar ascas al microscopio.

Hibridación. Para cepas homotálicas se recogieron las células esporuladas (ascas) con una espátula estéril, se resuspendieron en 0,3 ml de agua estéril, se añadieron 0,03 ml de helicasa (Suc *d'Helix pomatia*, L'Industrie Biologique Française, Clichy, Francia) y se incubó la suspensión a temperatura ambiente hasta observar al microscopio abundancia de esporas libres. Las esporas se lavaron con agua estéril, se recogieron por centrifugación, se contaron al microscopio en cámara de conteo y se mezclaron con un exceso de la cepa de laboratorio (proporción 1:3) MMY1 ó D517-4BC; tras 6 horas de incubación en YPD a 30° C se recogió la mezcla y se sembró en YNB con cicloheximida, donde sólo pueden crecer células híbridas entre esporas vínicas (prototrofas y sensibles al antibiótico) y levaduras de laboratorio (auxotrofas y con resistencia dominante a cicloheximida) (fig. 1A). Paralelamente se realizó una dilución apropiada y se sembró en medio YNB para estimar el número de esporas vínicas viables. Para cepas de auxotrofías complementarias (por ejemplo, FJF414-6 y cualquiera de las cepas de laboratorio), los híbridos se aislaron en medio YNB. Cuando se utilizaron cepas heterotálicas o de homotalismo retardado, la conjugación se realizó mezclando células vegetativas en lugar de esporas vínicas con la correspondiente cepa de laboratorio.

Conjugación sin fusión nuclear. Se realizó una mezcla de conjugación como la descrita para cepas homotálicas, pero mezclando las esporas vínicas con la cepa de laboratorio K5-5A *(rho–)*, incapaz de respirar por deficiencia en el ADN mitocondrial (8) y portadora de la mutación *kar1-1* que impide la

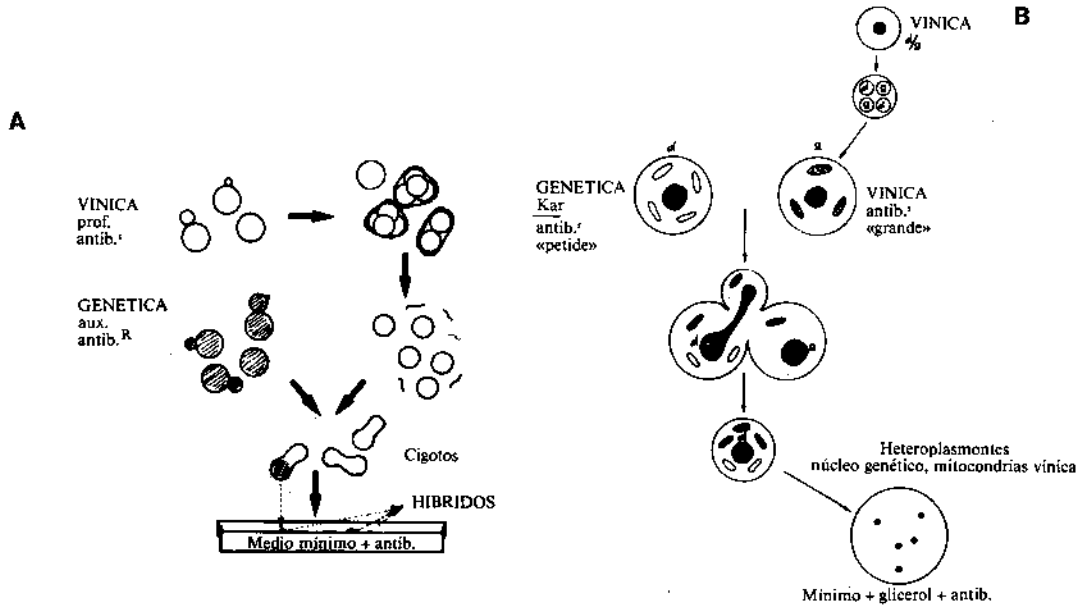

Fig. 1. A) Método de selección de híbridos entre cepas vínicas homotálicas y cepas de laboratorio heterotálicas. Las esporas de las cepas vínicas son prototrofas y sensibles al antibiótico. Las cepas de laboratorio son auxótrofas y poseen una resistencia dominante a dicho antibiótico. La selección de los híbridos se hace en medio mínimo YNB suplementado con el antibiótico. B) Método de selección de heteroplasmontes que poseen el núcleo de la cepa de laboratorio y las mitocondrias de la cepa vínica. La cepa de laboratorio es auxótrofa, incapaz de respirar *(rho–)* y posee una resistencia recesiva a un antibiótico. Las esporas de la cepa vínica son prototrofas, poseen mitocondrias *(rho+)* y son sensibles al antibiótico. La selección de los heteroplasmontes se hace en medio mínimo con una fuente de carbono respirable y el antibiótico.

fusión de núcleos tras la conjugación (1). Tras 6 horas de incubación, a 30° C, se sembró la mezcla de conjugación en YNBG suplementado con adenina, histidina y canavanina. En estas condiciones crecen todas las células de K5-5A que han conjugado con una espora vínica, adquiriendo vía citoplasma la capacidad de respirar sin que sus núcleos hayan fusionado (Fig. 1B). La canavanina impide el crecimiento de las esporas vínicas y el de los diploides que se hubieran podido formar por rezumancia de la mutación *kar*, ya que la resistencia a este antibiótico es recesiva.

Análisis de tetradas y aislamiento de cigotos. Los cigotos no seleccionables (como los autocigotos originados por homotalismo) se aislaron por micromanipulación en un microscopio invertido, con una microaguja de cristal, en un micromanipulador, siguiendo métodos descritos (12). De la misma forma se aislaron las esporas de las ascas mediante análisis de tetradas, sometiéndolas previamente a una digestión parcial con helicasa y colocando las cuatro esporas de la tetrada en lugares conocidos de una caja de YPD.

Resultados y discusión

En la Tabla 2 se recoge la frecuencia de esporas homotálicas que fueron capaces de conjugar y formar híbridos viables con las cepas de laboratorio MMY1 y D517, de sexos *α* y *a*, respectivamente (Tabla 2A). La conjugación se realizó con un exceso de células de la cepa de laboratorio, con lo que la frecuencia se refiere al número de esporas vínicas viables en la mezcla de conjugación.

En algunos casos, como en el de IFI256, la frecuencia es próxima a la que se esperaría por mutación espontánea del alelo *HO* a *ho*, de modo que la hibridación obtenida sería consecuencia de la

TABLA 2
FRECUENCIA DE HIBRIDOS (A) Y DE HETEROPLASMONTES (B) FORMADOS ENTRE LAS CEPAS GENETICAS Y LAS CEPAS VINICAS INDICADAS (POR 10^2 ESPORAS VINICAS)

	CEPA VINICA		
	IFI256	IFI82	ACA4
A) *Híbridos*			
—MMY_1 (α)	2	110	89
—D517-4BC *(a)*	0	12	4
B) *Heteroplasmontes*			
—K5-5A *(rho–)* (α)	26	1262	80

A) Número de híbridos formados por 10^6 esporas viables de las cepas vínicas indicadas y un exceso de la cepa de laboratorio MMY1 o D517-4BC en la mezcla de conjugación.
B) Número de heteroplasmontes K5-5A *(rho+)* obtenidos por 10^6 esporas vínicas viables en la mezcla de conjugación entre K5-5A *(rho–)* y esporas de la cepa indicada.

aparición espontánea de esporas heterotálicas. Sin embargo, los dos híbridos de IFI256 y los analizados para IFI82 y ACA4 generaron por meiosis tetradas completas con dos esporas que aisladas dieron lugar a células capaces de esporular y no de conjugar y otras dos esporas capaces de conjugar, pero no de esporular (segregación monogénica para el homotalismo). Los resultados, por tanto, indican que la hibridación procede de la conjugación de una espora homotálica de la cepa vínica y no de un mutante espontáneo heterotálico.

La proporción de híbridos obtenida fue unas 10 veces mayor con la cepa de laboratorio MMY1 (de sexo α) que con D517-4BC (de sexo *a*). Este sesgo sugiere que la eficiencia de hibridación entre cepas homotálicas y heterotálicas puede depender del sexo de la cepa heterotálica que se emplee. La feromona α se excreta constitutivamente, mientras que la feromona *a* se induce por la presencia de feromona α (5). De esta forma, una sobreabundancia de células *MAT*α heterotálicas en la conjugación podrían inducir más fácilmente la producción de feromona *a* en la espora vínica, favoreciendo la conjugación, pero no al revés. Adicionalmente la feromona α es muy inespecífica en su capacidad de actuar entre distintas especies de levaduras (Tabla 1), pero no así la feromona *a* (3). Una mayor eficiencia en el cambio de sexo de las esporas homotálicas de α a *a* que de *a* a α (6) generaría del mismo modo un sesgo en la conjugación como el observado (Tabla 2A). Alternativamente las diferencias observadas podrían deberse a la falta de isogenicidad entre las cepas MMY1 y D517-4BC. Sin embargo, el hecho de que cuando se utiliza la cepa MMY1 la proporción de híbridos obtenida sea mayor con cada una de las tres cepas vínicas utilizadas, genéticamente muy diferentes entre sí, sugiere que el determinante del sesgo es algo más que la falta de isogenicidad. Resultados preliminares (nos mostrados) obtenidos al cruzar las cepas heterotálicas ES1 (de sexo α) y ES2 (de sexo *a*) isogénicas, excepto para el locus sexual, con cepas vínicas parecen confirmar una mayor eficiencia de hibridación en la cepa ES1 de sexo α, probablemente por la acción conjunta de todos los mecanismos descritos.

Los híbridos obtenidos son consecuencia de una fusión celular seguida de una fusión de núcleos, generando híbridos viables. Para valorar separadamente ambos procesos se realizó la conjugación de esporas vínicas con la cepa de laboratorio K5-5A *(rho–)*, seleccionando posteriormente heteroplasmontes K5-5A *(rho+)* que poseen el núcleo de la cepa de laboratorio, pero mitocondrias de la espora vínica y cuantificando así la frecuencia con que se funden células homotálicas con heterotálicas, sin que necesariamente haya fusión nuclear y viabilidad del cigoto resultante. Como se observa en la Ta-

bla 2B para ACA4 esta frecuencia fue muy similar a la obtenida en la hibridación con MMY1, pero unas 10 veces mayor en el caso de las cepas IFI82 e IFI256. Los resultados sugieren que cuando los productos meióticos de ACA4 conjugan con la cepa MMY1 se forman núcleos viables con alta eficiencia; es decir, a toda fusión celular sigue la fusión nuclear y la formación de cigotos viables. En el caso de las cepas IFI82 e IFI256, los resultados indican que sólo una fracción de las esporas vínicas que conjugan con la cepa MMY1 llegan a fundir núcleos y formar híbridos viables. La formación de núcleos híbridos viables podría ser, pues, un factor que reduce la frecuencia de aparición de híbridos entre cepas homotálicas y cepas heterotálicas empleadas en este trabajo. También aquí podría aducirse que las diferencias observadas entre las frecuencias de heteroplasmontes y las de híbridos se debe a la falta de isogenicidad entre K5-5A y las cepas MMY1 y D517-4BC. Sin embargo, la limitación en la formación de núcleos diploides viables y no la falta de isogenicidad se ve apoyada por el hecho de que las frecuencias de heteroplasmontes son iguales o mayores, pero nunca menores, que las de híbridos en los tres casos estudiados.

Las cepas vínicas de «flor» generaron esporas haploides o próximas a la haploidía, de comportamiento heterotálico (7), con una alta eficiencia de conjugación cruzada, obteniéndose 1 híbrido con la cepa de laboratorio D517-4BC por cada 10 esporas viables en la conjugación.

Un análisis detallado realizado con la espora FSP414-6 reveló que, aunque esta espora es de comportamiento aparentemente heterotálico, dicha espora es portadora de un alelo *HO* de homotalismo. Se estudió la segregación para el alelo sexual de un híbrido FSP414-6/D517-4B. De este híbrido se obtuvieron inicialmente cuatro productos meióticos por asca capaces de conjugar. Dos de ellos conjugaban con células *MATα* y dos con células *MATa*, como se dedujo al observar al microscopio la formación de cigotos a partir de mezclas conjugantes. Sin embargo, en algunos de estos productos meióticos aislados se observaron células de morfología aberrante (Fig. 2). Estas células aberrantes se aislaron por micromanipulación en una caja de medio YPD y las colonias resultantes generaron células incapaces de conjugar y capaces de esporular, aunque con baja eficiencia (2-5 %) (la frecuencia de esporulación de la cepa FJF414 es del 82 %), lo que indica que esas células aberrantes eran autocigotos y que FSP414-6, aunque de fenotipo heterotálico, es portadora de un alelo *HO* de expresión ineficiente y retardada en algunos de los productos meióticos del cruce FSP414-6/D517-4B.

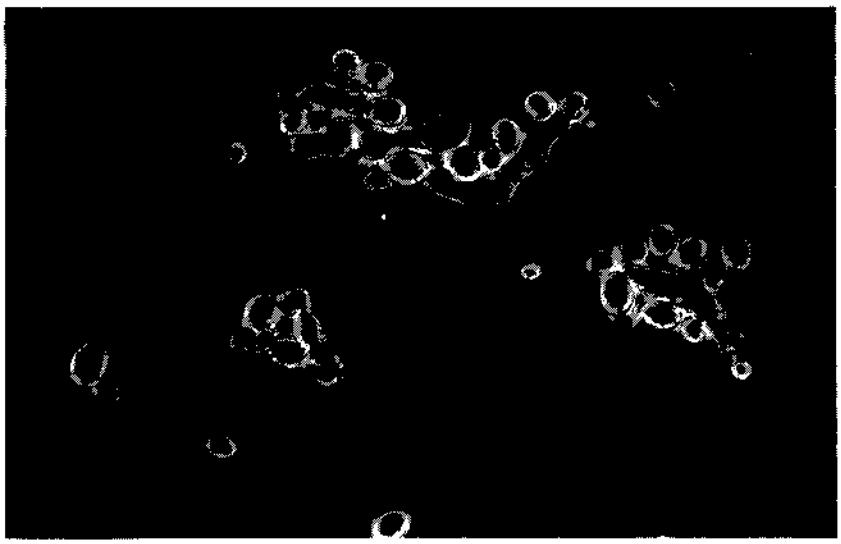

Fig. 2. Morgología aberrante que presentan los autocigotos que aparecen en algunos productos meióticos del híbrido FSP414-6/D517-4B.

TABLA 3
ANALISIS DE TETRADAS DEL HIBRIDO VINICO-GENETICO FJF414-6/D517-4B CON RESPECTO A SU CAPACIDAD (+) O NO (–) DE CONJUGAR CON LAS CEPAS MMY1 O ES6

Cepa heterotálica	ESPORA				Tetrada
	a	b	c	d	
MMY1 (α)	+	–	+	–	1
	+	–	–	+	2
	–	+/–	+	+	3
	–	+	+/–	+	4
	+	–	–	+	5
	+/–	+/–	+	+	6
ES6 *(a)*	+/–	+	–	+	1
	–	+	+	–	2
	+	+	–	?	3
	+	–	+	–	4
	–	+	+	+/–	5
	+	+	–	–	6

Crecimiento abundante (+), aparición de algunas colonias (+/–) o no crecimiento (–) en medio YNB a partir de la mezcla de conjugación entre las esporas del híbrido F414-6/D517-4B (tetradas 1 a 6, esporas a, b, c, d) y las cepas de laboratorio MMY1 o ES6.

A fin de estudiar la segregación de este tipo de homotalismo, se analizó la capacidad de formar colonias en YNB de una mezcla de conjugación entre los productos meióticos de FSP414-6/D517-4B (seis tetradas completas, todas con esporas portadoras de alguna auxotrofía) y las cepas MMY1 y ES6, seleccionando en medio mínimo YNB cualquier híbrido formado. Como se muestra en la Tabla 3, todas las tetradas segregaron *2a : 2α* para el gen *MAT*, con eficiencias de conjugación similares a las obtenidas entre los parentales FSP414-6 y D517-4B, pero algunas esporas fueron capaces de formar híbridos, aunque con baja eficiencia, con células de su propio tipo sexual (las esporas 1a, 3b, 4c, 5d, 6a y 6b). Los resultandos indican que estas esporas son portadoras de homotalismo retardado. De acuerdo con esta segregación, la presencia de tetradas con esporas heterotálicas: homotálicas en proporción 3:1 (tetradas 1, 3, 4 y 5), 2:2 (tetrada 6) y 4:0 (tetrada 2) indica que al menos dos genes están implicados en la expresión de este homotalismo retardado. En consecuencia, de acuerdo con la localización genética de los genes implicados en homotalismo (5), el heterotalismo de la cepa FSP414-6 se debe probablemente a una constitución genética *HMLα MATα HMRα HO*. Una situación similar ha sido previamente descrita, también para esporas de cepas de «flor» del tipo sexual *MATa* (13) y *MATα* (11).

La eficiencia de hibridación de estas esporas con homotalismo retardado evidencia que la probabilidad de hibridación cruzada con cepas homotálicas depende en gran parte del grado de homotalismo, siendo probablemente esto, la presencia de un *alelo HO* distinto y mucho menos eficiente que en las restantes cepas analizadas, la mayor fuente de dificultad en el cruzamiento entre cepas homotálicas y heterotálicas.

El retraso en la expresión del fenotipo homotálico podría jugar un papel decisivo en la capacidad de cruzar de cepas homotálicas, aunque en algunos casos la formación de núcleos híbridos viables tras la fusión celular también parece afectar a esta eficiencia. Esta sugerencia procede del hecho

de que tanto en IFI82 como en IFI256 se hayan obtenido 10 veces más heteroplasmontes que híbridos (Tabla 2). El éxito de la conjugación también podría depender del tipo sexual de la cepa heterotática que se emplee, en base a que se obtuvieron unas 10 veces más híbridos con la cepa MMY1, *MATα*, que con D517-4BC, *MATa*.

Agradecimientos

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Bibliografía

1. Conde, J., and Fink, G.R. (1976). A mutant of *Saccharomyces cerevisiae* defective for nuclear division. Proc. Nat. Acad. Sci. USA **73**, 3651-3655.
2. Del Castillo, L. (1984). Obtention of hetherothallic mutants from homothallic wine yeasts: application to hybridization for industrial purposes. Curr. Microbiol. **11**, 261-264.
3. Fujimura, M., and Yanagishima, H. (1983). Mating-type specific cell arrests and shmoo formation by α-pheromone of *Saccharomyces cerevisiae* in *Hansenula wingei*. Arch. Microbiol. **136**, 79-80.
4. Gjermanson, G., and Sigsgaard, P. (1981). Construction of a hybrid brewing strain of *Saccharomyces carlsbergensis* by mating of meiotic segregants. Carlsberg Res. Commun. **46**, 1-11.
5. Herskowitz, I., and Oshima, Y. (1981). Control of cell type in *Saccharomyces cerevisiae:* Mating type and mating type interconversion. *In:* J. N. Strathern, E. W. Jones and J. R. Broach (eds.). The molecular biology of the yeast *Saccharomyces cerevisiae*, pp. 181-209. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
6. Jansen, R. E., and Herskowitz, I. (1984). Directionality and regulation of cassette substitution in yeast. Cold Spring Harbor Symp. Quant. Bio. **49**, 87-104.
7. Jiménez, J., and Benítez, T. (1987). Genetic analysis of highly ethanol-tolerant wine yeasts. Curr. Genet. **12**, 421-428.
8. Jiménez, J., and Benítez, T. (1988). Yeast cell viability in high temperature and ethanol concentrations depends on the mitochondrial genome. Curr. Genet. **13**, 461-469.
9. Kostriken, R., and Heffron, F. (1984). The product of the *HO* gene is a nuclease: purification and characterization of the enzyme. Cold Spring harbor Symp. Quant. Biol. **49**, 89-96.
10. Russell, D. W., Jensen, R., Mark, J. Z.. Burke, J., Errede, B., Smith, M., and Herskowitz, I. (1986). Structure of the *Saccharomyces cerevisiae HO* gene and analysis of its upstream regulatory region. Mol. Cell Biol. **6**, 4281-4294.
11. Santa María, J., and Vidal, D. (1983). Genetic control of «flor» formation by *Saccharomyces*. J. Bacteriol. **113**, 1078-1080.
12. Sherman, F., Fink, G. R., and Lawrence, C.W. (1979). Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
13. Takano, Y., and Oshima, Y. (1967). An allele specific and a complementary determinant controlling homothallism in *Saccharomyces oviformis*. Genetics **57**, 875-879.

MICROBIOLOGIA SEM 4 (1988), 125 - 128 **NOTES**

Heat stability of the extracellular lipase from a *Pseudomonas* strain isolated from refrigerated raw milk

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Summary

A study on the thermal stability of the extracellular lipase of a *Pseudomonas* strain has been made. The D-values at 140° C ranged from 0.54 to 0.85 min, depending on the heating medium. The average Z-value and activation energy were 36.2° C and 8.18×10^4 J. mol^{-1}, respectively. The enzyme showed maximum activity at pH 8.5 and 37° C.

Key words: Psychrotrofs, lipase, heat stability.

Resumen

Se ha realizado un estudio sobre la lipasa extracelular de un cepa del género *Pseudomonas*. Los valores D a 140° C variaron entre 0,54 y 0,85 minutos, dependiendo del medio de calentamiento. Los valores medios de la energía de activación y del parámetro Z fueron de $8,18 \times 10^4$ J.mol^{-1} y 36,2° C, respectivamente. El enzima mostró su actividad máxima a pH de 8,5 y a una temperatura de 37° C.

Since the adoption of refrigerated systems for the collection and storage of raw milk there is a great opportunity for psychrotrophic bacteria to grow. They are, therefore, the predominant organisms in milk before processing. The psychrotrophic organisms found in refrigerated raw milk are mainly Gram-negative bacilli (14), being the pseudomonads the dominant ones usually (12). The pseudomonads are easily killed by mild heat treatment (e.g. pasteurization), but many strains produce extracellular enzymes (proteases and lipases) which can survive to heat treatments even those commonly used for commercial sterilization (12). Consecuently, although products may be commercially sterile, spoilage due to surviving enzyme activity may still occur.

Heat-resistant proteases and the organisms that produce them have been extensively studied (5, 12). Although there are several studies about the extracellular heat-resistant lipases produced by psychotrophic bacteria (1, 9), these enzymes have been studied much less extensively than heat-resistant proteases. The present work provides additional information about the heat-resistant lipase produced by a *Pseudomonas fluorescens* strain isolated from raw milk.

Ps. fluorescens-73 was isolated from refrigerated bulk milk. To produce lipase, several 1 liter conical flasks containing 250 ml each of Nutrient Broth (Difco) were inoculated with 1 ml from a 24 h subculture in the same medium. Cultures were incubated statically at 24° C for 2 days. Cells were removed by centrifugation (15,000 × g, 10 min). The proteins of the supernatant were precipitated with $(NH_4)_2SO_4$ at 50 % of saturation.

(*) Corresponding author.

The precipitate was redissolved in about 100 ml of distilled water which was concentrated 10-fold by ultrafiltration in a concentration cell, using a membrane with a nominal exclusion limit of 10,000 Da (Diaflo PM-10, Amicon). The concentrate was chromatographied through a column (132 × 60 cm) of Sepharose 4B. All fractions showing lipase activity were combined and lyophilized. This procedure gives an enzyme purification of about 300 times (unpublished data).

Lipase activity was determined by the method of Castberg and Solberg (6). The assay mixture consisted of 1 ml of tributyrin emulsion (10 % tributyrin in 10 % aqueous arabic gum) and 0.5 ml each of 1.0M TRIS-HCl, pH 8.5 and 1.1M NaCl. Enzyme solution and water were added to give a final volume of 5.0 ml. Standard assay conditions were an incubation of 1 hour at 37° C and pH 8.5 in a shaking water bath (50 strokes/min). To determine the optimun pH the TRIS-HCl buffer was substituted by the universal buffer of Britton and Robinson (8) at different pH values. The optimum temperature was determined under the standard assay conditions except that the incubation was carried out at different temperatures. The reaction was stopped by adding 2 ml of a solution of 0.1N H_2SO_4 in 1M NaCl to the reaction mixture. Liberated butyric acid was extracted with a 11:4 (v/v) mixture of diethyl ether:light petroleum. A sample of the upper phase was titrated with 0.005N KOH using thymol blue (0.1 % thymol blue in 60 % isopropanol) as indicator under continuous bubling with nitrogen.

For the determination of the heat stability of the lipase, portions (*c.a.* 50 μl) of the enzyme solution were sealed in capillary tubes (0.9-1 mm i.d., wall thickness 0.15 mm) and heated at several temperatures in a thermostatically controlled glycerol bath for various periods. Decimal reduction times (D-values) were calculated from the linear regression equations of the thermal inactivation curves. Heat stability was determined in several media: skim milk, synthetic milk salts (SMS) (11), SMS supplemented with 15 mM $CaCl_2$ and distilled water. The respective lipase solution was prepared before heating. For that, the lyophilizate was dissolved in distilled water and 1 volume of this solution was mixed with 9 volumes of each heating medium.

Table 1 shows the D-values of the partially purified lipase of *Ps. fluorescens*-73 in the four media used. It may be seen that, at the same temperature, the D-values in the different media were rather similar. Perhaps, the lipase was slightly more thermally stable (about 15 %) in skim milk than in SMS throughout the range of temperature tested. Similar observations have been made by other authors (9). Addition of 15 mM-$CaCl_2$ to the SMS buffer did not restore the stability to values comparable with those in SMS. In contrast with this fact, other authors (1,9) have observed that Ca ions exert some protective effect against heat desactivation of *Pseudomonas* spp. lipases but this effect was greater at temperatures below 100° C approximatively (9). It may be concluded that at temperatures in the range at which the commercial sterilization of milk is made (120-145° C) the calcium has no effect on the heat stability of lipase. This suggests that the heat stability mechanisms of extracellular lipases are different to those of extracellular proteinases of psychrotrophic bacteria. The latter enzymes are heat-stable at high temperatures only in systems containing calcium (5, 12).

TABLE 1

D-VALUES (mim) AT DIFFERENT TEMPERATURES, Z-VALUES (°C) AND ACTIVATION ENERGIES ($J.mol^{-1}$) OF THE EXTRACELLULAR LIPASE OF *PSEUDOMONAS*-73 IN SEVERAL HEATING MEDIA

Heating medium	D-values at				Z-values	Ea (x 10^4)
	110° C	120° C	130° C	140° C		
Distilled water	4.47	2.94	1.43	0.73	38.7	7.66
Skim milk	4.86	2.65	1.42	0.85	37.0	7.85
SMS	3.98	2.24	1.26	0.60	36.0	8.04
SMS-Ca	4.27	2.50	1.17	0.54	33.1	9.19

SMS, Synthetic milk salts. SMS-Ca, Synthetic milk salts with calcium

The plot of the logarithm of D-values versus the temperature of heating resulted in a straight line (decimal-reduction time curves) for the four suspension media used. As it can be seen in Table 1, the Z-value (temperature necessary to bring about a 10-fold change in the D-value) in the four media were quite close (average 36.2° C). This value is in agreement with those reported by others authors, e.g. Z = 37° C in nutrient broth and 38.9° C in skim milk for *Ps fluorescens* SIK W1 (4). Likewise, the activation energies (Ea) for inactivation calculated from the Arrhenius equation were quite similar (Ea average = 8.18×10^4 J.mol^{-1}). This Ea value obtained for the desactivation of the extracellular lipase of *Pseudomonas* 73 was similar to those found by other authors, e.g. Ea = 6.97×10^4 J.mol^{-1} in SMS for *Ps. fluorescens* AFT-36 (9).

Optimum temperature for *Ps. fluorescens*-73 lipase activity was 37° C and the optimun pH was 8.5 (fig. 1). Although it has been occasionally reported (13) an optimun pH of lipase activity of *Pseudomonas* sp. at a value of 6.5 most of the authors (1,9) reported optimum pH values in the range 8.0-9.0. Similarly, the optimun temperature found for *Ps. fluorescens*-73 is in the range observed for other *Pseudomonas* strains by the obove mentioned authors (1.9). At the pH of milk (6.7) and at normal room temperature (20-30° C), the lipase activity was lower than that observed at the optimum values of these two agents (Fig. 1), a 55 % and 50-60 % of the maximum activity, respectively.

The UHT (ultra-high-temperature) processes were envisaged to reduce the most heat-resistant bacterial spores populations to levels low enough to achieve a commercial sterilization. According to the heat-resistance parameters reported for *Bacillus stearotermophilus* (7) and *B. subtilis* (10) spores, wich are commonly used to determine the processing conditions required for commercial steriliza-

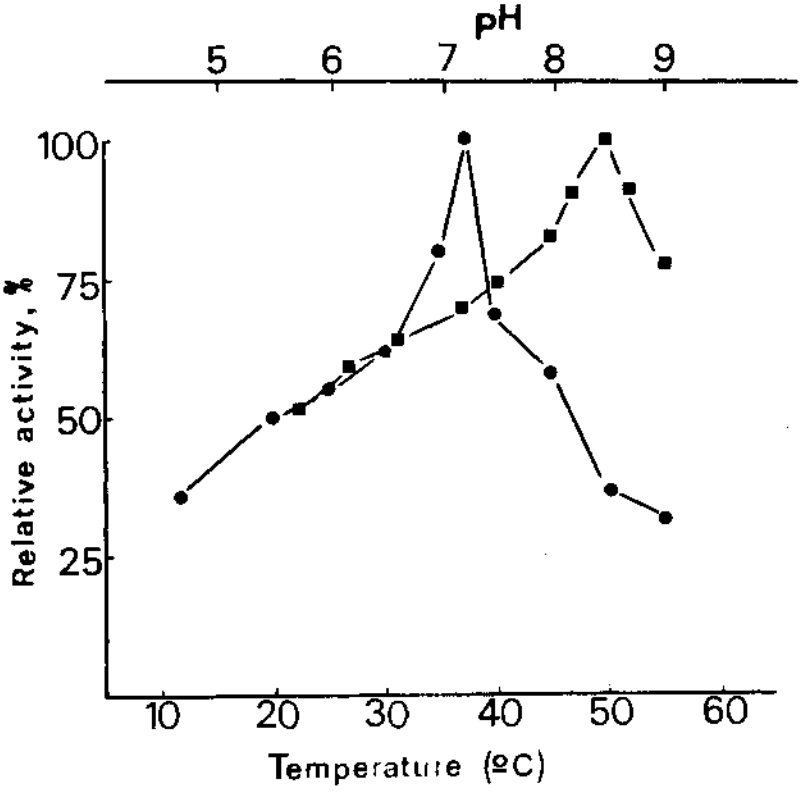

Fig. 1. Effect of pH (■) and temperature (●) on the activity of the extracellular lipase of *Pseudomonas*-73.

tion of milk, it may be deduced that the thermostability of lipase of *Ps. fluorescens*-73 is more than 200 and 1,000 times greater than spores from the two organisms mentioned above, respectively. Thus, an UHT treatment (140-145° C, 2-3 s.) would reduce the *B. stearothermophilus* and *B. subtilis* spore populations more than 15 and 80 log. cycles, respectively but *Ps. fluorescens* lipase would retain more than the 80 % of its original activity. Therefore, the enzyme may well attack the fat of UHT milk and other long-life milk products during storage yielding free fatty acids which are the responsible of off-flavors, such as rancid (fatty acids C4-C8) (15) or soapy (fatty acids C10-C14) (3). The role of lipase of psychrotrophic bacteria in spoilage has been recently reviewed (15).

References

1. Adams, D. M., and Brawley, T.G. (1981 a). Heat resistance bacterial lipases and ultra-high temperature sterilization of dairy products. J. Dairy Sci. **64**, 1951-1957.
2. Adams, D. M., and Brawley, T. G. (1981 b). Factors influencing the heat resistance of a heat resistant lipase of *Pseudomonas*. J. Food Sci. **46**, 673-676.
3. Al-Shabibi, M. M. A., Langner, E. H., Tobias, J., and Tuckey, S. L. (1964). Effect of added fatty acids on the flavour of milk. J. Dairy Sci. **47**, 295-296.
4. Anderson, R. E., Hedlund, C. B., and Jonnson, U. (1979). Thermal inactivation of a heat resistant lipase produced by the psychrotrophic bacterium *Pseudomonas fluorescens*. J. Dairy Sci. **62**, 361-367.
5. Barach, J. T., Adams, D. M., and Speck, M. L. (1976). Stabilization of a psychrotrophic *Pseudomonas* protease by calcium against thermal inactivation in milk at ultra-high temperature. Appl. Environ. Microbiol. **31**, 875-879.
6. Castberg, H. B., and Solberg, P. (1975). Tributyrate as a substrate for the determination of lipase activity in milk. J. Dairy Res. **42**, 247-253.
7. Davis, F. L., Underwood, M. H., Perkin, A. G., and Burton, H. (1977). Thermal death kinetics of *Bacillus stearothermophilus* spores at ultra-high temperatures. I. Laboratory determination of temperature coefficients. J. Food Technol. **12**, 115-129.
8. Dawson, M. L., Elliot, D. C., Elliot, W. H., and Jones, R. M. (1969). «Data for biochemical research». 2.ª ed. Oxford University Press. Oxford.
9. Fox, F., and Stepaniak, L. (1983). Isolation and some properties of extracellular heat stable lipases from *Pseudomonas fluorescens* strain AFT 36. J. Dairy Res. **50**, 77-89.
10. Franklin, J. G., Williams, D. J., and Clegg, L. F. L. (1958). Methods of assessing the sporicidal efficiency of and ultra-high temperature milk sterilizing plant. III. Laboratory determination of the heat resistance of spores of *Bacillus subtilis* in water and in milk. J. Appl. Bacteriol. **21**, 51-57.
11. Jenness R., and Koops, J. (1962). Preparation and properties of a salt solution wich simulates milk ultrafiltrate. Neth. Milk Dairy J. **16**, 153-164.
12. Law, B. A. (1979). Reviews of the progress of Dairy Science. Enzymes of psychrotrophic bacteria and their effects on milk and milk products. J. Dairy Res. **46**, 573-588.
13. Law, B. A., Sharpe, M. E., and Chapman, H. R. (1976). The effect of lipolytic Gram-negative psychrotrophs in stored milk on the development of rancidity in Cheddar cheese. J. Dairy Res. **43**, 459-468.
14. Muir, D. D., Phillips, J. D., and Dalgleish, D. G. (1979). The lipolytic and proteolytic activity of bacteria isolated from blended raw milk. J. Soc. Dairy Technol. **32**, 19-23.
15. Stead, D. (1986). Microbial lipases: their characteristics, role in food spoilage and industrial uses. J. Dairy Res. **53** (3), 481-505.


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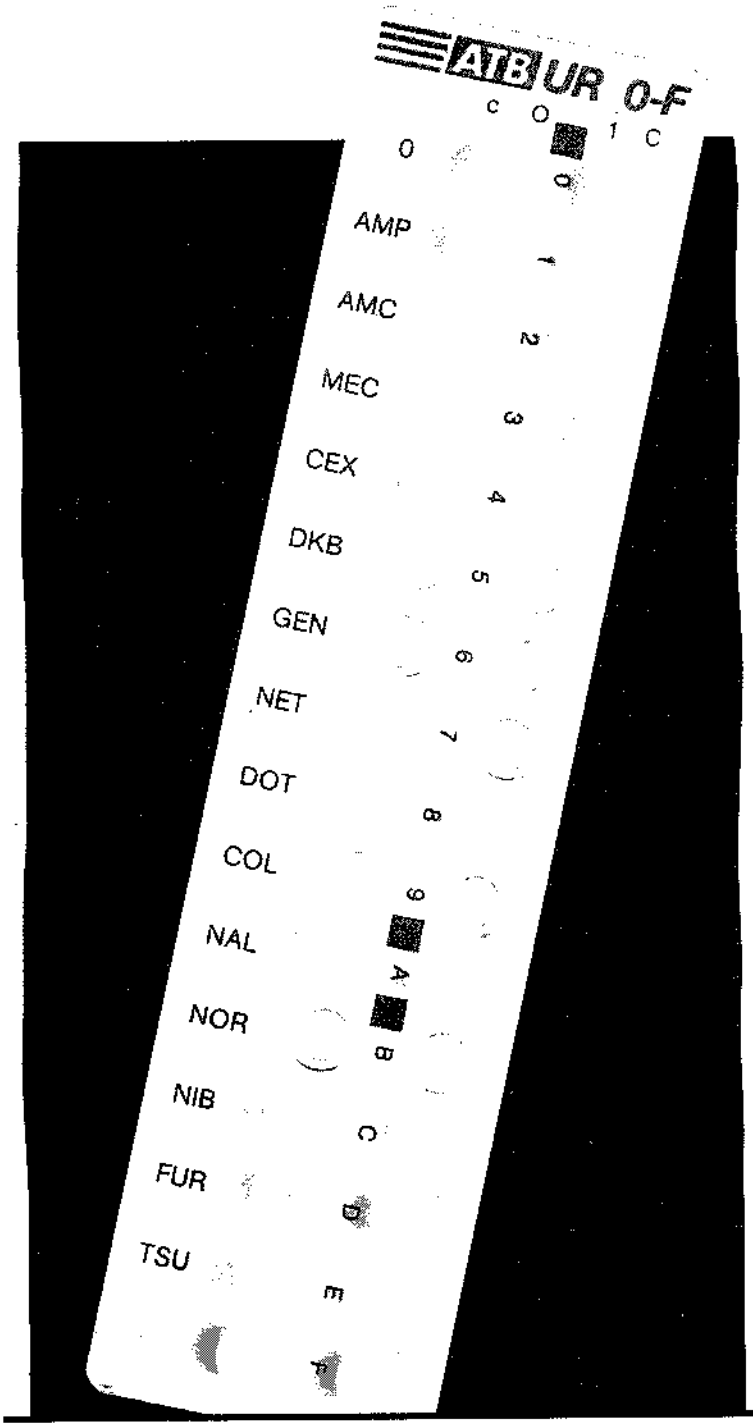

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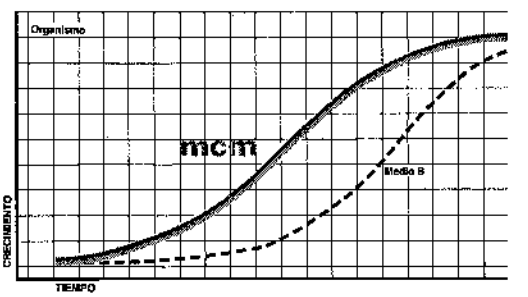

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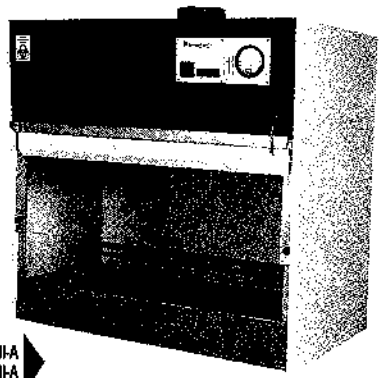

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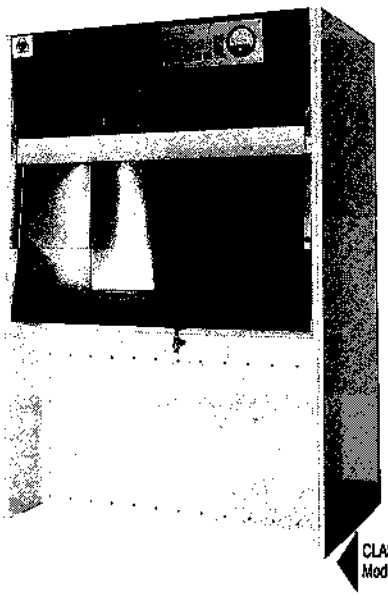

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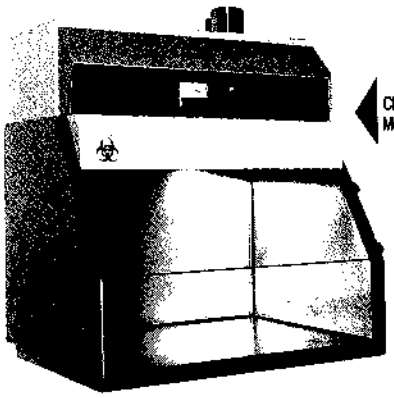

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