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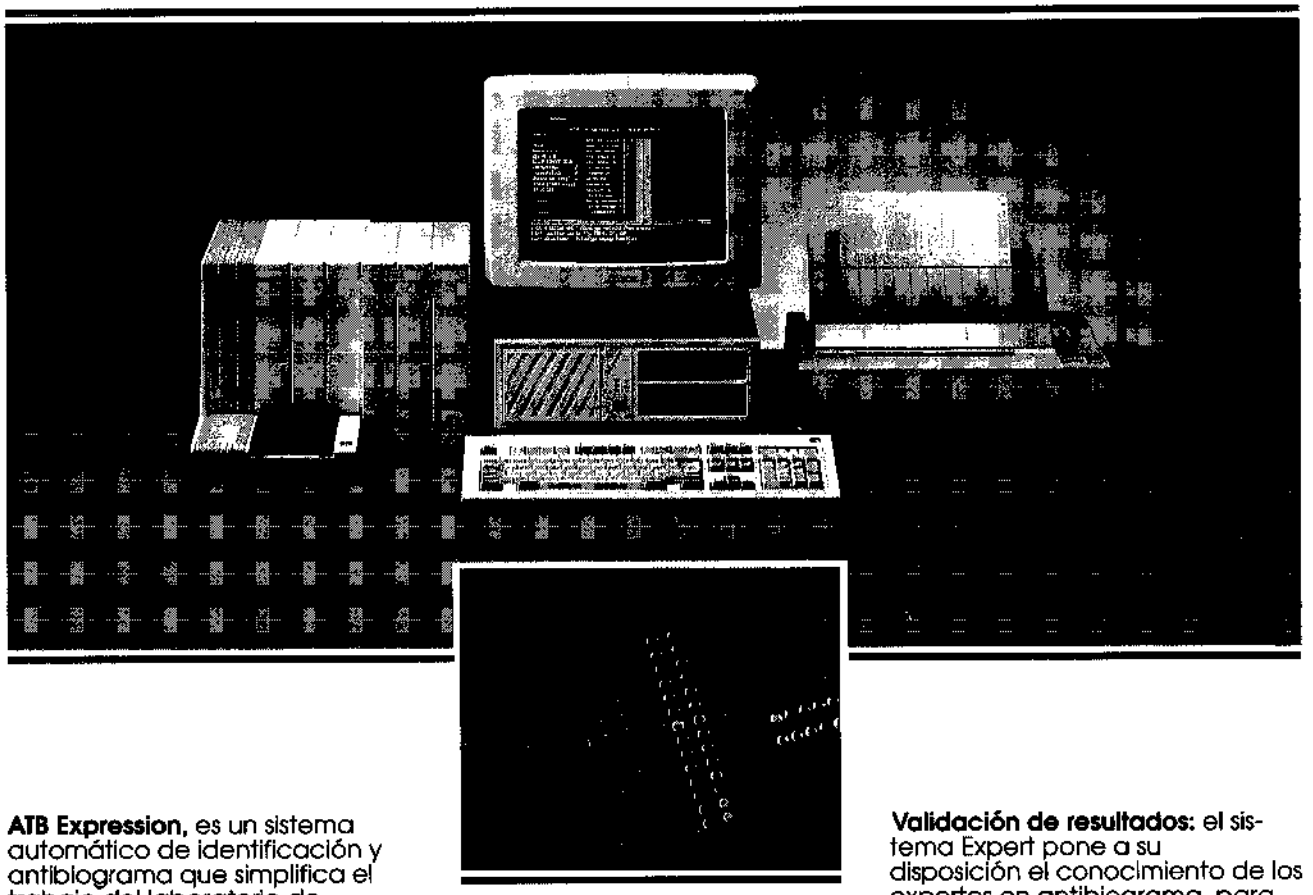

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MICROBIOLOGIA SEM **8** (1992), 49-62 **MINIREVIEW**

Regulation of gene expression in *Streptomyces*

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Introduction

Members of the genus *Streptomyces* are obligate aerobic Gram positive soil bacteria with a life cycle that includes a complex differentiation process as a consequence of their adaptation to the saprophytic life style (18). In parallel to the morphological changes a physiological differentiation occurs, the so called secondary metabolism, which is normally associated with the formation of aerial mycelium. These two processes may share similar regulatory elements (15). The secondary metabolism involves the biosynthesis of products which are not essential for vegetative growth, but are of great industrial interest. These compounds include antibiotics (more than 60 % of the approximately 5000 known antibiotics, antiviral, antitumoral, antihelminthics and antiparasitic agents, enzyme inhibitors, plant growth promoters, herbicides and compounds with other modes of action (56).

Genomic organization

The use of modern electrophoretic techniques, such as PFGE, indicate that the *Streptomyces* genome has an approximate size of 6400-8500 kb (36,49). The DNA has a very high G + C content (70-74 %).

The existence of extrachromosomal genetic elemets in *Streptomyces* is a very frequent phenomena. Their sizes and copy number are quite variable (35, 40). In most of cases plasmids are detectable in the ccc form, but also lineal plasmids have been described. Some plasmids can be isolated either in the linear or circular form, depending on the culture conditions (40, 49). Plasmids of up to 580 kb have been reported and in at least one case, the production of the pigment-antibiotic methylenomycin, there is a clear association of a megaplasmid with antibiotic production (48).

The Actinomycetes show a large degree of genetic mobility (24). This genetic instability can affect different facets of their life cycle such as differentiation, production and resistance to antibiotics, formation and secretion of enzymes and the appearance of auxotrophic strains. In some cases regions which have been naturally deleted can be located close to DNA regions susceptible to amplification (AUD) (38, 40). Recently, small segments of the *Streptomyces* genome have been described to act as transposons: IS110 (19), ØKC591 (54), Tn 4556 (23), IS493 (78), IS117 (previously called the 2.6 kb minicircle) (31) and IS116 (51).

(*) Corresponding author.

Regulation of genetic expression in Streptomyces

The regulation of the genetic expression is a process which allows the selective expression of the genetic information codified for in the DNA. This aspect is particularly important in microorganisms such as *Streptomyces,* which go through drastic processes of morphological differentiation and dramatic changes in their cellular metabolism. These morphological and physiological differentiation processes are blocked during certain stages of the growth cycle. The cloning of an increasing number of *Streptomyces* genes is allowing for a more detailed analysis of the multiple mechanisms which control the genetic expression in these microorganisms.

Transcription

Genetic expression is controlled in part by the level of DNA to RNA transcription. The synthesis of RNA is regulated by a variety of elements: promoters, proteins that interact with the DNA, RNA polymerase and sigma factors.

Promoters

A large number of *Streptomyces* DNA fragments with promoter activity have been identified and characterized (40, 57). The use of vectors for the search of promoters has been of great utility in cloning DNA fragments involved in the initiation of transcription. A promoter probe vector contains a gene without a promoter —a «reporter gene»—, which is used as expression marker in order to measure the strength as promoters of the DNA fragments located upstream. The genes commonly used to measure promoter strength in *Streptomyces* are:

a) The *neo* gene, derived from Tn5, which has been traditionally the most used reporter gene, as part of the promoter-probe vectors pIJ486 and pIJ487 (88).
b) The *xyl*E gene, which derives from the TOL plasmid of *Pseudomonas putida* (41). The cathecol dioxygenase activity encoded by *xyl*E is easily detectable on plates, as well as in cell-free extracts from liquid cultures, by the degradation of cathecol to the yellow product 2-hydroxymuconic semialdehyde. There is no background activity in the host strain.
c) The system based on the *lux*AB gene, derived from *Vibrio harveyi* (74), produces luminiscence in the presence of N-decanal vapors. The main advantage of this system is that these activities do not exist naturally in the host cell and thus permit one to determine the timing and spatial localization of the gene expression.
d) The *lac*Z gene of *Escherichia coli* is poorly expressed in *Streptomyces.* Thus the *lac* gene of *Streptomyces lividans* has been used as an expression indicator in the construction of promoter cloning vectors in *Streptomyces* (80, Asturias *et al.,* 1992 in preparation). However the elimination of the ß-galactosidase activity in the host strain is required.
e) There are other genes which have been used in the construction of promoter-probe vectors in *Streptomyces.* These include: the *Streptomyces coelicolor* gene encoding for a brown diffusable pigment (27), the *vph* gene of *Streptomyces vinaceus,* which codifies a phosphotranferase involved in viomycin resistance; the *cat86* gene of *Bacillus pumilis* encoding a chloramphenicol acetyltransferase or the *E. coli amp*C and *gal*K genes for ß-lactamase and galactokinase activities (41). Recently a gene encoding a thermostable malate dehydrogenase (MDH) from *Thermoactinomyces flavus* has been used as a reporter gene by measuring the MDH activity after heat inactivation of the natural activity in the host strain (86).

Most of these genes fall in two groups: genes for resistance to antibiotics and genes whose products are chromogenic, the latter being further developed as they permit the detection of fragments

with promoter activity of genes expressed only in the late phase of the cellular cycle (secondary metabolism).

These promoter cloning vectors need a transcriptional terminator upstream of the cloning site to impede reading through from putative promoters present in the vector (see below). Recently bifunctional *E. coli-Streptomyces* vectors and vectors which permit the detection of bidirectional promoters have been developed (3, 64).

There are no optimum criteria, such as the association of these sequences with certain RNA polymerase factors, for the classification of *Streptomyces* promoters. Thus, the criteria most often used are the homology to the *E. coli* promoters, the similarity of genes expressed from such promoters or the cellular state during which the expression occurs.

Some promoters have a sequence similar to the consensus sequence for procaryotic promoters. Among these, the best known are the *gyl*P1, *gyl*P2 and *gyl*Rp promoters of the *gyl* operon (for glycerol utilization) from *S. coelicolor;* they have a strong homology with *E. coli* promoters associated with the σ^{70} factor, for vegetative growth which are transcribed by σ^{70}. These type of promoters have been called SEP promoters *(Streptomyces-E. coli* type promoters) (45). There are *Streptomyces* promoters *(tsr, aph)* which only show homology with the «-10» region of the *E. coli* consensus promoters, while the «-35» region is quite different. A similar situation exists with the positively regulated procaryotic promoters which require protein activators (21).

Some *Streptomyces* promoters show promoter activity in *E. coli.* These include: the constitutive P_2 promoter from the *S. lividans* galactose operon, the P_c and pIJ101A promoters from the pIJ101 plasmid; the P_{xp55} promoter from the XP55 gene and the P_{saf} promoter from *S. griseus* (25, 40). In all these cases the transcription is initiated at the same nucleotide in both microorganisms, indicating that the interaction of the *E. coli* RNA polymerase is very similar to that of *Streptomyces.* There are also *S. coelicolor* promoters which show activity in *B. subtilis* (68). However, the large mayority of *Streptomyces* promoters do not act as such when they are introduced into *E. coli.* The high G+C content of *Streptomyces* DNA may explain this fact, although it certainly is not the only reason.

The existence in *Streptomyces* of regions with multiple promoters or with tandem promoters has been well documented and includes the promoter regions of genes for glyecerol utilization, production of α–amylase and agarase, synthesis of ribosomal RNA, catabolism of galactose, biosynthesis of streptomycin and erythromycin, synthesis of the subtilisin inhibitor (ssi) by *S. albogriseolus* and the promoters of antibiotic resistance genes such as those for erythromycin *(erm*E*),* neomycin *(aph),* viomycin *(vph),* thiostrepton *(tsr)* and streptomycin *(str)* (9, 42, 43, 44, 83). The presence of multiple promoters provides the cell with mechanisms for the modulation of the genetic expression.

Regions with multiple promoters in opposite directions have been described in the *erm*E gene of *Saccharopolyspora erythraea,* the *sph* gene of *Streptomyces glauscescens* (40), the *red*D gene of *S. coelicolor* (63) and the repressor of the temperate actinophage ØC31 (77). These can be related to a type of regulation by antisense RNA.

An extreme example of how complicated the structure and function of *Streptomyces* promoters can get to be is the case of a promoter in the actinomycete *Micromonospora echinospora,* which only requires 5 bp upstream of the transcription initiation site in order for the latter to begin (8).

The great heterogeneity and complexity of the *Streptomyces* promoter squences may be due in part to the existence of multiple forms of the RNA polymerase.

RNA polymerases

RNA polymerases have been purified from *Streptomyces antibioticus* (46), *Streptomyces hygroscopicus* (59), *Streptomyces ambofaciens* (76), *Streptomyces granaticolor* (79), *S. coelicolor* (13), *S. lividans* (J. Schmit, personal communication) and *S. griseus* (A. T. Marcos, personal communication).

TABLE 1.
SIGMA FACTORS PRESENT IN *STREPTOMYCES* AND OTHER EUBACTERIA

Sigma factor	Recognition sequence	Microorganism	Target
70	TTGACA (17) TATAAT (6) + 1	*E. coli*	Vegetative growth
	TTGACA (17) TATAAT (6) + 1	*B. subtilis*	Vegetative growth
35	TTGACA (18) TAGGAT (6) + 1	*S. coelicolor*	Vegetative growth
32	CTTGAA (13-15) CCCCAT-TA (7) + 1	*E. coli*	Heat shock
37	AGG-TT (13-16) GG-ATTG-T (6) + 1	*B. subtilis*	Unknown
49	Similar to *B. subtilis* σ^{37}	*S. coelicolor*	Catabolic genes *(endoH, dagAp3)*
28	Unknown	*S. coelicolor*	Agarase *(dagAp32)*
32	AAATC (14-17) CATATT (8-10) + 1	*B. subtilis*	Unknown
29	TT-AAA (14-17) CATATT (8-10) + 1	*B. subtilis*	Sporulation
30	GCAGGA (17) GAATT (?) + 1	*B. subtilis*	Sporulation
spollAC	Unknown	*B. subtilis*	Sporulation
whiG	Unknown	*S. coelicolor*	Sporulation
28	CTAAA (16) CCGATAT (7) + 1	*B. subtilis*	Motility
flbB, flal	TAAA (15) GCCGATAA (?) + 1	*E. coli*	Motility
	TTGGCCC (5) TTGCA (6-11) + 1	*C. crescentus*	Motility
54	CTGGCAC (5) TTGCA (6-11) + 1	*E. coli*	Nitrogen regulated genes
Gen 28	T-AGGAGA-A (15-16) TTT-TTT (4-7) + 1	SPO1/*B. subtilis*	SPO_1 middle genes
Genes 33/34	CGTTAGA (17-19) GATATT (?) + 1	SPO1/*B. subtilis*	SPO_1 early genes
Gen 55	(No –35) TATAAATA (3-6) + 1	T4/*E. coli*	SPO_1 late genes

In several procaryotic organisms RNA polymerases have been demonstrated to bind several factors, which confers different promoter specificities to the holoenzyme as well as a great potential in the control of the expression of a large number of genes. This is also the case in *Streptomyces* (15). Besides this type of control, in *E. coli* the ß subunit of the RNA polymerase is involved in the stringent response. A similar phenomena has been suggested in *Streptomyces* (65) as well as the modulation of the RNA polymerase transcription specificity in *S. griseus* by posttranscriptional ADP-rybosylation (7).

The heterogeneity of *Streptomycces* RNA polymerases was first discovered by Westpheling *et al.* in *S. coelicolor* (89). They identified two different factors: σ^{35} recognizes the *Bacillus subtilis veg* promoter, which would be equivalent to the *E. coli* σ^{70}, and σ^{49} which recognizes the *ctc* promoter of *B. subtilis* and the promoter of the *Streptomyces plicatus endo* 4 gene. This may explain why the *S. lividans* RNA polymerase may recognize the *B. subtilis* Ø29 phage promoters (71). The *dag A* gene from *S. coelicolor* is subject to complex transcriptional regulation, being transcribed from 4 promoters by at least 3 different RNA polymerases. The *dagAp2* promoter is recognized by a new factor, σ^{28}, the promoter *dagAp3* by σ^{49} and σ^{35} recognizes the *dagAp4* promoter (14). The *S. coelicolor* galactose operon is controlled by two promoters, *galp*1 *galp*2. The *galp*2 promoters is also controlled by σ^{28}, while the *galp*1 is recognized by a different holoenzyme which has not yet been characterized (90).

Sequences with strong analogy to the *rpo*D gene, which codifies for the principal sigma factors of *E. coli* (σ^{70}) and *B. subtilis* (σ^{A}), have been found in different *Streptomyces* species (84). Three of the four *S. coelicolor* genes *(hrd*B, *hrd*D, and *hrd*A*)* homologous to the *rpo*D gene of *E. coli* have been cloned and sequenced (16, 86). Mutants for *hrd*B were never obtained from *S. coelicolor* by gene disruption, suggesting that *hrd*B codifies for a factor homologous to the *E. coli* σ^{70} responsible for the transcription of housekeeping genes in the cell (16). This interpretation is consistent with

the observation that of the four genes of *S. lividans* homologous to *rpo*D, only *hrd*B is present in all the *Streptomyces* species studied (84).

The *whi*G gene of *S. coelicolor* codifies for the *whi*G factor, which plays a fundamental role in the initiation of sporulation. However this factor does not seem to be essential for the expression of genes involved in the production of antibiotics (20).

The great transcriptional flexibility which results from the capability to synthesize different sigma factors is very well established in *Streptomyces*, although except for in the cases of σ^{whiG} and σ^{hrdB} the role in the biology of *Streptomyces* of the other factors has not yet been elucidated (Table 1).

Transcription termination and messenger processing

Transcription termination in *Streptomyces* seems to be similar to that in *E. coli*, as *rho*-independent transcriptional terminators also produce termination in *Streptomyces* (33). In *Streptomyces* the most widely used terminators are the *fd* terminator from the *fd* coliphage (10) and the t_{oop} terminator derived from λ (39). In parallel, a *Streptomyces* terminator acts as such in *E. coli* (26). Inverted repeated sequences which originate secondary hairpin structures are responsible for this type of termination and are found at the 3' ends of many *Streptomyces* messengers. In the cluster of genes for methylenomycin biosynthesis, an inverted sequence acts as a transcriptional terminator for the convergent transcripts of both the *mmr* and *mmy* genes. In some cases these structures correspond to termination sites, but they may also be sites for processing the transcripts (21) such as is found in *E. coli* (67). The U-rich regions located at the 3' end of the typical hairpin loop of this type of terminators in *E. coli* and *B. subtilis*, does not exist in the *Streptomyces* terminators studied up to now (21). There is even less information on protein dependent *Streptomyces* terminators, similar to the *rho* factor dependent terminators in *E. coli*, as they do not have easily recognizable sequences.

Antitermination

The transcription of some genes depends on the ability of the RNA polymerase to read through terminators located on the 3' end of the genes. This depends on the existence of a gene codifying for an antiterminator that modifies the RNA polymerase so that it will not recognize the termination signal.

In *Streptomyces* it has been proposed that the *strR* gene of *S. griseus* codifies for an antiterminator which allows the reading of genes located downstream of terminators, such as *strA (aph*D*)*, the streptomycin resistance gene, and *strB*, which codifies for a key streptomycin biosynthetic enzyme (70). A similar situation has been described for the *nsh* gene which confers resistance to the nosiheptide thiopeptide produced by *Streptomyces actuosus* (52). This strategy of antitermination in this case allows the producer microorganisms to protect themselves from the antibiotic being produced.

Genetic regulation by antisense RNA

This mechanism of regulation is based on the existence of two complementary RNA transcripts, the antisense RNA and the mRNA transcript being regulated, which are originated from two close promoters present in opposite directions. The antisense RNA blocks the mRNA ribosome binding site and prevents its translation.

A mechanism of regulation by antisense RNA could be involved in the control of the expression of some *Streptomyces* genes such as the *aph* gene, codifying for neomycin resistance in *S. fradiae* and the *erm*E gene for the methylase involved in erythromycin resistance in *S. erythraea*. Both promoter regions contain several divergently transcribed promoters.The absence of clear SD se-

TABLE 2
SOME *STREPTOMYCES* SECONDARY METABOLITES BIOSYNTHETIC GENES

Gene	Regulation	Strain	Secondary metabolite	Function
brpA	Positive	*S. hygroscopicus*	Bialaphos	Enhances the transcription of *bar* (for bialaphos resistance) and 6 *bap* genes (for bialaphos biosynthesis).
?	Positive	*S. fradiae*	Tylosin	Enhances the transcription of *tylF* and other *tyl* biosynthetic genes.
$dnrR_1$	Positive	*S. peuceticus*	Daunorubicin	
actII	Positive	*S. coelicolor*	Actinorhodin	Increases 30-40 fold actinorhodin production.
?	Negative	*S. coelicolor*	Methylenomycin	Mutants with deletions or inactivating insertions in this *locus* superproduce methylenomycin.
strR	Positive	*S. griseus*	Streptomycin	Codifies for a protein (antiterminator) required for the expression of *strA* and *strB*.
red	positive	*S. coelicolor*	Undecylprodigiosin	Enhances the transcription of *red*.
afsR	positive	*S. lividans* and *S. coelicolor*	A factor and pigment-antibiotics	The phosphorylated protein AfsR increases the transcripts of regulatory genes for pigment-antibiotics.

quences in the mRNA's produced from these promoters has led to the suggestion that antisense regulation may be involved, however the problem remains non elucidated (12).

Transcription regulation by interaction of DNA with proteins

A different type of transcriptional regulation is carried out by proteins which are able to interact with the DNA. One of the characteristics of these proteins is their secondary structure, a «helix-turn-helix» type. The interaction of regulatory proteins with DNA is not as simple as positive or negative regulation, in some cases —such as the mercury resistance in *E. coli* (66)—, a single protein positively and negatively regulates different genes.

The adoption of positive or negative control for a particular metabolic pathway may differ according to the bacterial group. The genes for glycerol utilization are negatively controlled by the product of the *glpR* gene in *E. coli*, positively by the *Glp1* protein in *B. subtilis* and probably both positively and negatively by the product of the *gylP* gene in *S. coelicolor* (21).

The studies on the organization and expression of the genes involved in the biosynthesis of secondary metabolites in *Streptomyces* have allowed for the location and characterization of regulatory genes (34, 57) (Table 2). These genes fall into two general types: those which are clustered with the antibiotic biosynthetic genes that they regulate (i. e. *actII, milbII, redD, mmy, brpA, strR* and *dnrlR*), and genes which may have pleiotropic phenotypes, acting as high hierarchy regulatory genes and are not necessarily clustered with genes for secodary metabolites *(afsA, afsB, afsR, whiG, bldA* and *asaA)* (81).

The modulation of the expression of genes by DNA-protein interactions has been described to

occur during the replication of plasmid pIJ101 in *S. lividans* (80), and in the expression of the *mel* gene of *S. glaucescens* (28); the *tipA* gene expression also is activated by a dimeric protein of 60 kDa (61, J. L. Caso and C. J. Thomson, personal communication) as occurs with the *tsrpl* promoter of the *tsr* gene of *Streptomyces azureus* (44).

Translation

The mechanism of translation seems to be very conserved in all the eubacteria. Generally, the genes are preceded by a short region located upstream of the translation initiation site. This region is called the ribosome binding site (RBS) or the Shine-Dalgarno sequence (SD) and results in an mRNA complementary to an RNA sequence close to the 3' end of the 16S rRNA.

In *S. lividans* the sequence on the 3' end of the 16S rRNA is 5'GAUCACCUCCUUUCU-OH-3' (11). The analysis of SD sequences in *Streptomyces* shows that it is not really essential for the mRNA and the ribosome sequences to be totally complementary, an aspect more similar to *E. coli* than to *Bacillus* or *Staphylococcus* (33, 57). Not all of the *Streptomyces* genes transcripts have a conventional SD sequence; eight genes which have their transcription initiation sites close to or on the initiation codon have been described. In general they are genes involved in antibiotic biosynthesis or resistance and in cellular differentiation. They are the genes encoding the acetyltransferases responsible of streptomycin, aminocyclitol and chloramphenicol resistance *(sta*P, *aac*7, *cat)* in *S. lavendulae, S. rimosus* and *S. acrimycini,* the phosphotransferases encoded for *aph* and *sph* confering resistance to aminocyclitol or ribostamycin in *S. fradiae* and *S. ribosidificus var paromomycinus,* the methylase encoded by *ermE* for resistance to eythromycin in *S. erythraea,* and *nsk* which confers resistance to nosiheptide in *S. actuosus* and *afs*A a gene involved in factor A formation in *S. griseus.*

The transcription initiation codon most often used in *E. coli* is AUG (97 %), while GUG is only used with a 3 % frequency. Due to the high G+C content of *Streptomyces* DNA the GUG codon is used more frequently (20 %) in this microorganism. Likewise, in *Streptomyces,* the utilization of codons in 90 % of the cases is slanted toward codons having G or C in the third position.

Streptomyces contains all the tRNA's required to translated the 61 possible sense codons, but it has been found that the relative cellular content of some tRNA's controls the level of translation in particular genes. This is the case of the *bld*A gene, which codifies for a tRNA gene that recognizes the rare UUA codon for leucine. Mutants *bld*A negative are unable to produce aerial mycelim except when grown in cultures with a low phosphate concentration (29). The UUA codon has been found only in a few genes involved in differentiation such as *car*B, for resistance to carbomycin in *Streptomyces thermotolerans;* the ORF 1590, codifying for a protein required for sporulation in *S. griseus* or *sph* and *hyg,* codifying phosphotransferases for hydroxystreptomycin and hygromycin resistance (5, 21). The proteins codified by these last two genes appears to be especially dependent on the expression of the *bldA* gene, as both of these genes have UUA codons at the beginning of the codifying region and in front of a possible RBS. Thus when limiting amounts of tRNA are available, the translation of this gene is halted at the UUA codon (21).

Attenuation

A model for translational attenuation has been described for the *S. fradiae erm*SF gene. This gene confers induced resistance to the macrolide-lincosamide-streptogramin B group of antibiotics (47). Deletion analysis of a 385 nucleotide 5' leader sequence upstream of the *erm*E support the existence of inverted complementary repeated sequences capable of assuming alternative conformations. Two putative Shine-Dalgarno sequences for a leader peptide and for the erythromycin methylase encoded by *erm*E regulate the standstill or the movement of the ribosome along the

mRNA in response to the presence of erythromycin. The model is similar to that proposed for the *erm*C gene from *Staphylococcus aureus.*

Regulation of genes involved in antibiotic biosynthesis in Streptomyces

The two processes which characterize the final steps of morphological and physiological differentiation in *Streptomyces,* sporulation and antibiotics production, are subject to some form of common control, as shown by the isolation of mutants of many *Streptomyces* species blocked simultaneously in both processes. In *S. coelicolor* the *bld* mutants are unable to produce antibiotics and aerial mycelia. At least 7 different classes of this type of *bld* mutants have been described: *bld*A-D and *bld*F-H, with the *bld*B locus being composed of at least 2 different genes (22, 30).

Other mutants unable to produce antibiotics are *afs*B, *afs*C, *abs*A and *abs*B (1, 17). By complementation of *afs*B mutants (18), an additional regulatory gene, *afs*R has been found. It codifies for a protein of 105 kDa, with an ATP binding site in the aminoterminal end and a DNA binding site at the carboxyl terminal end (37). This phosphorylated protein acts as a transcriptional activator of the *act*II gene, which is involved in the biosynthesis of the pigment-antibiotic actinorhodin (32). The presence of extra copies of *afs*R stimulates the production of both pigments actinorhodin and undecylprodigiosin in *S. lividans,* while the *afs*R mutants practically do not produce any of the pigments (37). The *abs*A mutation blocks the production of antibiotics, but not the sporulation, suggesting the existence of some type of specific regulator for the biosynthesis of antibiotics (1). Both *abs*A and *abs*B mutants are complemented by multiple copies of the carboxyl terminal fragment of the *afs*R protein (17).

Several pleiotropic effectors have been described in *Streptomyces* which affect both antibiotic production and sporulation. The best studied is the A factor [(-)2-isocaproyl-(3R)-hydroxymethyl-γ-butyrolactone] which positively controls the biosynthesis and resistance to streptomycin and the sporulation in *S. griseus* and *Streptomyces bikiniensis.* The A factor acts by binding and inactivating a repressor protein (60).

In summary, the biosynthesis of antibiotics in *Streptomyces* is controlled by a complex regulatory network which is still not completely elucidated although different models for coordinated regulation have been proposed (22).

Phosphate regulation

Phosphate is an important nutrient as it is indispensable to form nucleotides for DNA and RNA synthesis, and ATP as energy supply in the whole cellular metabolism. Furthermore, most of the metabolic precursors in the metabolism are phosphorylated compounds. The study of phosphate metabolism is therefore of great importance. The complex media used in the industry for the production of any type of metabolites usually has a plentiful supply of phosphate resulting in a high specific growth rate of the strains. However, the formation of many metabolites decreases due to regulation by phosphate.

Regulation by phosphate in Escherichia coli: pho *regulon*

Phosphate metabolism has been thoroughly studied in *E.coli,* both physiological and genetical. The *pho* regulon includes a complex network of not less than 20 genes for phosphatases (*pho*A, *aap*A), proteins for phosphate binding and transport (*pho*E, *pho*S, *pst* operon), transport of phosphorylated sugars and alcohols (*glp* regulon, *uhp*ABCT, *pgt* clusters of genes) and regulatory proteins (*pho*B, *pho*R, *pho*T) which are controlled by the phosphate content present in the medium (72).

Under phosphate starvation conditions the product of the *pho*R gene phosphorylates the *pho*B protein; this modified form of *pho*B acts as a transcriptional activator by binding the phosphate box

TABLE 3
PHOSPHATE REGULATION OF ENZYMES INVOLVED IN SECONDARY METABOLISM

Antibiotic	Poducer organism	Enzyme	Type of regulation
Candicidin	*Streptomyces griseus*	*p*-Aminobenzoate synthase*	R
Cephalosporin	*Acremonium chrysogenum*	Deacetoxicephalosporin C synthetase*	D
Cephamycin	*Streptomyces clavuligerus*	Aminoadipyl-cysteinyl-valine synthetase*	R
		Deacetoxycephalosporin C synthetase*	RI
		Isopenicillin N synthase*	RI
Cephamycin	*Nocardia lactamduruns*	Deacetoxycephalosporin C synthetase*	I
Gramicidin S	*Bacillus brevis*	Gramidicin S synthase*	D
Neomycin	*Streptomyces fradiae*	Neomycin P phosphotranferase	R
Streptomycin	*Streptomyces griseus*	Streptomycin-6-P phosphotransferase	R
Tetracyclin	*Streptomyces aureofaciens*	Anhydrotetracyclin oxygenase*	R
Tylosin	*Streptomyces fradiae*	Valine dehydrogenase*	D
		Methyl malonyl-CoA: pyruvate transcarboxylase*	D
		Propionyl-CoA carboxylase*	D
		Protilonolide synthetase*	D
	Streptomyces T59.235	dTDP-D-glucose-4,6-dehydratase*	R
		dTDP-mycarose synthetase*	R
		Macrocin O-methyltransferase*	R

[a] R, repression; I, inhibition; D, the enzymatic activity decreases, but the mode of regulation is unknown.
* Enzymes catalyzing reactions in which phosphate is not involved.

(*pho* box), a sequence present near the operator in genes related to the phosphate regulon (55). A similar regulation mechanism has been found in other enterobacteria (50, 58), *Pseudomonas aeruginosa* (2) and *Bacillus subtilis* (75).

Phosphate regulation in Streptomyces

Phosphate control of the biosynthesis of secondary metabolites is a widely observed phenomena in bacteria, fungi and plants (56). In *Streptomyces* the phosphate content in the culture media plays an important role in the starting time and the level of production of secondary metabolites, such as antibiotics. This explains the interest in knowing how the cell controls the differential expression of growth and secondary metabolism. It is not yet known if phosphate is the final effector or simply regulates the level of an intermediate effector which controls the final expression of the antibiotic production genes. As possible effectors ATP (56) or the phosphorylate nucleotides ppGpp and pppGpp (65) have been considered. However the level of these compounds does not seem to be sufficient to explain the regulation by phosphate (82).

Effect of phosphate on primary metabolism and secondary metabolism

As a rule, the key enzymes for primary metabolism are stimulated by phosphate. This is the case of the phosphofructokinase and glucose-6-phosphate dehydrogenase in *S. griseus* IMRU 3570 (56).

In constrast, as indicated above, the biosynthesis of a large number of antibiotics and secondary metabolites is regulated by phosphate. This kind of control is found in almost all of the structural groups: aminoglycosides (streptomycin), tetracyclines (chlorotetracyclin), macrolides (tylosin), polyenes (candicidin) or ionophores (monensin). The biosynthesis of the ß-lactam antibiotics, and in general peptide antibiotics (i.e. gramicidin S), is less sensistive to phosphate than the aminogly-

cosidic or polyketide types are (56). The effect of phosphate on secondary metabolites production is occasionally due to interference with enzymes of the primary metabolism which originate precursors needed for secondary metabolism. Such is the case of the methylmalonyl-CoA-pyruvate dehydrogenase, propionyl-CoA carboxylase and valine dehydrogenase, which are sensitive to the phosphate concentration and lead to a lower production of tylosin in *S. fradiae.*

Effect of phosphate on phosphatases and specific enzymes of secondary metabolism

Specific phosphatases in *Streptomyces* act forming inactive phosphorylated intermediates during antibiotic production and are retroregulated or inhibited by phosphate. In the biosynthesis of streptomycin (89), viomycin (69) and neomycin (6) phosphorylated intermediates lacking antibiotic activity exist. Similar cases seem to be responsable for the phosphate regulation of the biosynthesis of vancomycin by *Streptomyces orientalis* and of ristomycin in *Proactinomyces fructiferi* var. *ristomicini,* although phosphorylated intermediates have not been found in this case (56).

In the biosynthesis of various antibiotics there are enzymatic steps in which phosphate is not involved, but the enzymes are nevertheless repressed by phosphate. The mechanism of action of these enzymes is rather different (Table 3) (53, 56). In the case of the PABA synthase of *S. griseus* 3570 it is known that phosphate affects the transcription of the *pab*S gene, and mutants insensible to phosphate have been shown to be deregulated in the transcription (4, Asturias, J. A., 1991. Ph.D. Thesis, University of León).

The isolation of promoters regulated by phosphate is of great interest in order to understand the molecular mechanisms which control phosphate regulation in *Streptomyces.* The promoter P_{114} (73) has an 18 nt sequence with a 66 % homology with the phosphate box of *E. coli.* This promoter appears to bind a protein as shown by gel retardation experiments (Asturias, J. A. 1991. Ph.D. Thesis, University of León). The purification and study of proteins which modulate the expression of promoters regulated by phosphate, or the elucidation of possible sigma factors that interact with this type of promoters is of great interest.

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References

1. Adamidis, T., Riggle, P. and Champness, W. (1990). Mutations in a new *Streptomyces coelicolor* locus which globally block antibiotic biosynthesis but not sporulation. J. Bacteriol. **158,** 2962-2969.
2. Anba, J., Bidaud, M., Vasil, M. L. and Lazdunski, A. (1990). Nucleotide sequence of the *Pseudomonas aeruginosa phoB* gene, the regulatory gene for the phosphate regulon. J. Bacteriol. **172,** 4685-4689.
3. Asturias, J. A., Liras, P. and Martín, J. F. (1990). A bifunctional *Streptomyces-Escherichia coli* promoter-probe vector. FEMS Microbiol. Lett. **68,** 65-68.
4. Asturias, J. A., Liras, P. and Martín, J. F. (1990). Phosphate control of *pabS* gene transcription during candicidin biosynthesis. Gene **93,** 79-84.
5. Babcock, M. J. and Kendrick, K. E. (1990). Transcriptional and translational features of a sporulation gene of *Streptomyces griseus.* Gene **95,** 57-63.
6. Bandyopadhayay, S. K. and Madjumdar, S. K. (1974). Regulation of the formation of alkaline phosphatase during neomycin biosynthesis. Antimicrob. Agents Chemother. **5,** 431-434.
7. Barabás, G., Szabó, I., Penyige, A. and Ensing, J. C. (1986). Posttranscriptional modification by ADP-ribosylation in *Streptomyces griseus* as a possible bioregulation way. *In*: Abstracts of the 5th International Symposium of Genetics of industrial microorganisms. pp. 245. Split, Yugoslavia.

8. Baum, E. Z., Buttner, M. J., Lin, L. S. and Rothstein, D. M. (1989). The P_1 promoters of *Micromospora echinospora* do not require extensive upstream DNA sequences. J. Bacteriol. **171,** 6503-6510.
9. Baylis, H. A. and Bibb, M. J. (1988). Transcriptional analysis of the 16S rRNA gene of the *rrnD* gene set of *Streptomyces coelicolor* A3 (2). Mol. Microbiol. **2,** 569-579.
10. Beck, B., Sommer, R., Averswald, E. A., Kurz, C. H., Zink, B., Osterburg, G., Schaller, H., Sugimoto, K., Sugisaki, H., Okamoto, T. and Takanami, M. (1978). Nucleotide sequence of bacteriophage *fd* DNA. Nucleic Acids Res. **5,** 4495-4504.
11. Bibb, M. J. and Cohen, S. N. (1982). Gene expression in *Streptomyces:* construction and application of promoter-probe plasmid vector in *Streptomyces lividans.* Mol. Gen. Genet. **187,** 265-277.
12. Bibb, M. J. and Janssen, G. R. (1987). Unusual features of transcription and translation of antibiotic resistance genes in antibiotic-producing *Streptomyces. In:* M. Alacevic, D. Hranueli and Z. Toman (eds.). Genetics of Industrial Microorganisms, Part B, pp. 309-318. Pliva, Zagreb.
13. Buttner, M. J. and Brown, N. L. (1985). RNA polymerase-DNA interaction in *Streptomyces in vitro* studies of a *Streptomyces lividans* plasmid-promoter with *Streptomyces coelicolor* RNA polymerase. J. Mol. Biol. **185,** 177-188.
14. Buttner, M. J., Smith, A. M. and Bibb, M. J. (1988). At least three different RNA polymerase holenzymes direct transcription of the agarose gene (*dag A*) of *Streptomyeces coelicolor* A3(2). Cell **52,** 599-607.
15. Buttner, M. J. (1989). RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). Mol. Microbiol. **3,** 1613-1659.
16. Buttner, M. J., Chater, K. F. and Bibb, M. J. (1990). Cloning, disruption and transcrptional analysis of three RNA polymerase sigma factor genes of *Streptomyces coelicolor* A3(2). J. Bacteriol. **172,** 3367-3378.
17. Champness, W., Riggle, P. and Adamidis, T. (1990). Loci involved in regulation of antibiotic synthesis. J.Cell. Biochem. **14A**-88.
18. Chater, K. F. (1984). Morphologial and physiological differentiation in *Streptomyces. In:* R. Losick and L. Shapiro (eds.). Microbial development, pp. 89-115. CSH Laboratory, New York.
19. Chater, K. F., Bruton, C. J., Foster, S. G. and Tobeck, I. (1985). Physical and genetic analysis of *IS110,* a transposable element of *Streptomyces coelicolor* A3 (2). Mol. Gen. Genet. **200,** 235-239.
20. Chater, K. F., Bruton, C. J., Plaskitt, K. A., Buttner, M. J., Méndez, C. and Helmann, J. D. (1989). The developmental fate of *Streptomyces coelicolor* hyphae depends crucially on a gene product homologous with the motility sigma factor of *Bacillus subtilis.* Cell **59,** 133-144.
21. Chater, K. F. and Hopwood, D. A. (1989). Diversity of bacterial genetics. *In:* D. A. Hopwood and K. F. Chater (eds.). Genetics of bacterial diversity, pp. 24-26. Academic Press, New York.
22. Chater, K. F. (1990). The improving prospects for yield increase by genetic engineering in antibiotic-producing *Streptomyces.* Bio/Technology **8,** 115-121.
23. Chung, S. T. (1987). *Tn4556,* a 6.8 kilobase-pair transposable element in *Streptomyces fradiae.* J. Bacteriol. **169,** 4436-4441.
24. Cullum, J., Altenbuchner, J., Flett, F. and Piendl, W. (1986). DNA amplification and genetic instability in *Streptomyces.* Biotech. Gen. Engin. Rev. **4,** 59-78.
25. Daza, A., Martín, J. F., Vigal, T. and Gil, J. A. (1991). Functional and regulatory analysis of the promoter region of the *saf* gene, a *Streptomyces griseus* gene that increases the production of several extracellular enzymes. Gene **108,** 63-71.
26. Deng, Z., Kieser, T. and Hopwood, D. A. (1987). Activity of *Streptomyces* transcriptional terminator in *Escherichia coli.* Nucleic Acids Res. **15,** 2665-2675.
27. Feitelson, J. S. (1988). An improved plasmid for the isolation and analysis of *Streptomyces* promoter. Gene **66,** 59-162.
28. Geistlich, M., Irniger, S. and Hütter, R. A. (1989). Localization and functional analysis of the regulated promoter from the *Streptomyces glaucescens* mel operon. Mol. Gen. Genet. **222,** 337-344.
29. Guthrie, E. P. and Chater, K. F. (1990). The level of a transcript required for production of a *Streptomyces coelicolor* antibiotic is conditionally dependent on a tRNA gene. J. Bacteriol. **172,** 6189-6193.
30. Harasyma, M., Zhang, L. H., Chater, K. F. and Piret, J. (1990). The *Streptomyces coelicolor* A3 (2) *bldB* region contains at least two genes involved in morphological development. J. Gen. Microbiol. **136,** 1543-1550.
31. Hendersson, D. J., Brolle, D. F., Kieser, T., Melton, R. E. and Hopwood, D. A. (1990). Transformation of *IS117* [the *Streptomyces coelicolor* A3 (2) mini-circle] to and from a cloned target site and into secondary chromosomal sites. Mol. Gen. Genet. **224,** 65-71.
32. Hong, S. K., Kito, M., Beppu, T. and Horinouchi, S. (1991). Phosphorilation of the AfsR products, a global regulatory protein for secondary-metabolite formation in *Streptomyces coelicolor* A3 (2). J. Bacteriol. **173,** 2311-2318.
33. Hopwood, D. A., Bibb, M. J., Chater, K. F., Janssen, G. R., Malpartida, F. and Smith, C. P. (1986). Regulation of gene expression in antibiotic-producing *Streptomyces. In:* I. R. Booth and C. F. Higgins (eds.). Regulation of gene expression-25 years on, pp. 251-277. Cambridge University Press. United Kingdom.
34. Hopwood, D. A.(1987). Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. Proc. R. Soc. Lond. **235,** 121-138.
35. Hopwood, D. A., Bibb, M. J.,Chater, K. F. and Kieser, T. (1987). Plasmid and phage vectors for cloning and gene analysis in *Streptomyces.* Methods Enzymol. **153,** 116-165.
36. Hopwood, D. A. (1988). Understanding the genetic control of antibiotic biosynthesis and sporulation in *Streptomyces. In:* Y. Okami, T. Beppu and M. Ogawara (eds.). Biology of actinomycetes, pp. 3-10. Japan Scientific Societies Press, Tokyo.

37. Horinouchi, S., Kito, M., Nishiyama, M., Furuya, K., Hong, S. K., Miyake, S. and Beppu, T. (1990). Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces*. Gene **95,** 49-56.
38. Hornemann, U., Otto, C. J. and Zhang, X. Y. (1989). DNA amplification in *Streptomyces achromogenes* subsp. *rubrans* is accompanied by a deletion and the amplified sequences are conditionally stable and can be eliminated by two pathways. J. Bacteriol. **171,** 5817-5822.
39. Howard, B. H., de Crombrugghe, B. and Rosemberg, M. (1977). Transcription *in vitro* of bacteriophage lambda 4S RNA: studies on termination and *rho* protein. Nucleic Acids Res. **4,** 827-842.
40. Hütter, R. A. and Eckhardt, T. (1988). Genetic manipulation. *In:* M. Goodfellow, S. T. Williams and M. Mordarski (eds.). Actinomycetes in biotechnology, pp. 89-185. Academic Press, New York.
41. Ingram, C., Brawner, M., Yougman, P. and Westpheling, J. (1989). *xylE* functions as an efficient reporter gene in *Streptomyces* spp.: use for the study of *galP1*, a catabolite-controled promoter. J. Bacteriol. **171,** 6617-6624.
42. Janssen, G. R. and Bibb, M. J. (1988). Tandem promoters transcribe the thiostrepton resistance gene from *Streptomyces azureus* and the viomycin resistance gene from *Streptomyces vivaceus*. *In:* J. Okami, T. Beppu and H. Ogawara (eds.). Biology of Actinomycetes '88, pp. 374-379. Japan Scientific Societies Press, Tokio.
43. Janssen, G. R., Ward, J. M. and Bibb, M. J. (1989). Unusual transcriptional and translational features of the aminoglycoside phosphotransferase gene *(aph)* from *Streptomyces fradiae*. Genes Dev. **3,** 415-429.
44. Janssen, G. R. and Bibb, M. J. (1990). Tandem promoters, *tsrp1* and *tsrp2*, direct transcription of the thioestrepton resistance gene *(tsr)* of *Streptomyces azureus* transcriptional interactions from *tsrp2* occurs after deletion of the -35 region. Mol. Gen. Genet **221,** 339-346.
45. Jaurin, G. and Cohen, S. N. (1985). *Streptomyces* contain *Escherichia coli*-type A+T-rich promoters having novel structural features. Gene. **139,** 191-201.
46. Jones, G. H. (1979). Purification of RNA polymerase from actinomycin producing and non producing cells of *Streptomyces antibioticus*. Arch. Biochem. Biophys. **198,** 195-204.
47. Kamimiya, S. and Weisblum, B. (1988). Translational attenuation control of *ermSF,* an inducible resistance determinant encoding rRNA n-methyltransferase from *Streptomyces fradiae*. J. Bacteriol. **170,** 1800-1811.
48. Kinashi, H. and Shimaj-Murayama, T. (1991). Physical characterization of SCP1, a giant linear plasmid for *Streptomyces coelicolor*. J. Bacteriol. **173,** 1523-1529.
49. Leblond, P., Francou, F. X., Simonet,J. M. and Decaris, B. (1990a). Pulsed-field gel electrophoresis analysis of the genome of *Streptomyces ambofaciens* strains. FEMS Microbiol. Lett. **72,** 79-88.
50. Lee, T., Makino, K., Shinagawa, H., Amemura, M. and Nakata, A. (1989). Phosphate regulon in members of the family Enterobacteriaceae: comparison of the *phoB-phoR* operons of *Escherichia coli, Shigella dysenteriae* and *Klebsiella pneumoniae*. J. Bacteriol., **171,** 6593-6599.
51. Leskiw, B. K., Mevarech, M., Bessitt, L. S., Jensen, S. E., Hendersson, D. J., Hopwood, D. A., Bruton, C. J. and Chater, K. F. (1990). Discovery of an insertion sequence from *Streptomyces clavuligerus* and its relatedness to two transposable elements of *Streptomyces coelicolor*. J. Gen. Microbiol. **136,** 1251-1258.
52. Li, Y., Dosch, D. C., Strohl, W. R. and Floss, H. G. (1990). Nucleotide sequence and transcriptional analysis of the nosiheptide-resistance gene from *Streptomyces actuosus*. Gene **91,** 9-17.
53. Liras, P., Asturias, J. A. and Martín, J. F. (1990). Phosphate control sequences involved in transcriptional regulation of antibiotic biosynthesis. Trends Biotechnol. **8,** 184-189.
54. Lydiate, D. J., Henderson, D. J., Ashby, A. M. and Hopwood, D. A. (1987). Transposable elemets of *Streptomyces coelicolor* A3 (2). *In:* M. Alacevic, D. Hranueli and Z. Toman (eds). Genetics of industrial microorganisms, part B, pp. 49-56. Zagreb, Yugoslavia.
55. Makino, K., Shinagawa, H., Amemura, M., Kamamoto, T., Yamada, M. and Nakata, A. (1989). Signal transduction in the phosphate regulon of *Escherichia coli* involves phosphotransfer between PhoR and PhoB proteins. J. Mol. Biol. **210,** 551-559.
56. Martín, J. F. (1989). Molecular mechanisms for the control by phosphate of the biosynthesis of antibiotics and other secondary metabolites. *In:* S. Shapiro (ed.). Regulation of secondary metabolism in *Streptomyces*, pp. 213-237. CRC Press, Florida.
57. Martín, J. F. and Liras, P. (1989). Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. Ann. Rev. Microbiol. **43,** 173-206.
58. McCarter, L. L. and Silverman, M. (1987). Phosphate regulation of gene expression in *Vibrio parahaemolyticus*. J. Bacteriol. **169,** 3441-3449.
59. Meyer, J., Gantschi, J., Stalhammar-Carlemalm, M., Störl, J. and Klaus, S. (1983). DNA of the *Streptomyces* phage SH10: binding sites for *Streptomyces hygroscopicus* RNA polymerase and *in vitro* transcript map. Gene . **23,** 25-34.
60. Miyake, K., Kuzuyama, T., Horinouchi, S. and Beppu, T. (1990). The A-factor binding protein of *Streptomyces griseus* negatively controls streptomycin production and sporulation. J. Bacteriol. **172,** 3003-3008.
61. Murakami, T., Holt, T. G. and Thompson, C. J. (1989). Thiostrepton-induced gene expression in *Streptomyces lividans*. J. Bacteriol. **171,** 1459-1466.
62. Murray, I. A., Gil, J. A., Hopwood, D. A. and Shaw, W. V. (1989). Nucleotide sequence of the chloramphenicol acetyltransferase gene of *Streptomyces acrimycini*. Gene **85,** 283-291.

63. Narva, K. E. and Feitelson., J. S. (1990). Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A3 (2). J. Bacteriol. **172,** 326-333.

64. Nazarov, V., Zatkovic, P. and Godany, A. (1990). New shuttle promoter-probe vectors for *Escherichia coli* and *Streptomyces.* Biotech. Lett. **12,** 639-644.

65. Ochi, K. (1987). Metabolic initiation of differentiation and secondary metabolism by *Streptomyces griseus:* significance of the stringent response (ppGpp) and GTP content in relation to A factor. J. Bacteriol. **169,** 3608-3616.

66. O'Halloran, T. V., Frantz, B, Shin, M. K., Ralston, D. M. and Wright, J. G.(1989). The MerR heavy metal receptor mediates positive activator in a topologically novel transcription complex. Cell. **56,** 119-129.

67. Owolabi, J. B., and Rosen, B. P. (1990). Differential mRNA stability controls relative gene expression within the plasmid-encoded arsenical resistance operon. J. Bacteriol. **172,** 2367-2371.

68. Parro, V. and Mellado, R. P. (1990). Heterologous control of procaryotic gene expression. Proceedings of the 3rd Spanish Congress of Biotechnology, pp. 3-8. Murcia, Spain.

69. Pass, L. and Raczynska-Bojanowska, K. (1968). Inhibition mecanism of viomycin synthesis by inorganic phosphate. Actaz Biochim. Pol. **15,** 355-367.

70. Piepersberg, W., Distler, J., Ebert, A., Heinzel, P., Mansouri, K., Kayer, G. and Pissoootzi, K. (1968). Expression of genes for streptomycin biosynthesis. *In:* H. Okami, T. Beppu and H. Ogawara (eds.). Biology of actinomycetes '88, pp. 86-91. Japan Scientific Societies Press, Tokyo.

71. Pulido, A., Jiménez, A., Salas, M. and Mellado, R. P. (1986). *Bacillus subtilis* phage ø29 main promoters are efficiently recognized *in vitro* by the *Streptomyces lividans* RNA polymerase. Gene **172,** 377-382.

72. Rao, N. N. and Torriani, A. (1990). Molecular aspects of phosphate transport in *Escherichia coli.* Mol. Microbiol. **4,** 1083-1090.

73. Rebollo, A., Gil, J. A., Liras, P., Asturias, J. A. and Martín, J. F. (1989). Cloning and characterization of a phosphate regulated promoter involved in phosphate control of candicidin biosynthesis. Gene **79,** 47-58.

74. Schauer, A., Ranes, M., Santamaría, R., Guijarro, J., Lawlor, E., Mendes, C.,Chater, K. and Losick, R. (1988). Visualizing gene expression in time and space in the filamentous bacteria *Streptomyces coelicolor.* Science **240,** 768-772.

75. Seki, T., Yoshikawa, H., Takahashi, H. and Saito, H. (1987). Cloning and nucleotide sequence of *phoP*, the regulatory gene for alkaline phosphatase and phosphodiesterase in *Bacillus subtilis.* J. Bacteriol. **169,** 2913-2916.

76. Simuth, J., Sternbach, H., Zelinka, J., Chromutor, R. M. and Nedospasov, A. A. (1987). DNA dependent RNA polymerase from the chlorotetracycline producing strain of *Streptomyces aureofaciens.* FEBS Lett. **218,** 163-166.

77. Sinclair, R. and Bibb, M. J. (1989). Transcriptional analysis of the repressor gene of the temperate *Streptomyces* phage. øC31. Gene **85,** 275-282.

78. Solenberg, P. J. and Burgett, S. G. (1989). Method for selection of transposable DNA and characterization of a new insertion-sequence. *IS493,* from *Streptomyces lividans.* J. Bacteriol. **171,** 4807-4813.

79. Spizek, J., Smardora, J., Janecek, J. and Strastrea, J. (1991). DNA dependent RNA polymerase from *Streptomyces granaticolor.* Purification and properties. Abstract of the ISBA. P2-102. Madison, USA.

80. Stein, D. S., Kendall, K. J. and Cohen, S. N. (1989). Identification and analysis of transcriptional regulatory signals for the *kil* and *kor* loci of *Streptomyces* plasmid pIJ101. J. Bacteriol. **171,** 5768-5775.

81. Stein, D. S. and Cohen, S. N. (1989). A cloned regulatory gene of *Streptomyces lividans* can suppress the pigment deficiency phenotype of different developmental mutants. J. Bacteriol. **171,** 2258-2261.

82. Strauch, E., Takano, E., Baylis, H. A. and Bibb, M. J. (1991). The stringent response in *Streptomyces coelicolor* A3 (2). Mol. Microbiol. **5,** 289-298.

83. Taguchi, S., Nishiyama, K., Kumagai, I. and Miura, K. (1989). Analysis of transcriptional control regions in the *Streptomyces* subtilisin-inhibitor-encoding gene. Gene **34,** 279-286.

84. Tanaka, K., Shiina, T. and Takahashi, H. (1988). Multiple principle sigma factor homologs in Eubacteria: identification of the «*rpoD* box». Science **242,** 1040-1042.

85. Tanaka, K., Shiina, T. and Takahashi, H. (1991). Nucleotide sequences of genes *hrd*A *hrd*C and *hrd*D from *Streptomyces coelicolor* having similarity to *rpo*D genes. Mol. Gen. Genet. **229,** 334-340.

86. Vujaklija, D., Ueda, K., Hong, S. K., Beppu, T. and Horinouchi, S. (1991). Identification of an A-factor dependent promoter in the streptomycin biosynthetic gene cluster of *Streptomyces griseus.* Mol. Gen. Genet. **229,** 119-128.

87. Walker, M. S. and Walker, J. B. (1971). Streptomycin biosynthesis separation and substrate specificities of phosphatases acting on guanidine deoxyscyllo-inositol phosphate and streptomycin (streptidine) phosphate. J. Biol. Chem. **246,** 7034-7040.

88. Ward, J. M., Jansen, G. R., Kieser, T., Bibb, M. J. and Bibb, M. J. (1986). Construction and characterization of a series of

multi-copy promoter probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. Mol. Gen. Genet. **203,** 468-479.

89. Westpheling, J., Ranes, M. and Losick, R. (1985). RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature **513,** 22-27.

90. Westpheling, J. and Brawner, M. (1989). Two transcribing activities are involved in expression of the *Streptomyces* galactose operon. J. Bacteriol. **171,** 1355-1361.

MICROBIOLOGIA SEM **8** (1992), 63-75 **MINIREVIEW**

Microbial corrosion of stainless steels

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Summary

Stainless steels, developed because of their greater resistance to corrosion in different aggressive environments, have proved to be affected, however, by various processes and types of corrosion. Some of these types of corrosion, mainly pitting, is activated and developed in the presence of microorganisms, which acting in an isolated or symbiotic way, according to their adaptation to the environment, create a favorable situation for the corrosion of these steels.

The microorganisms that are involved, mainly bacteria of both the aerobic and anaerobic type, modify the environment where the stainless steel is found, creating crevices, differential aeration zones or a more aggresive environment with the presence of metabolites. In these circumstances, a local break of the passive and passivating layer is produced, which is proper to these types of steels and impedes the repassivation that is more favorable to corrosion.

In the study and research of these types of microbiologically influenced corrosion are found electrochemical techniques, since corrosion is fundamentally an electrochemical process, and microbiological techniques for the identification, culture, and evaluation of the microorganisms involved in the process, as well as in the laboratory or field study of microorganism-metal pairs. Microstructural characterization studies of stainless steels have also been considered important, since it is known that the microstructure of steels can substantially modify their behavior when faced with corrosion. As for surface analysis studies, it is known that corrosion is a process that is generated on and progresses from the surface.

The ways of dealing with microbiologically influenced corrosion must necessarily include biocides, which are not always usable or sucessful, the design of industrial equipments or components that do not favor the adherence of microorganisms, using microstructures in steels less sensitive to corrosion, or protecting the materials.

Key words: Microbiologically influenced corrosion, MIC, stainless steels, SS, case histories, mechanisms, testing, electrochemical methods, microbiological methods, control, prevention.

Resumen

Los aceros inoxidables, desarrollados precisamente por su carácter de mayor resistencia a la corrosión en diferentes ambientes agresivos, se han visto afectados, sin embargo, por diferentes

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procesos y tipos de corrosión. Alguno de estos tipos de corrosión, principalmente el de picaduras, se ve potenciado en su génesis y progreso por la presencia de microorganismos, que actuando aislada o simbióticamente, según su adaptación al medio, crean una situación favorable para el proceso de corrosión de estos aceros.

Los microorganismos involucrados, principalmente bacterias, tanto aerobias como anaerobias, modifican el entorno ambiental en que se encuentra el acero inoxidable, creando resquicios, zonas de aireación diferencial o un medio más agresivo con la presencia de metabolitos. En estas circunstancias se produce la rotura local de la capa pasiva y pasivante, propia de estos tipos de aceros, se impide la repasivación y se facilita la corrosión.

En el estudio e investigación de estos tipos de corrosión inducida por microorganismos se utilizan técnicas electroquímicas, por ser fundamentalmente la corrosión un proceso electroquímico, y técnicas microbiológicas para la identificación, cultivo y evaluación de los microorganismos involucrados en el proceso, así como para el estudio en laboratorio o en campo de los pares microorganismos-metal. Se han manifestado también como muy importantes los estudios de caracterización microestructural de los aceros inoxidables, ya que la microestructura de los aceros puede modificar sustancialmente su comportamiento frente a la corrosión, y los estudios de análisis superficial, ya que la corrosión es un proceso que se genera y progresa desde la superficie.

Las formas de actuación contra la corrosión inducida por microorganismos pasan por el empleo de biocidas, no siempre utilizables o con éxito, por el diseño de equipos o componentes industriales que no favorezcan la fijación de los microorganismos, por la utilización de microestructuras en los aceros menos sensibles a la corrosión o por la protección de los materiales.

Stainless steels

The fight against metal corrosion has been developed on very different fronts. One of these has been the development of alloys that are less sensitive to environmental effects than the traditional ones. It was in this field that the so-called stainless steels, which are iron-based alloys that contain variable amounts of different alloy elements (the basic element, chromium, is added in quantities of more than 12%). Since chromium can spontaneously form an oxidized surface layer which produces the passivation of the steel, the corrosion process becomes more difficult to step (9).

The variation in the content of the different elements of the alloy (Cr, Ni, Mo, etc.) is what makes up the different types of stainless steels, fundamentally characterized by their microstructure. It is in this way that martensitic, ferritic, austenitic, and austeno-ferritic stainless steels can be obtained. Another group, the precipitation hardening stainless steels, refers to the type of heat treatment that gives them their superior tensile properties (1).

The previously mentioned passive surface layer protects these steels against the most habitual type of corrosion that attacks common steels: generalized corrosion (89), although it is this very layer which makes stainless steels sensitive to various types of localized corrosion in environments that are sufficiently aggressive and under certain microstructural conditions (102). Corrosion in the form of pitting is one of the types of localized corrosion which appears most frequently. It is generally accepted that the pitting starts at the local break of the passive layer which creates a zone of active metal that can repassivate itself immediately, or can form an electrochemical cell in which the anode is a small area of active metal. Meanwhile, the large area of passive metal that surrounds it constitutes the cathode. The notable difference in potential characterized by this active-passive cell (0.5 V for austenitic stainless steels that belong to the 300 series) has a considerable flow of current and starts the rapid corrosion process of the small anode (97). The resistance to corrosion of the passive metal surrounding the anode, and the very activity of the co-

rrosion products within the pitting, increase its tendency to penetrate the metal rather than to extend itself on the surface. It is because of an autocatalytic process that the corrosion process within the pitting produces conditions that both stimulate and continue the pitting activity (2).

This corrosive process is very dangerous since it can happen very quickly (31), thus making the element useless in very little time, despite the small weight loss that is associated (for example, perforation of a pressure vessel). Other important corrosive processes in stainless steels are intergranular corrosion, stress corrosion cracking, or corrosion fatigue, phenomena which are very important because of their frequency and the harm they cause, but which will not be treated in great detail in this paper since their association to microbiologically influenced corrosion (MIC) is not as clear as corrosion by pitting. In general, these other localized corrosion processes are related to incorrect microstructural states and aggressive environments, and at times, to external actions such as constant or variable stress (fatigue) that allow for the attack and localized advances through preferential zones in the mass of the material. This can render the part useless rather quickly (2, 89).

Microbial corrosion of stainless steels

The participation of microorganisms in the corrosion of buried metals or those submerged in water has been known since the beginning of this century (30). The first cases that were studied in a systematic way referred to corrosion of buried ferrous metals. Afterwards, the first mechanisms used to explain the corrosion of buried metals caused by the action of sulfate reducing bacteria (SRB) were proposed (109). At present, numerous cases have been mentioned in relation to MIC in common steels, since a great deal of research has been devoted to this theme (101, 106, 107).

In the case of stainless steels, their generalized use came much later, thus the discovery that their corrosion could be influenced by microorganisms. In fact, very little was written about the microbial corrosion in stainless steels before 1976 (81).

In almost all cases, this type of corrosion appears in the form of pitting frequently localized under the biological deposit material in adjacent zones to welds (heat affected zones and weld metal). On occasions, stress corrosion cracking under biofilms and biodeposits was also detected (15, 44, 75, 96).

Defects caused by corrosion have been produced in various locations such as in waste water treatment plants (98, 115), power generating plants (26, 46, 47, 92, 93, 114), chemical plants (39, 90, 100), the paper industry (57, 98), etc.

Case histories referring to MIC make references to stainless steels coming in contact with different types of water: fresh (72, 80, 100), demineralized (96), and seawater (16, 33, 95). Although water low in chlorides (20 ppm) should not be corrosive, it has been proved that together with the action of microorganisms, corrosion can occur.

In terms of the types of stainless steels affected by this problem, references have been made of attacks in austenitic steels of the 300 series both in normal grades, such as the AISI 303, 304, 316 (6, 44, 74, 80, 95, 98) or in low carbon grades, such as AISI 304L (6, 43, 74) and 316L (6, 43, 44), as well as titanium stabilized grades, such as AISI 321 (5). In austenitic stainless steel welds, the filler metal, once deposited, has an austenoferritic structure. In some cases, the austenite is preferentially attacked, whereas in others, it is the ferrite that corrodes by preference to the austenite (6, 8, 42, 43, 46, 48, 93). Besides the austenitic types of stainless steels, others have shown they are susceptible to MIC such as, for example, martensitic stainless steel with a 13% Cr and 1% Ni content (72), or ferritic stainless steel, such as AISI 409 (115).

Although the microorganisms involved in the corrosion of stainless steels were not identified at first, it was observed that aerobic and anaerobic bacteria act symbiotically in the process. Iron-

oxidizing aerobic bacteria of the *Gallionella* (6, 43, 72), *Sphaerotillus* (72, 98) genera, and of manganese of the *Leptothrix* (57, 72) and *Siderocapsa* (70) genera have been identified in tubercles or pustules with corrosion products found on stainless steels. Moreover, slime forming bacteria (57) of many genera, including *Pseudomonas, Flavobacterium* and *Bacillus* are also part of the tubercles (73, 98). Below these are often found iron sulfide blackish deposits where sulfate reducing anaerobic bacteria (44, 70, 72, 80, 98, 100) of the *Desulfovibrio* and *Desulfotomaculum* genera, and sulfite reducing anaerobic bacteria (70, 98) of the *Clostridium* genus have been isolated.

Mechanisms involved in MIC of stainless steels

In the most common case, sulfate reducing bacteria, the generally accepted mechanism which produces microbial corrosion, starts with the formation of a biofilm on the stainless steel, specially in regions where there is a low speed flow, such as joints or other areas where there is stagnant water. The biofilm is usually formed by aerobic microorganisms, if the conditions of the current are favorable (13, 14). The growth of the biofilm progresses, catching particles that are pulled into the current. These include iron oxides and planktonic iron-oxidizing bacteria which develop in these deposits, consume the oxygen, and produce anaerobic conditions in the interphase with the steel. In this zone, the anaerobic bacteria present can grow and concentrate themselves, thus forming tubercles. It is below these tubercles that the pitting is developed (32, 99). In this aspect, a certain controversy exists in the sense that the contribution of the biomaterials and the corrosion could simply be the creation of crevice zones, which later produce corrosion because of the effects of differential aeration. The most widely accepted opinion states that the corrosion rate is accelerated in the presence of microorganisms (35, 77).

This biological action in the case of aerobic microorganisms does not only limit itself to the consumption of oxygen, which creates oxygen concentration cells. CO_2 is also produced, resulting in the appearance of carbonic acid which is corrosive to stainless steels (76).

It has also been proved that there is a relation between sulfate reducing anaerobic bacteria such as *Desulfovibrio* and *Desulfotomaculum* and the corrosion of stainless steels (43, 98). It has been shown that the metabolic products produced in sulfate reduction, particularly with sulfides, make the pitting attack of stainless steel more likely (37, 60, 110), possibly by breaking the stability of the passive layer (11), and increasing the aggresive action of chlorides (63). There are also references with respect to the role of these bacteria in cathodic depolarization reactions which facilitate the corrosive process (112).

Another possible mechanism is applied through the action of certain aerobic bacteria, such as *Gallionella* and *Sphaerotilus*, which seem to be able to fix the Fe^2/Fe^3 or Mn^2/Mn^4 redox potential on the metal surface. It seems that they polarize the surface of the metal to potentials where Fe^3 or Mn^4 are present.

This means mangenese or iron soluble ionsare converted into insoluble ferric or manganesic ions, which gives rise to the precipitation of de posits rich in Fe or Mn. The deposits are not specially corrosive in themselves, except for the possible effects of crevice corrosion. However, in the presence of chlorides, the $FeCl_3$ and the $MnCl_4$ that result are aggressive for stainless steels and thus cause pitting corrosion (74, 103). It has been proved that *Gallionella* is frequently found on stainless steels in the zones close to welds (98).

In aerobic systems where the reduction of oxygen is the cathodic reaction, corrosion can be accelerated because of the catalysis produced by the microorganisms in the reduction of oxygen (87). In this case, it has been shown that the acceleration of the cathodic semireaction raises the corrosion potential of stainless steel in water by some decimals, which can be enough to introduce the steel into the pitting potential range (12).

On occasions, various anaerobic bacteria are present, which is justified by a symbiosis between them (64). In this way the sulfate reducing bacteria produce, in their own reduction to sulfides, intermediate compounds (78) and among them sulfides, which are susceptible of being used by the microorganisms of the *Clostridium* genus (incapable of metabolizing sulfates).

Test methods

The study and evaluation of MIC is carried out using different techniques of the electrochemical, microbiological, surface analysis and metallurgical types. The objective of the application of these techniques can be diverse, from the detection of the problem to the selection of resistant materials or MIC control methods, or to system monitoring. In view of its importance and specificity, the electrochemical and microbiological techniques will be basically dealt with in this section.

Electrochemical methods

The electrochemical test methods are particularly adequate, given that the mechanisms through which MIC is produced are naturally electrochemical (23, 24).

These electrochemical methods can be divided into three groups: a) Methods without external perturbation to the system, b) Direct current methods, and c) Alternating current methods.

a) Methods without perturbation to the system

These methods are undoubtedly attractive since they allow one to get information on the state of the system without ever having to interact with it, and in that way, produce alterations. In this group of methods can be found the corrosion potential measurement (Ecorr), the split-cell technique, and the electrochemical noise technique. Among these, the most widely used is the corrosion potential measurement, which is used both in laboratory (61, 87), and in field (17, 108). Studies in this latter case are used as a monitoring method for the state in which the material is found. This is practically the only electrochemical monitoring technique that is used on stainless steels.

The Ecorr measurement is the simplest electrochemical technique. It is carried out by determining the potential difference between the metal surface of the object being studied, and an appropriate reference electrode. Of these measurements, important information is obtained on the state of activity or passivity and the influence of the biofilms on the corrosion of stainless steels (50, 51, 52). The materials on which a biofilm is developed have higher or more noble Ecorr values than those obtained in the absence of biofilm (41, 61). These values increase with time, making the stainless steels more susceptible to pitting attack. As previously mentioned, the Ecorr measurement has been suggested as a monitoring technique. When its value is too high, pitting can occur, therefore action should be taken to reduce further pitting (12).

The other two methods are promising, but have been used very little in the research of stainless steels, basically because of the difficulty of interpreting the results obtained, which fluctuate depending on the characteristics of the localized corrosion mechanisms produced in those steels.

The split-cell technique uses a special type of cell that has two compartments with identical electrodes. One of these is inoculated with the microorganisms under study coming in contact with the semipermeable membrane of the sterile semi-cell (49). The current flow between both cells is studied in short circuit, but in localized corrosion (pitting) types, the analysis of the fluctuations is very complicated (18, 19).

In the electrochemical noise technique, the changes are measured in potential or current in function of time, and the results must be subjected to a statistical analysis of the frequency and amplitude of the fluctuations (56). Complications also arise in treating these results, but it seems that this technique is able to distinguish between the different types of corrosion (generalized, localized in pitting, stress corrosion cracking, etc.) (18, 19, 40, 62).

b) Direct current methods

In this section a series of techniques will be grouped together that are consistent in carrying out the polarization of the material under study through the use of potential scans both in increasing and decreasing scales so that Potential-Intensity diagrams can be obtained. Among these techniques polarization curves, Tafel diagrams, polarization resistance tests (Rp), as well as pitting potential determination (Ep) and repassivation (Er) tests can be obtained.

Among the techniques mentioned, the most widely used on stainless steels is the Ep and Er potential determination test (3, 7, 82, 97). The test basically consists in carrying out a scan of increasing potentials, using a sample of the object under study as the electrode, and an adequate solution, that frequently contains chlorides as the electrolyte, or even metabolites from the cultures of the microorganisms being studied. The potential is increased until the intensity that circulates through the sample rises sharply. This happens when the passive layer is locally broken, that is to say, when some pitting occurs. This potential is known as the pitting potential. On other occasions, the increasing potential scan is followed until a predetermined level of circulating intensity is reached, at which moment, the potential scan is inverted, and decreasing potentials are obtained. The potential whose intensity is of the same order as the intensity which circulated through the sample before the pitting appeared (passivation landing intensity), is known as the repassivation potential because it is assumed that the pitting which was active to high potentials becomes passive. This test is related to the type of corrosion to which the stainless steel is effectively subjected to in a rather direct way. This also allows one to get quantitative data which makes it possible to make a direct comparison among the materials, or among the level of aggressiveness of various environments. These types of tests allow, for example, to determine the increasing aggressiveness in a culture medium, when it contains metabolites produced by sulfate reducing bacteria (63), which increases the chances of a pitting attack in austenitic (60) and martensitic (37, 65) stainless steels. Polarization curves give important information, besides the Ep and Er already mentioned, when they are carried out in an anodic way and reach rather high potentials. In this way, the supply information about the influence of biofilms in the cathodic reaction through polarization curves in a cathodic direction. It has been observed that the cathodic current densities increase depending on how long they are exposed to water (61, 88, 104). Frequently, the effect of microorganisms on stainless steels not only induces localized corrosion at more active potentials, but it makes the repassivation of the pitting more difficult than in a sterile medium. This can be seen in the potentiodynamic curves by observing the density of the current in the passive state, which is higher in the presence of microorganisms (66, 82). This suggests that the layer is less protective than one which is formed in a sterile medium.

Tafel diagrams and polarization resistance tests consist on potential scans around the Ecorr, by extrapolation or various mathematical calculations, the intensity of corrosion that circulates in a specific case can be deduced (94). By using the Faraday Law, and by supposing that the corrosion is generally uniform, it is possible to establish the corrosion rate. The Tafel and polarization resistance tests have been used very infrequently with stainless steels (20, 68, 105), possibly because they are of a localized corrosion type, and given the characteristics of the surface layers formed, the data obtained is meaningless, or the measurements do not reflect the real corrosion rate (18, 19).

c) Alternating current methods

The electrochemical impedance spectroscopy will be presented in this section. This technique is particularly effective in the case of existing non-conductor or semi-conductor surface layers, such as metal oxide layers, or biofilms (18, 19). The technique basically consists in introducing a low alternating current signal in the corroding system. The frequency is varied so that its behavior can be studied as if one was dealing with an electronic circuit with a determined impedance (21, 91). The analysis of the results is complicated, but from this, an equivalent circuit to the system in corrosion can be obtained that is not only composed of resistances (to the medium, polarization...), but of capacitive components that can represent the effect of the surface layers (55). The smallness of the signal, which supposes a minimum disturbance, and the amount of information obtained with respect to the corrosion mechanisms that are acting upon the system, are making this method very common (22, 29). It is important to note that there are some inconveniences involved when it is applied to MIC, such as the fact that the biological layers tend to be dynamic entities (28), that are not always continuous or of the same thickness, making it difficult to interpret the results.

Laboratory microbiological methods

In order to carry out laboratory studies of microbiologically influenced corrosion of stainless steels, immersion tests have been developed in: batch, continous and semicontinuous cultures, and recirculation systems.

In the batch cultures, the culture medium is not renovated during the test, whereas in the continuous and semicontinuous ones, the culture medium is periodically renewed. In these latter two types, the dilution rate will depend on the growth rate that each microorganism has in specific culture conditions (medium, pH, temperature, reactor volume, etc.). The recirculation systems consist in having the medium circulate through a test loop. It has mainly been used for natural media once the current conditions of the system are known.

In all laboratory tests, stainless steels are exposed to a culture medium (natural, artificial or mixed) where microorganisms are developed. The natural sterile media (by filtration or autoclave) have been supplemented with nutritional substances in order to facilitate the growth of isolated microorganisms from the environment. Among these artificial culture media the Postgate C medium or similar ones are highlighted (Beckwith, BTZ-4, etc.). This medium, on occasions, has been supplemented with chlorides in order to simulate a marine environment.

The fact that resistance to corrosion of stainless steels has improved notably influences the duration of exposure time needed to find corrosion. These corrosion acceleration tests in laboratory settings are not as quick as one would wish: 39 days (28), 60 days (53, 63, 112), 120 days (83, 86), 146 days (85), and 260 days (70).

Microbiological field methods

Field studies constitute the most adequate technique to determine how stainless steels are attacked by microorganism and they can establish a precise scale of their resistance to corrosion.

In all the filed tests that have been carried out, stainless steels are exposed to the environment that generally coincides with the one which is in contact with a service material that is equal to or has similar characteristics to the one being tested. In these conditions, the periods of exposure can, in general, go from one to several years.

The materials submerged in natural environments are rapidly colonized by microorganisms that form biolfilms in their first stages, and biofouling later on. The majority of the work carried out on stainless steels, whether in or out of laboratory, has supplied researchers with more information on the phenomena of biofilm and biofouling than on the corrosion process itself.

MIC Control

Corrosion control methods can act in various ways. The first method consists in eliminating the bacteria. This can theoretically be carried out by adding the right amount of biocides (45, 71). Biocides have proved to be effective against planktonic microbiological populations (10, 54, 84), however they are not able to control sessile populations (25, 69), which are the ones which contribute to MIC. The use of biodispersants has increased in the last few years so microorganisms can be dispersed in the mass, making them more susceptible to the action of biocides (4, 45, 79). The elimination of biofilms and the softening of inorganic deposits are translated in the elimination of anaerobic zones in the system, and as a result, a reduction in the action of MIC. It is possible, in theory, that the action of some biocides favors the growth of other microorganisms, promoting the growth of biolayers which later cause MIC (77). To these questions must also be added the fact that their massive treatment can produce ecological problems (59).

A second control method consists in making the adherence of the microorganisms more difficult, which means that care must be taken in the geometric design of the equipments, by eliminating joints, crevices, and areas of stagnant water in general, which is not always possible to do. It is recommendable for the surfaces to have a smooth finish (58).

The third method acts on the quality of the material. It has been proved that certain microstructural states make stainless steels more susceptible to MIC, which is why it is convenient to control the heat treatments of the materials (60, 65), or to avoid welding (6) on materials that can be strongly sensitized (38). In some cases, a method to reduce MIC is cladding or coating with alloys rich in chromium or nickel. The coating of the materials should be carried out in such a way that there is no local break, since this would accelerate the corrosion process. Moreover, the coating should not be able to give nutrients to the microorganisms. In extreme cases, resorting to a change of material is essential. In these cases, special stainless steels are used with a high (6%) molybdenum content (36).

Another method used on occasions is cathodic protection, although there is no unanimity in terms of its convenience. The potential used to reach an effective protection must be stronger than when carbon steels are used [–1,100 mV (SCE) compared to –750 mV (SCE)]. This can produce fragilization because of the hydrogen found in ferritic stainless steels (67). Moreover, an increase in the SRB population was registered close to buried materials subject to this type of protection, which can produce an increase corrosion in case of an imperfect cathodic protection or of a temporal interruption (34).

Development and tendencies in the future

Research is necessary in two main areas: technology and science. The first includes the requirements and appropriate means to identify, mitigate and/or prevent MIC. The second deals with the knowledge of the specific mechanisms involved and the development of new procedures and means to combat MIC.

Technological area

It is necessary to develop means to detect MIC in field studies than are simple, reliable, and as inexpensive as possible. On site test kits must be developed. More data must be gathered when case histories are compiled (service time, work parameters, biocide concentration, environment characterization, etc.) so that expert monitoring and control systems can be fed. In this respect,

the requirements set by the American Nuclear Regulatory Commission (NRC) should be mentioned because they require licensees of nuclear power plants to have a maintenance program for the piping system and its components in order to prevent MIC. Another program which should be established is one which does a routine inspection and maintenance for the detection, treatment, and prevention of MIC (113).

It is urgent and necessary to develop standards in order to establish a classification for the resistance of stainless steels to MIC. Until recently, both the information supplied and the characterization, with a few exceptions, are very insufficient and deficient. Because of that, evaluation and comparation of the behavior obtained in different tests by different authors are very difficult, and in some cases, even impossible. It is important to take into account that even the slightest microstructural variation of two similar kinds of stainless steels makes a big difference in the way each one will behave to different types of corrosion.

Scientific area

It is necessary to have a more thorough knowledge of the mechanisms that make microorganisms induce corrosion on stainless steels. It is for this reason, that an in-depth study of the microorganisms involved requires biochemical and genetic studies of them and of their associations. In the past, more emphasis was put on finding solutions to the problems rather than on studying them. This was true because adequate and rapid means in service were lacking, and the interdisciplinary characteristic of this area required researchers to add their knowledge of microorganisms to that of the materials in corrosion (27).

It is necessary to be able to have more information on the capacity of microorganisms for action in their environment since they excrete polysaccharides in function of the material on which they are found (rough, heat treatment, etc.). With this knowledge, methods to prevent them from adhering to the material can be developed. It would also be convenient to determine the capacity of the microorganisms to fix electrochemical potentials on the surface of stainless steels.

It is necessary to make a model of the microorganisms-environment-material, possibly in an electrochemical context, so as to get further knowledge of the mechanisms in interaction and to be able to evaluate, in certain cases, the resistance of these materials to MIC aggressiveness.

It is necessary to develop antifouling agents and mud dispersants, and to carry out more in-depth research on the use of biocides in connection with those biodispersants, so that better results at lower costs and with less impact on the environment can be found. In this sense, biological solutions to the problems caused by MIC, for example using bacteriophages, enzymatic treatments, etc., can be researched.

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References

1. ASM (1987a). Properties and Selection: Stainless steels, tool materials and special-purpose metals. Metals Handbook, ninth edition. Vol. **3.** Ed. American Society for Metals International. Metals Park. Ohio.
2. ASM (1987b). Corrosion. Metals Handbook, ninth edition, Vol. **13.** Ed. American Society for Metals International. Metals Park. Ohio.

3. ASTM (1978). Conducting ciclyc potentiodynamic polarization measurements for localized corrosion. Book of Standards, Vol. **03.02,** G-61. ASTM, Philadelphia. Pennsylvania.
4. Beech, I. B., Moreno, D. A. and Ranninger, C. (1992). A simple scanning electron microscopy method for preliminary assessment of the biocide treatment on removal of SRB-biofilms. *In:* C. A. C. Sequeira and A. K. Tiller (eds.). Microbial corrosion The Institute of Materials, U. K., pp. 139-145.
5. Bibb, M. (1986). Bacterial corrosion in the South African power industry. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 96-101.
6. Borenstein, S. W. (1988). Microbiologically influenced corrosion failures of austenitic stainless steel welds. Mater. Performance **27** (8), 62-66.
7. Borenstein, S. W. and White, D. C. (1989). Influence of welding variables on microbiologically influenced corrosion of austenitic stainless steel weldments. Corrosion/89, paper 183. NACE, Houston, Texas.
8. Boresntein, S. W. (1991). Microbiologically influenced corrosion of austenitic stainless steel weldments. Mater. Performance **30** (1), 52-54.
9. Caprio, G. di. (1987). Los aceros inoxidables. Ed. S. A. Ebrisa. Barcelona. (Gli Acciai Inossidabili. Ed. Ulrico Hoepli, 1981.)
10. Costerton, J. W. and Lashen, E. S. (1984). Influence of biofilm on efficacy of biocides on corrosion-causing bacteria. Mater. Performance **23** (2), 13-17.
11. Cristofaro, N. B. de, Acosta, C. A., Salvarezza, R. C. and Videla, H. A. (1986). The effect of sulfides on the electrochemical behavior of AISI 304 stainless steel in neutral buffered solutions. Corrosion **42** (4), 240-242.
12. Cubicciotti, D. and Licina, G. J. (1989). Electrochemical aspects of MIC. Corrosion/89, paper 517. NACE, Houston, Texas.
13. Characklis, W. G. (1981). Fouling biofilm development: a process analysis. BIBIA **XXIII,** 1923-1960.
14. Characklis, W. G. (1984). Biofilm development: a process analysis. In: K. C. Marshall (ed.). Microbial Adhesion and Aggregation. Springer-Verlag, pp. 135-157.
15. Das, C. R. and Mishra, K. G. (1986). Biological corrosion of welded steel due to marine algae. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 114-117.
16. Dexter, S. C., Lucas, K. E. and Gao, G. Y. (1986). The role of marine bacteria in crevice corrosion initiation. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 144-153.
17. Dexter, S. C. and Gao, G. Y. (1988). Effect of seawater biofilms on corrosion potential and oxygen reduction of stainless steel. Corrosion **44,** 717-723. Discussion on this paper by F. Mansfeld and B. Little in Corrosion **45** (10), 786-789.
18. Dexter, S. C., Siebert, O. W., Duquette, D. J. and Videla, H. A. (1989). Use and limitations of electrochemical techniques for investigating microbiological corrosion. Corrosion/89, paper 616. NACE, Houston, Texas.
19. Dexter, S. C., Duquette, D. J., Siebert, O. W. and Videla, H. A. (1991). Use and limitations of electrochemical techniques for investigating microbiological corrosion. Corrosion **47** (4), 308-318.
20. Dowling, N. J. E., Guezennec, J. and White, D. C. (1986). Facilitation of corrosion of stainless steel exposed to aerobic seawater by microbial biofilms containing both facultative and absolute anaerobes. *In:* E. C. Hill, J. L. Shennan and R. J. Wilkinson (eds.). Microbial Problems in the Offshore Oil Industry. Institute of Petroleum, London, pp. 27-38.
21. Dowling, N. J. E., Franklin, M., White, D. C., Lee, C. H. and Lundin, C. (1989). The effect of microbiologically influenced corrosion on stainless steel weldments in artificial seawater. Corrosion/89, paper 187. NACE, Houston, Texas.
22. Dowling, N. J. E., White, D. C., Buchanan, R. A., Danko, J. C., Vass, A. and Brooks, S. (1990). Microbiologically influenced corrosion of 6% molybdenum stainless steels and AISI 316: comparison with ferric chloride testing. Corrosion/90, paper 532. NACE, Houston, Texas.
23. Duquette, D. J. (1985). Electrochemical techniques for evaluation of microbiologically influenced corrosion processes. Proceedings of the Argentine-USA Workshop on Biodeterioration (CONICET-NSF). La Plata (Argentina), 21-27 April, 1985. Ed. Aquatec Química, S. A., Sao Paulo, Brazil, pp. 15-32.
24. Duquette, D. J. and Ricker, R. E. (1986). Electrochemical aspects of microbiologically induced corrosion. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 121-130.
25. Eagar, R. G., Leder, J., Stanley, J. P. and Theis, A. B. (1988). The use of glutaraldehyde for microbiological control in waterflood systems. Mater. Performance **27** (8), 40-45.
26. EPRI CS-5495 (1987). Microbial corrosion in fossil-fired power plants. A study of microbiologically influenced corrosion and a practical guide for its treatment and prevention. Palo Alto, California.
27. Fellers, B. D. (1989). An interdisciplinary perspective on microbiological influenced corrosion. Corrosion/89, paper 510. NACE, Houston, Texas.
28. Feron, D. (1991). Chemical and electrochemical aspects of the corrosion of stainless steels in the presence of sulphate reducing bacteria. *In:* N. J. Dowling, M. W. Mittleman and J. C. Danko (eds.). Microbially Influenced Corrosion and Biodeterioration. The University of Tennessee, Knoxville, **5***I* pp. 73-83.
29. Ferrante, V. and Feron, D. (1991). Microbially influenced corrosion of steels containing molybdenum and chromium: a biological and electrochemical study. *In:* N. J. Dowling, M. W. Mittleman and J. C. Danko (eds.). Microbially Influenced Corrosion and Biodeterioration. The University of Tennessee, Knoxville, **3***I* pp. 55-63.
30. Gaines, R. H. (1910). Bacterial activity as a corrosive influence in the soil. Industrial and Engineering Chemistry **2,** 128-130.

31. Galvele, J. R. (1982). Pitting corrosion. *In:* J. C. Scully (ed.). The Principles of Corrosion. Pergamon, New York.
32. Griffin, R. B., Cornwell, L. R., Seits, W. and Estes, E. (1988). Localized corrosion under biofouling. Corrosion/88, paper 400. NACE, Houston, Texas.
33. Guezennec, J. (1988). Biocorrosion of stainless steels in natural sea water comparison tests in field and in lab. *In:* C. A. C. Sequeira and A. K. Tiller (eds.). Microbial Corrosion **1.** Elsevier Applied Science, London, pp. 183-187.
34. Guezennec, J., Dowling, N. J. E., Conte, M., Antoine, E. and Fiksdal, L. (1991). Cathodic protection in marine sediments and the aerated seawater column. *In:* N. J. Dowling, N. W. Mittleman and J. C. Danko (eds.). Microbially Influenced Corrosion and Biodeterioration. The University of Tennessee, Knoxville, **6/** pp. 43-50.
35. Hamilton, W. A. (1985). Sulphate-reducing bacteria and anaerobic corrosion. Ann. Rev. Microbial. **39,** 195-197.
36. Hanford, R. (1990). Nuclear plant remedies for MIC with «super» stainless steel. Corrosion/90, paper 535. NACE, Houston, Texas.
37. Ibars, J. R., Moreno, D. A., Lloret, M. and Ranninger, C. (1988). Microstructural effects on the electrochemical behaviour of AISI 410 in microbiological media. *In:* C. A. C. Sequeira and A. K. Tiller (eds.). Microbial Corrosion **1.** Elsevier Applied Science, London, pp. 287-299.
38. Ibars, J. R., Moreno, D. A. and Ranninger, C. (1992). MIC of stainless steels: a technical review on the influence of microstructure. International Biodeterioration and Biodegradation **29** (3 & 4), 343-355.
39. Iverson, W. P. and Olson, G. J. (1984). Problems related to sulfate-reducing bacteria in the petroleum industry. *In:* R. M. Atlas (ed.). Petroleum Microbiology. Macmillah Publishing Company. New York, pp. 619-641.
40. Iverson, W. P., Olson, G. J. and Heverly, L. F. (1986). The role of phosphorus and hydrogen sulfide in the anaerobic corrosion of iron and the possible detection of this corrosion by an electrochemical noise technique. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 154-161.
41. Johnsen, R. and Bardal, E. (1986). The effect of a microbiological slime layer on stainless steel in natural sea water. Corrosion/86, paper 227. NACE, Houston, Texas.
42. Kearns, J. R. and Borenstein, S. W. (1991). Microbially influenced corrosion testing of welded stainless alloys for nuclear power plant service water systems. Corrosion/91, paper 279. NACE, Houston, Texas.
43. Kobrin, G. (1976). Corrosion by microbiological organisms in natural waters. Mater. Performance **15** (7), 38-43.
44. Kobrin, G. (1986). Reflections on microbiologically induced corrosion of stainless steels. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 33-46.
45. Lamot, J. E. (1988). Role of biocides in controlling microbial corrosion. *In:* C. A. C. Sequeira and A. K. Tiller (eds.). Microbial Corrosion **1.** Elsevier Applied Science, London, pp. 224-234.
46. Licina, G. J. (1988). A review of microbial induced corrosion in nuclear power plant systems. Corrosion/88, paper 268. NACE, Houston, Texas.
47. Licina, G. J. (1989). An overview of microbiological influenced corrosion in nuclear power plant systems. Mater. Performance **28** (19), 55-60.
48. Licina, G. J. (1990). Microbiologically influenced corrosion in nuclear power plants: a review of susceptibility and mitigation methods. Corrosion/90, paper 527. NACE, Houston, Texas.
49. Little, B. J., Wagner, P. A., Gerchakov, S. M., Walch, M. and Mitchell, R. (1986). The involvement of a thermophilic bacterium in corrosion processes. Corrosion **42** (9), 533-536.
50. Little, B. J., Wagner, P. A., Characklis, W. G. and Lee, W. (1990). Microbial Corrosion. *In:* W. G. Characklis and K. C. Marshall (eds.). Biofilms. John Wiley & Sons, Inc., New York, pp. 635-670.
51. Little, B. J., Ray, R., Wagner, P., Lewandowski, Z., Lee, W. C., Characklis, W. G. and Mansfeld, F. (1990). Electrochemical behavior of stainless steels in natural seawater. Corrosion/90, paper 150. NACE, Houston, Texas.
52. Little, B., Ray, R., Wagner, P., Lewandowski, Z., Lee, W. C., Characklis, W. G. and Mansfeld, F. (1991). Impact of biofouling on the electrochemical behaviour of 304 stainless steel in natural seawater. Biofouling **3** (1), 45-59.
53. Lloret, M., Moreno, D. A., Aparicio, M. L. and Ranninger, C. (1988). Stainless steel corrosion analysis in semicontinous culture of sulphate-reducing bacteria. *In:* C. A. C. Sequeira and A. K. Tiller (eds.). Microbial Corrosion **1.** Elsevier Applied Science, London, pp. 335-360.
54. Lunden, K. C. and Stastny, T. M. (1985). Sulfate reducing bacteria in oil and gas production. Corrosion/85, paper 296. NACE, Houston, Texas.
55. Mansfeld, F., Tsai, R., Shih, H., Little, B., Ray, R. and Wagner, P. (1990). Results of exposure of stainless steels and titanium to natural seawater. Corrosion/90, paper 109. NACE, Houston, Texas.
56. Mansfeld, F. and Little, B. (1991). A technical review of electrochemical techniques applied to microbiologically influenced corrosion. Corrosion Science **32** (3), 247-272.
57. Marmo, S. A., Nurmiaho-Lassila, E. L., Varjonen, O. A. and Salkinoja-Salonen, M. S. (1991). Biofouling and microbially induced corrosion on paper machines. *In:* N. J. Dowling, M. W. Mittleman and J. C. Danko (eds.). Microbially Influenced Corrosion and Biodeterioration. The University of Tennessee, Knoxville, **4/** pp. 33-38.
58. McCoy, W. F. (1987). Strategies for the treatment of biological fouling. *In:* M. W. Mittelman and G. G. Geesey (eds.). Biological fouling of industrial water systems: a problem solving approach. Water Micro Associates, San Diego, pp. 247-268.
59. Meitz, A. (1991). Environmental concerns and biocides. Corrosion/91, paper 306. NACE, Houston, Texas.

60. Mele, M. F. L. de, Moreno, D. A., Ibars, J. R. and Videla, H. A. (1991). Effect of inorganic and biogenic sulfide on localized corrosion of heat treated type 304 stainless steel. Corrosion **47** (1), 24-30.
61. Mollica, A., Trevis, A., Traverso, E., Ventura, G., de Carolis, G. and Dellepiane, R. (1989). Cathodic performance of stainless steels in natural seawater as a function of microorganism settlement and temperature. Corrosion **45** (1), 48-56.
62. Moosavi, A. N., Dawson, J. L. and King, R. A. (1986). The effect of sulphate-reducing bacteria on the corrosion of reinforced concrete. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Textas, pp. 291-308.
63. Moreno, D. A. (1990). Análisis de la susceptibilidad a la corrosión microbiana de los aceros inoxidables en diferentes estados microestructurales. Ph Tesis. Universidad de Alcalá de Henares, Madrid, Spain.
64. Moreno, D. A. and Laborda, F. (1988). Sulphate- and sulphite-reducing bacteria symbiosis in microbial corrossion. *In:* C. A. Sequeira and A. K. Tiller (eds.). Microbial Corrosion **1.** Elsevier Applied Science, London, pp. 84-90.
65. Moreno, D. A., Mele, M. F. L. de, Ibars, J. R. and Videla, H. A. (1991). Influence of microstructure on the electrochemical behavior of type 410 stainless steel in cloride media with inorganic and biogenic sulfide. Corrosion **47** (1), 2-9.
66. Moreno, D. A., Ibars, J. R., Ranninger, C. and Videla, H. A. (1992). Technical note: Use of potentiodynamic polarization to assess pitting of stainless steels by sulfate-reducing bacteria. Corrosion, **48** (3), 226-229.
67. Nekoksa, G. and Gutherman, B. (1991). Determination of cathodic protection criteria to control microbially influenced corrosion in power plants. *In:* N. J. Dowling, M. W. Mittleman and J. C. Danko (eds.). Microbially Influenced Corrosion and Biodeterioration. The University of Tennessee, Knoxville, **6/** pp. 1-8.
68. Nivens, D. E., Nichols, P. D., Henson, J. M., Geesey, G. G. and White, D. C. (1986). Reversible acceleration of the corrosion of AISI 304 stainless steel exposed to seawater induced by growth and secretions of the marine bacterium *Vibrio natriegens.* Corrosion **42** (4), 204-210.
69. Obuekwe, C. O., Westlake, D. W. S., Cook, F. D. and Costerton, J. W. (1981). Surface changes in mild steel coupons from the action of corrosion-causing bacteria. Appl. Environ. Microbiol. **41** (3), 766-774.
70. OCIDE (1989). Corrosión bacteriana en aceros inoxidables. Final Report, Proj. No. 120.11. Madrid, Spain.
71. Paulus, W. (1988). Developments in microbicides for the protection of materials. *In:* D. R. Houghton, R. N. Smith and H. O. W. Eggins (eds.). Biodeterioration **7.** Elsevier Applied Science, London, pp. 1-19.
72. Pintado, J. L. and Montero, F. (1986). Corrosión microbiológica en centrales hidroeléctricas. Rev. Iberoamer. Corros. Prot. **17** (5), 361-366.
73. Poole, A. B. (1989). Trending of the extent of damage to stainless steel pipe welds affected by microbiologically induced corrosion (MIC) related to strength reserve factor. Corrosion/89, paper 515. NACE, Houston, Texas.
74. Pope, D. H. (1986). A study of microbiologically influenced corrosion in nuclear power plants and a practical guide for countermeasures. Electric Power Research Institute. Final Report EPRI NP-4582s. Research Project 1166-6, May, 1986. Palo Alto, California.
75. Pope, D. H., Soracco, R. J. and Wilde, E. W. (1982). Studies on biologically induced corrosion in heat exchanger systems at the Savannah River Plant, Aiken, SC. Mater. Performance **21** (7), 43-50.
76. Pope, D. H., Duquette, D. J., Johannes, A. H. and Wayner, P. C. (1984). Microbiologically influenced corrosion of industrial alloys. Mater. Performance **14** (4), 14-18.
77. Pope, D. H., Duquette, D., Wayner, P. C. and Johannes, A. H. (1989). The case for microbiologically influenced corrosion of stainless steels. In: Microbiologically Influenced Corrosion: A State-of-the-Art Review, second edition, Publication 13, Materials Technology Institute of the Chemical Process Industries, Inc. Columbus, Ohio.
78. Postgate, J. R. (1984). The sulphate-reducing bacteria. Second edition. Ed. Cambridge University Press.
79. Poulton, W. I. J. and Lutey, R. W. (1991). Mitigation of microbiologically influenced corrosion in open recirculating cooling water system. *In:* N. J. Dowling, M. W. Mittleman and J. Danko (eds.). Microbially Influenced Corrosion and Biodeterioration. The University of Tennessee, Knoxville, **6/** pp. 29-36.
80. Puckorius, P. R. (1983). Massive utility condenser failure caused by sulfide producing bacteria. Mater. Performance **22** (12), 19-22.
81. Ranninger, C. (1989). Corrosión microbiológica de aceros inoxidables. Revisión y análisis de casos prácticos. Proceedings of the XIIth Congreso Nacional de Microbiología, 24-27 September, 1989. Pamplona, Spain. Vol. **1,** pp. 315-317.
82. Ringas, C. and Robinson, F. P. A. (1988). Corrosion of stainless steel by sulfate-reducing bacteria. Electrochemical techniques. Corrosion **44** (6), 386-396.
83. Ringas, C. and Robinson, F. P. A. (1988). Corrosion of stainless steel by sulfate-reducing bacteria. Total immersion test results. Corrosion **44** (9), 671-678.
84. Ruseska, I., Robbins, J. and Costerton, J. R. (1982). Biocide testing against corrosion-causing oil-field bacteria helps control plugging. Oil and Gas J. **80,** 253-264.
85. Scott, P. J. B. and Davies, M. (1989). Microbiologically influenced corrosion of high molybdenum austenitic stainless steel. Corrosion/89, paper 186. NACE, Houston, Texas.
86. Scott, P. J. B., Goldie, J. and Davies, M. (1991). Ranking alloys for susceptibility to MIC: A preliminary report on high-Mo alloys. Mater. Performance **30** (1), 55-57.
87. Scotto, V., di Cintio, R. and Marcenaro, G. (1985). The influence of marine aerobic microbial film on stainless steel corrosion behaviour. Corrosion Science **25** (3), 185-194.

88. Scotto, V., Alabiso, G. and Marcenaro, G. (1985). The behaviour of stainless steels in natural seawater. In: Bioelectrochemistry and Bioenergetics **16,** pp. 347-355. A section of J. Electroanal. Chem., and constituting Vol. 212.
89. Sedriks, A. J. (1979). Corrosion of stainless steels. Ed. John Wiley and Sons. New York.
90. Silva, A. J. N., Tanis, J. N., Silva, J. O. and Silva, R. A. (1986). Alcohol industry biofilms and their effect on corrosion of 304 stainless steel. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 76-82.
91. Silverman, D. C. (1986). Primer on the AC Impedance Technique. In: R. Baboian (ed.). Electrochemical Techniques for Corrosion Engineering. NACE, Houston, Texas, p. 73.
92. Sinha, U. P., Wolfram, J. H. and Rogers, D. (1991). Microbially influenced corrosion of stainless steels in nuclear power plants. *In:* N. J. Dowling, M. W. Mittleman and J. C. Danko (eds.). Microbially Influenced Corrosion and Biodeterioration. The University of Tennessee, Knoxville, **4/** pp. 51-60.
93. Soracco, R. J., Pope, D. H., Eggers, J. M. and Effinger, T. N. (1988). Microbiologically influenced corrosion investigations in electric power generating stations. Corrosion/88, paper 83. NACE, Houston, Texas.
94. Stern, M. and Geary, A. L. (1957). Electrochemical polarization. I. A theoretical analysis of the shape of polarization curves. J. Electrochem. Soc. **104** (1), 56-63.
95. Stoecker, J. G. (1984). Guide for the investigation of microbiologically induced corrosion. Mater. Performance **23** (8), 48-55.
96. Stoecker, J. G. and Pope, D. H. (1986). Study of biological corrosion in high temperature demineralized water. Corrosion/86, paper 126. NACE, Houston, Texas.
97. Szklarska-Smialowska, Z. (1986). Pitting corrosion of metals. Ed. NACE, Houston, Texas.
98. Tatnall, R. E. (1981a). Case histories: bacteria induced corrosion. Mater. Performance **20** (8), 41-48.
99. Tatnall, R. E. (1981b). Fundamentals of bacteria induced corrosion. Mater. Performance **20** (9), 32-38.
100. Tiller, A. K. (1983). Is stainless steel susceptible to microbial corrosion? In: Microbial Corrosion. Ed. The Metals Society, London. Publ. No. 303, pp. 104-107.
101. Tiller, A. K. (1986). A review of the European research effort on microbial corrosion between 1950 and 1984. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 8-29.
102. Tousek, J. (1985). Theoretical aspects of the localized corrosion of metals. *In:* D. J. Fisher and F. H. Wohlbier (eds.). Material Sciences Surveys.
103. Tverberg, J. (1990). The role of manganese fixing bacteria on the corrosion of stainless steel. Corrosion/90, paper 151. NACE, Houston, Texas.
104. Varjonen, O. A., Hakkarainen, T. J., Nurmiaho-Lassila, E. L. and Salkinoja-Salonen, M. S. (1988). The brackish water biofilm on stainless steels: an electrochemical and morphological study. *In:* C. A. C. Sequeira and A. K. Tiller (eds.). Microbial Corrosion **1.** Elsevier Applied Science, London, pp. 164-178.
105. Varjonen, O. A., Hakkarainen, T. J. and Marmo, S. A. (1991). Observations on biofilms and corrosion resistance of stainless steels in paper machine environments. Corrosion/91, paper 190. NACE, Houston, Texas.
106. Videla, H. A. (1990). Biological corrosion research in Latin America past, present and future. Biodeterioration Abstracts **4** (1), 1-11.
107. Videla, H. A. (1991). Corrosión microbiológica. *In:* S. Feliú and M. C. Andrade (eds.). Corrosión y Protección Metálicas. Colección Nuevas Tendencias, Vol. **15.** Consejo Superior de Investigaciones Científicas, Madrid, Spain. Vol. 1, pp. 117-137.
108. Videla, H. A., Mele, M. F. L. de, and Brankevich, G. (1989). Biofouling and corrosion of stainless steel and 70/30 copper-nickel samples after several weeks of immersion in seawater. Corrosion/89, paper 291. NACE, Houston, Texas.
109. Von Wolzogen Kühr, C. A. H. and Van der Vlugt, L. S. (1934). De grafiteering van gietijzer als electrobiochemisch proces in anaerobe gronden. Water, Den Haag **18,** 147-165.
110. Webster, B. J., Newman, R. C. and Kelly, R. G. (1991). SRB-Induced localized corrosion of stainless steels. Corrosion/91, paper 106. NACE, Houston, Texas.
111. Weimer, P. J. and Ng, T. K. (1988). Use of two-stage continous culture to assess biocorrosion by sulfate-reducing bacteria. Corrosion/88, paper 90. NACE, Houston, Texas.
112. Williams, R. E., Ziomek, E. and Martin, W. G. (1986). Surface stimulated increases in hydrogenase production by sulfate-reducing bacteria: consequences in corrosion. *In:* S. C. Dexter (ed.). Biologically Induced Corrosion. NACE, Houston, Texas, pp. 184-192.
113. Witt, F. J. (1991). A regulatory perspective on microbiologically influenced corrosion. *In:* N. J. Dowling, M. W. Mittleman and J. C. Danko (eds.). Microbially Influenced Corrosion and Biodeterioration. The University of Tennessee, Knoxville, **7/** pp. 1-3.
114. York, J. (1990). Inspecting a raw cooling water system in a nuclear plant for MIC damage-case history. Corrosion/90, paper 131. NACE, Houston, Texas.
115. Zamanzadeh, M., O'Connor, K. and Bavarian, B. (1989). Case histories of corrosion problems in waste-water facilities. Mater. Performance **28** (9), 43-48.

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Thiobacillus ferrooxidans detection using immunelectron microscopy

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Summary

A specific, fast and very sensitive immunelectron microscopy method was developed to morphologically and serologically distinguish different cultures of ironoxidizers. Bacteria isolated from the acidic waters of «Matahambre» and «Mina Delita» mines (Cuba) were characterized. An antiserum specific to *Thiobacillus ferrooxidans* did not react with other bacteria also present in the acidic waters of mine drainage. Our results suggest the occurrence of some strains of *Thiobacillus ferrooxidans, Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* in these waters.

Key words: Thiobacillus, *iron oxidation, immunelectron microscopy.*

Resumen

La técnica de inmunomicroscopia electrónica desarrollada por nosotros es específica, rápida y capaz de caracterizar bacterias morfológica y serológicamente. Aquí se presenta su aplicación al estudio de microorganismos presentes en las aguas de las minas de «Matahambre» y «Mina Delita» (Cuba). El antisuero obtenido, específico para *Thiobacillus ferrooxidans,* no reconoció a otras bacterias que habitan normalmente en las aguas ácidas de drenaje de las mismas. Los resultados de este trabajo sugieren la posible presencia de cepas de *Thiobacillus ferrooxidans, Thiobacillus thiooxidans* y *Leptospirillum ferrooxidans* en estas aguas.

Introduction

The descomposition of certain minerals is due to the combined action of different species of *Thiobacillus* and some others of *Leptospirillum.* The association of a spiral bacterium to *T. ferrooxidans* has been recently described. It is an autotrophic bacterium which uses ferrous iron as energetic source, but it is unable to oxidize either elemental sulphur or related sulphur compounds; it is a highly pleomorphic organism (2).

Classical identification methods are not suitable for these bacteria. They are not easy to stain

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using routine microbiological procedures, since they have a complex cell envelope, similar to that observed in other Gram negative bacteria (6). Serological methods are commonly used in microbial diagnosis since they are fast and efficient; however, there are few data concerning *T. ferrooxidans* in this aspect (1, 4, 5). The present paper describes the use of immunelectron microscopy (IEM) and antibodies labelled with colloidal gold, to characterize iron-oxidizing bacteria.

Materials and methods

Samples

Samples of the acidic water and muds from Matahambre and Mina Delita mines (Cuba) were used for the isolation of bacteria.

The different cultures were identified as 1H, 2H, 3H, 8H, 22M, 24Ha, 24Hb. Numbers represent the *depth* (expressed as meters); H and M mean water and mud, respectively. In the case of sample 24, two different places, a and b, were checked. *T. ferrooxidans,* strain 2321, from the American Type Culture Collection was used as reference.

Samples were cultured in Touvinen and Kelly (TK) medium (7) until amber colour appeared, and on plates of agarose with thiosulphate (3). Cultures were observed by optical and contrast phase microscopy.

Chemical and immunological analyses

The iron oxidizing capacity of the cultures was analyzed by complexometric technique, using sodium ethyldiamine-tetraacetate and 10% sulphosalycilic acid (7).

For the preparation of the immunogen, a pure culture of *T. ferrooxidans* ATCC 2321 cells grown on ferrous iron was harvested by centrifugation. The pellet was washed three times using acidic water and, finally, the concentration was estimated by measuring the absorbance at 540 nm in a Spekol-11 spectrophotocolorimeter.

For the antiserum preparation, a New Zeland white rabbit was injected subcutaneously with 1 ml of *T. ferrooxidans* ATCC 2321 cell suspension mixed with an equal amount of Freund complete adjuvant, according to the following protocol:

Day	0	7	14	21	28	35
Inoculation	1	2	3	4	5	—
Absorbance of antigen suspension	(*) 0.4	0.4	0.9	0.9	1.0	—
Bled	—	—	—	—	—	+

* The first inoculation (day 0) was made with heat-inactivated cells.

Five weeks after the first inoculation the rabbit was bled by intracardiac puncture. The serum was preserved in 0.02% sodium azide and kept at –4° C until use.

Immunelectron microscopy (IME)

The antibodies against *T. ferrooxidans* were detected by immunelectron microscopy. A cellular suspension of *T. ferrooxidans* ATCC 2321 was used as antigen source. In order to know the antiserum specificity, different antigens from the acidic water of the mine drainage were tested.

TABLE 1
IRON-OXIDIZING ACTIVITY, ANTIGEN-ANTIBODY REACTION AND MORPHOLOGY OF SAMPLES FROM MINE WATER

Sample	Fe-oxidizing activity	Reaction with anti-T.f.	Morphology
T.f. ATCC	+++	+++	Straight rod
1H	—	—	Straight rod
2H	—	—	Straight rod
3H	—	—	Straight rod
8H	+++	+++	Straight rod
22M	+++	+++	Straight rod
24Ha	+++	—	Curved rod, pleomorphic
24Hb	+++	—	Curved rod, pleomorphic

Results and discussion

Microbial oxidant activity

The cultures 8H, 22M, 24Ha and 24Hb were able to oxidize ferrous sulphate to ferric iron, but 1H, 2H and 3H were not (Table 1). The time-course of ferric iron concentration for some of the samples is shown in Fig. 1. In culture 3H no significant increase of ferric iron concentration

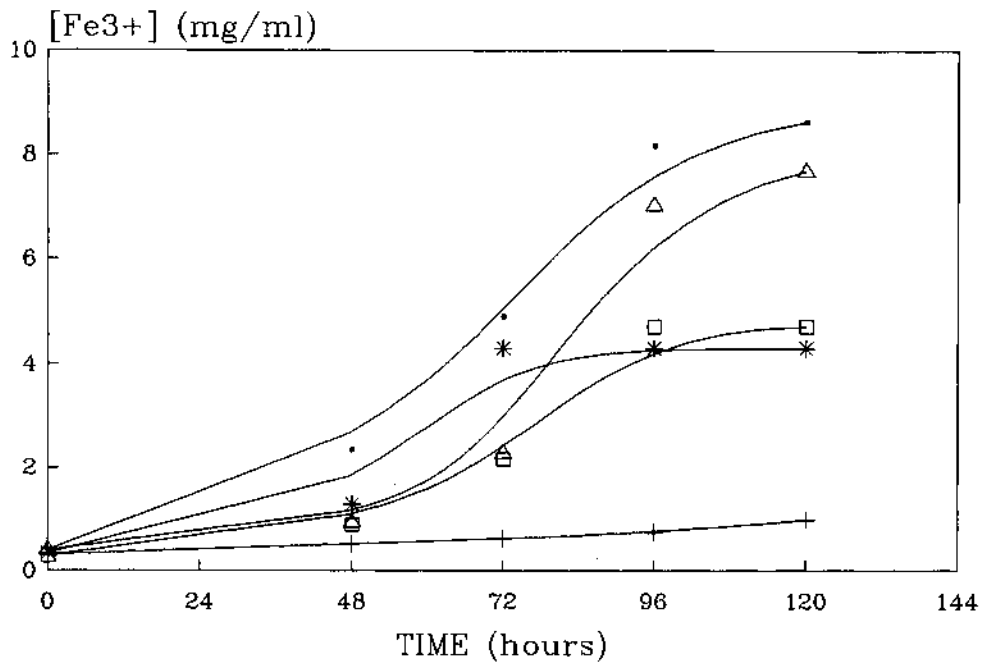

Fig. 1. Time-course of ferric iron concentration in cultures of some isolates of bioleaching-related bacteria. (–•–) *T. ferrooxidans* ATCC 2321; (– + –) 3H; (– ∗ –) 8H; (– □ –) 22M; (– ▵ –) 24 Ha.

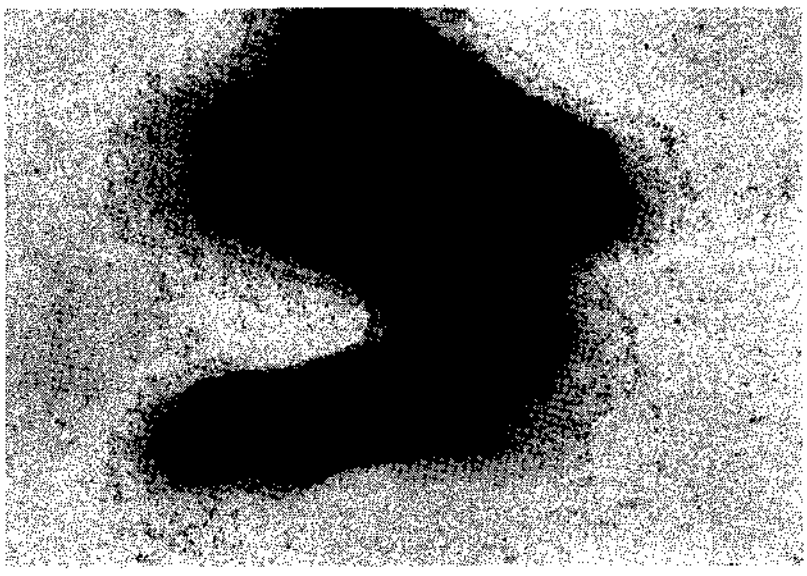

Fig. 2. Immunelectron microscopy of *T. ferrooxidans* ATCC 2321, labelled with colloidal gold (50,000 ×).

was observed; the measured ferric iron levels (0.5 mg/ml) can be explained on the basis of atmospheric oxidation at low pH. Similar concentrations were obtained with both control (not inoculated) and cultures 1H and 2H (results not presented).

Microbial strain isolation

After several passes through liquid medium (TK), cultures were plated on agarose-thiosulphate. In this way, isolated colonies could be obtained.

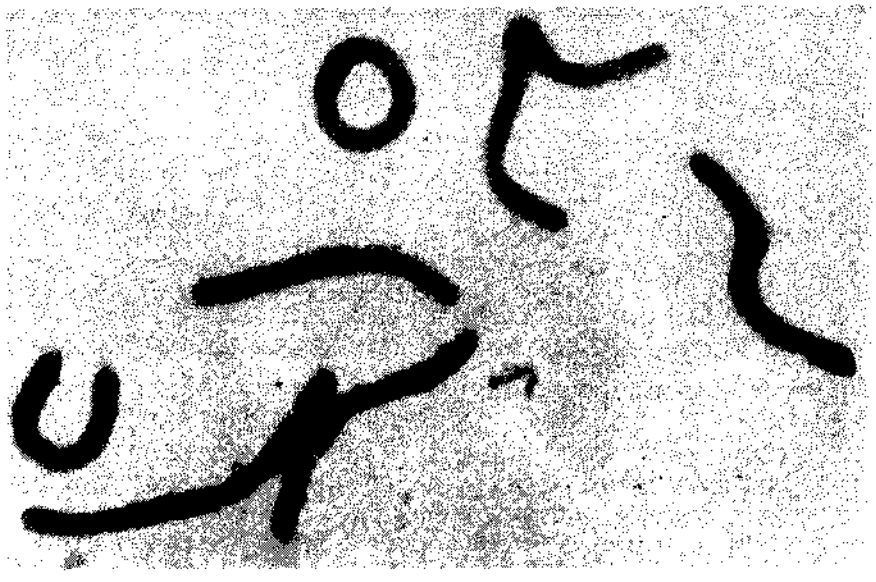

Fig. 3. Immunelectron microscopy of cells in culture 24Ha (10,000 ×).

Immunelectron microscopy

The *T. ferrooxidans* ATCC 2321 antigen reacted with its specific antiserum (Table 1). This bacterium could be observed by IEM after colloidal gold labelling (Fig. 2), showing the antigen-antibody binding. The antiserum titer was 1/2000 when faced to their homologous antigen.

All the cultures under study were used as sources of antigen for IEM. 8H and 22M cultures gave positive reaction with *T. ferrooxidans* antiserum, while cells in cultures 24Ha and 24Hb were not labelled by antibodies against *T. ferrooxidans* (Table 1). Among iron-oxidizing cultures some morphological differences were also observed. Fig. 2 shows the rod shape of *T. ferrooxidans,* and Fig. 3 shows the curved rod and polimorphism of cells in culture 24Ha. These cells and those of 24Hb culture appeared morphologically and serologically different from cells in 8H, 22M and *T. ferrooxidans* ATCC cultures, as revealed by IEM.

These results (Table 1) suggest that the iron oxidizing properties are not related to their antigenicity. Muyzer *et al.* (1987) reported cross-reaction between an anti-*T. ferrooxidans* antiserum and a *Leptospirillum ferrooxidans* culture by using an ELISA analysis. They later demonstrated that it was an erroneous identification since the culture analyzed was in fact a coculture of *T. ferrooxidans* and *L. ferrooxidans.* Although ELISA is as sensitive as IME, it is unable to differentiate morphological characteristics among organisms. This aspect is considered a very important one in the identification of iron-oxidizing bacteria.

From the results described in this paper, cells in cultures 8H and 22M characterized as *T. ferrooxidans* because they oxidize ferrous sulphate and are recognized by antiserum anti-*T. ferrooxidans.* Three samples: 1H, 2H and 3H were neither recognized by this antiserum (Table 1) nor able to oxidize ferrous sulphate (Fig. 1); they might belong to the species *T. thiooxidans.* Finally, cells in cultures 24Ha and 24Hb are iron-oxidizers but are not recognized by *T. ferrooxidans* specific antiserum (Table 1). Because of its curve shape and polymorphism (Fig. 3) they could be related to *Leptospirillum ferrooxidans* species.

The use of colloidal gold as immunochemical tracer to differentiate iron-oxidizing cultures constitutes an original method for the characterization of these bacteria. It is possible to predict their potential use for the fast and direct detection and for the identification of the microorganisms occurring in the acidic waters of mine drainages.

Acknowledgments

We thank Dr. J. Perera, from the University Complutense of Madrid, for his counsel and help.

References

1. Apel, W., Dugan, P., Talppi, J. and Rheins, M. (1976). Detection of *Thiobacillus ferrooxidans* in acid mine environments by indirect fluorescent antibody staining. Appl. Environ. Microbiol. **32,** 1.
2. Bravckmann, M. B. (1986). Untersuchungen zur Biozonose des Bergwerks biotops «Altes Lager» Erzbergwerk remmlesberg. Erzmetall. **39,** 613-617.
3. Hutchinson, M., Johnstone, K. I. and White, D. (1969). Taxonomy of the genus *Thiobacillus:* the outcome of numerical taxonomy applied to the group as a whole. J. Gen. Microbiol. **57,** 397-410.
4. Jerez, C. A., Peirano, I., Chamorro, D. and Campos, G. (1986). Immunological and electrophoretic differentiation of *Thiobacillus ferrooxidans* strains. Appl. Biohydrometallurgy. **8,** 443.
5. Muyzer, G., Brayn, A. C., Schemedding, D. J. M., Bos, P., Westbreak, P. and Juenen, G. I. (1987). A combined immunofluorescence-DNA fluorescence staining technique for enumeration of *Thiobacillus ferrooxidans* in a population of acidophilic bacteria. Appl. Environ. Microbiol. **53,** 4.

6. Pichuantes, S., Garrido, J., Leighton, V. and Rodríguez M. (1986). Studies on native strains of *Thiobacillus ferrooxidans*. Biotechnol. Appl. Biochem. **8,** 284-291.
7. Touvinenn, O.H. and Kelly, D. P. (1973). Studies on the growth of *Thiobacillus ferrooxidans*. Use of membrane filters and ferrous iron agar to determine viable numbers and comparison with CO2 fixation. Ach. Microbiol. **88,** 285.

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Identification of yeast cloned genes by genetic analysis

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Summary

Gene cloning in yeast is usually carried out by complementation of recessive mutations. However, the fact that a DNA fragment is able to complement a mutation in a certain gene does not necessarily mean that it contains that gene. The identification of a cloned gene can involve the use of Molecular and/or Classical Genetics techniques. In this paper we describe the strategy to be followed in order to establish the identity of a cloned gene, by using genetic crosses and tetrad analysis. As a practical example of the use of this strategy, we describe the cloning of the *THR1* gene which codes for the homoserine kinase in *S. cerevisiae*. This gene has been isolated from a yeast genomic library by complementation of a *thr1* mutation. The complementing DNA fragment has been subcloned and integrated into the yeast genome. By genetic crosses and tetrad analysis it has been demonstrated that integration has ocurred at the *THR1* locus. Since in this organism integration takes place mainly by homologous recombination, it can be inferred that we have, in fact, cloned the *THR1* gene. Biochemical analysis of the transformant that carries multiple copies of the cloned gene confirms this result. It shows that this strain presents a homoserine kinase activity about 60 times higher than that of the wild type.

Key words: yeast, gene cloning, genetic analysis.

Resumen

En levaduras, la clonación de genes suele llevarse a cabo mediante complementación de mutaciones recesivas. Sin embargo, el hecho de que un fragmento de ADN sea capaz de complementar una mutación en cierto gen no implica necesariamente que contenga dicho gen. La identificación del gen clonado puede realizarse utilizando técnicas tanto de Genética Molecular como de Genética Clásica. En este artículo se describe la estrategia a seguir para establecer la identidad de un gen clonado utilizando análisis genético de cruzamientos en los que interviene el mismo. Como ejemplo práctico de dicha estrategia se presenta la clonación del gen *THR1* que determina la homoserina quinasa de *S. cerevisiae*. Este gen ha sido aislado de una genoteca genómica mediante complementación de un marcador *thr1*. El fragmento que complementa dicha mutación ha sido subclonado e integrado en el genomio de una cepa de levadura. Mediante cruzamiento y análisis de las tétradas derivadas de él se ha podido establecer que la integración había tenido lugar en el

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locus *THR1*. Dado que en este organismo la integración se produce mayoritariamente por recombinación homóloga, este resultado implica que, efectivamente, se ha clonado *THR1*. El análisis bioquímico del transformante que lleva el gen en alto número de copias confirma este resultado, dado que revela que esta cepa presenta una actividad homoserina quinasa unas 60 veces mayor que la del tipo silvestre.

Introduction

The yeast *Saccharomyces cerevisiae* is an experimental organism that has been extensively used both in Basic, and in Applied Biology. The main properties that make this yeast particulary suitable for biological studies include rapid culture and easy manipulation in the laboratory, and a highly elaborated genetic system. In the last decades, it has also demonstrated to be very well suited for Genetic Engineering studies. The transformation methods are very simple, a great variety of cloning vectors are available, and cloned genes are expressed rather efficiently.

Yeast transformation was first reported by Hinnen *et al.* (9). They transformed a Leu2$^-$ strain with a ColE1 plasmid containing the *LEU2* yeast wild type allele, that had been previously isolated by complementation of a *leuB* mutation in *E. coli* (19). All yeast transformants carried a *LEU2* wild type allele integrated into the genome. It could also be demonstrated that, in the vast majority of the cases, integration had taken place by homologous recombination between sequences present in the plasmid and in the genome.

The high efficiency of homologous recombination in mitosis is a unique property of this organism, which has allowed the development of a panoply of sophisticated genetic tools. Allele rescue, gene replacement, or construction of null mutants are techniques currently used in the New Yeast Genetics which, at present, have no parallel in other organisms (3, 21). These techniques involve the use of both Molecular and Classical Genetics approaches. In this paper, we present another example of «old» and «new» genetic techniques used together, such as the demonstration of the identity of a cloned gene. The most common strategy used to clone a gene is to isolate it from a genomic DNA library by complementation of a recessive marker (20). The gene library is constructed by ligating restriction fragments of the yeast genome to an autonomously replicating plasmid vector. By conventional genetic crosses, a recipient strain is constructed that contains a recessive mutation in the gene of interest as well as a non-reverting chromosomal allele of the selectable marker carried on the vector. That strain is transformed with the gene bank and transformants are selected by complementation of the mutation in the gene of interest and/or in the plasmid marker. This approach can be used for most genes in which recessive mutations are available. It is more difficult for genes that have shown to be lethal at multiple copies, to inhibit the growth of *E. coli*, or to be linked to yeast centromeres or telomeres (20).

Complementation of recessive mutations does not necessarily mean that the cloned DNA fragment corresponds to the same gene (20). Translational suppression by a tRNA gene, or phenotypic suppression by an otherwise silent copy of the gene or by another gene, could also account for the complementation (4). The complete characterization of a cloned gene should, thus, establish the identity between that gene and a previously defined mutation.

Several strategies can be used to identify a cloned gene, most of which are rather complicated (20, 24). The most straightforward approximation consists in integrating the complementing fragment into the genome and to locate the integration site. Since integration occurs mainly by homologous recombination, location of the integration site at the chromosomal locus of the gene, would be indicative of the homology between both DNA fragments.

Mapping of the integration site of a cloned gene can be approached both by classical and by

TABLE I
YEAST STRAINS USED IN THIS WORK

Strain	Genotype	Source
AJ483	MATa leu2-3,112 his4-417 can1	AJ
AN33	MATα thr1 arg1 gal2 SUC2 mal	YGSC
DBY747	MATa leu2-3,112 his3-Δ1 ura3-52 trp1-289	YGSC
MMY1	MATα ura3-52 Cyh^R	RB
S288C	MATα SUC2 mal mel gal2 CUP1	YGSC
TMR10	MATα Leu2-3,112 his3-Δ1 his4-417 ura3-52 thr1::pMR10	This work
XMR7-4D	MATα leu2-3,112 his4-417 thr1 arg1	This work
XMR9-9A	MATα leu2-3,112 his4-417 thr1	This work
XMR24-3A	MATa leu2-3,112 his4-417 his3-Δ1 ura3-52 thr1	This work

AJ: Antonio Jiménez, Centro de Biología Molecular, Madrid. YGSC: Yeast Genetic Stock Center, Berkeley, California, USA. RB: Richard Bailey, Solar Energy Research Institute, Golden Co., USA.

recombinant DNA techniques. The cloned gene can be used as a probe in hybridization experiments carried out with whole chromosomes or with chromosome restriction fragments; it can also be used for chromosome fragmentation (7). However, conventional genetic analysis is still the preferred system.

In this paper we present a practical example on how tetrad analysis can contribute in the cloning and identification of a certain gene, i.e.*THR1*. This gene encodes the homoserine kinase (EC 2.7.1.39), responsible for the transformation of homoserine into homoserine phosphate in the threonine biosynthetic pathway (for a review, see 11).

Materials and methods

Strains and plasmids. Saccharomyces cerevisiae strains used in this work are listed in Table 1. *Escherichia coli* strain DH5 α (8) was used for the plasmids maintenance and recovery.

YIp5, an integrative vector (26), was used for subcloning.

The gene bank constructed by Nasmyth and Reed (16) was kindly provided by Dr. Aguilera (New York University). It consists of 5-20 kb DNA fragments obtained by partial digestion with *Sau3A* of *S. cerevisiae* AB320 total DNA; these fragments were inserted into the *BamHI* cloning site of YEp13.

Media. Formulations used for synthetic minimal medium (SD), synthetic minimal medium with appropriate supplements for a given strain (SC), and for rich medium (YEPD) for yeasts, are described in Sherman *et al.* (25). Nutrient broth (LB) for *E. coli* was prepared as described in Maniatis *et al.* (13); ampicillin 50-100 μg/ml was added when necessary.

Transformation. Yeast transformation was carried out basically by following the spheroplasts method as described by Hinnen *et al.* (9). About 40 μg of salmon sperm DNA, previously sonicated and boiled (13), was added as carrier DNA in each transformation. The transformants were selected in minimal medium supplemented as indicated in each case.

E. coli was transformed using the Rubidium Chloride treatment as described by Hanahan (8).

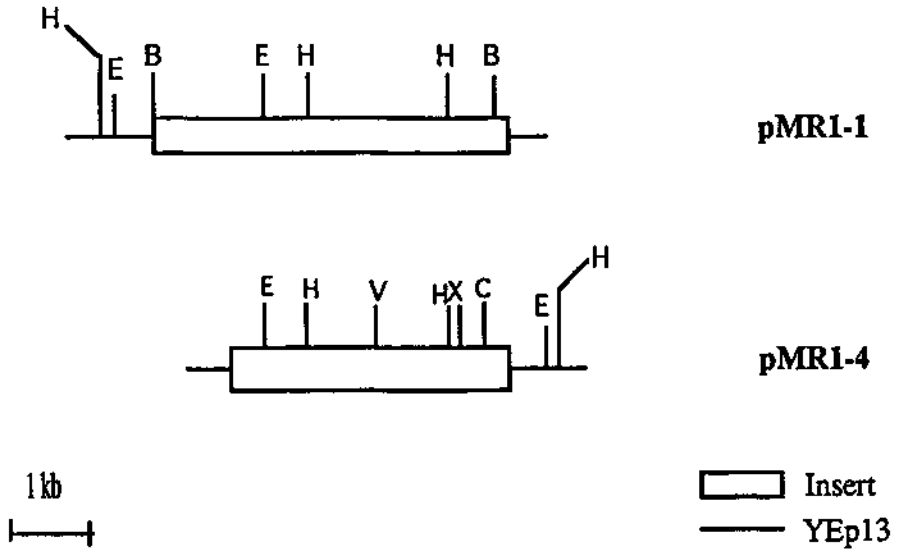


Fig. 1. Restriction map of the *thr1* complementing fragment of plasmids pMR1-1 and pMR1-4. (Note the opposite orientation of the inserts, C, X and V were not tested with pMR1-1). E, *EcoRI;* H, *HindIII;* B, *BamHI;* C, *ClaI;* X, *XbaI* and V, *EcorV.*

In vitro *DNA manipulation.* Total yeast DNA was prepared following the procedure of Davis *et al.* (6). Plasmid DNA was prepared from *E. coli* as described in Birnboim and Doly (3). When necessary, plasmid extraction was followed by a CsCl centrifugation gradient as described in Maniatis *et al.* (13). Restriction analysis and ligation of DNA fragments were done as described by Maniatis *et al.* (13) and following the procedures provided by the enzyme supplier (Boehringer Mannheim, Germany). The size of the resulting fragments was determined by agarose gel electrophoresis according to Maniatis *et al.* (13). Gel purification of DNA fragments was performed by «Geneclean», following the instructions provided by the supplier (AMS, Biotecnología, Spain).

Enzyme purification and assay. The homoserine kinase activity was partially purified according to Ramos *et al.* (18) after ammonium precipitation of the crude extract, and assayed by monitoring ADP production using the pyruvate kinaselactate dehydrogenase coupled assay (18). One unit (U) of activity was defined as the amount of partially purified extract required to convert 1 μmol of ATP to ADP per minute at 30° C. Total protein concentrations were determined according to Lowry *et al.* (12).

Genetic analysis. Conventional procedures were used for strain mating, diploid selection and tetrad analysis (23, 25).

Results and discussion

Cloning of the THR1 *gene*

Spheroplast transformation is a very efficient technique that requires the use of an appropriated strain. The «transformability» of a strain depends on its capacity for generating spheroplasts, and on the capability of these spheroplasts on taking up DNA and regenerate viable cells. Since the number and type of genetic factors involved in this character are unknown (10), trans-

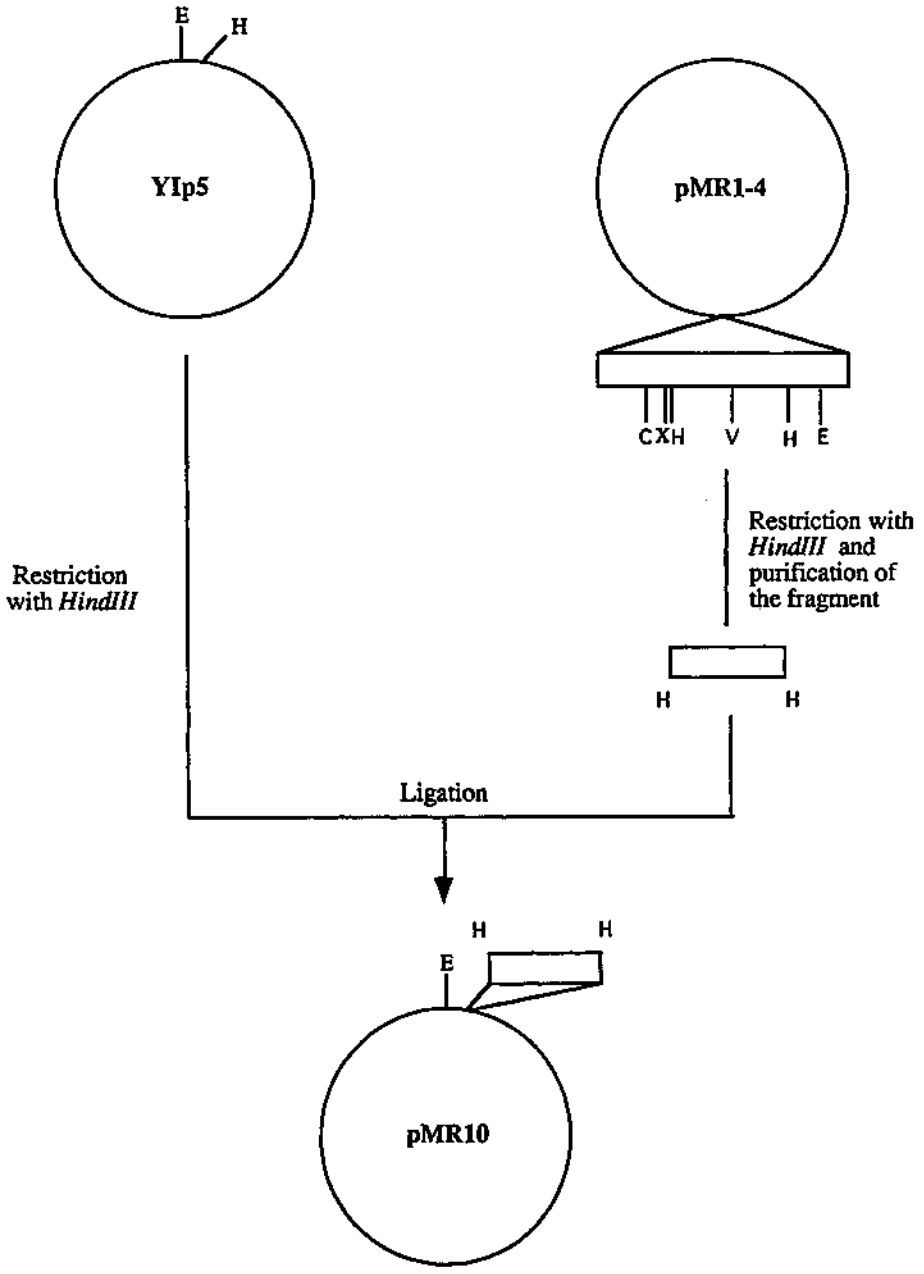
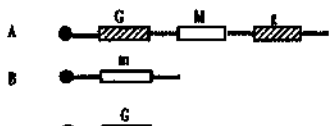
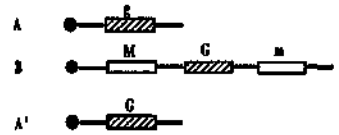
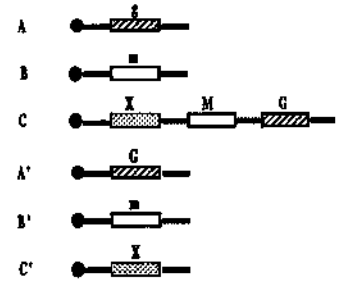


Fig. 2. Subcloning and construction of the integrative plasmid pMR10 [YIp5-*THR1*]. E, *EcoRI*; H, *HindIII*; C, *ClaI*; X, *XbaI* and V, *EcoRV*.

formable strains with the desired markers are usually obtained by repeated backcrosses to a well-known transformable strain. In this work, the transformable strain XMR9-9A was constructed by crossing strain AN33 with the transformable AJ483, and strain XMR7-4D (a meiotic derivate of this cross) again with AJ483. Strain XMR9-9A was chosen among the meiotic products of the lat-

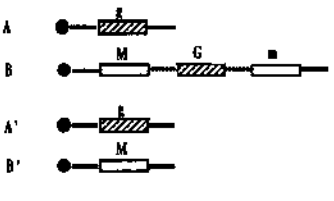
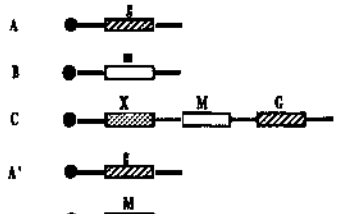
TABLE 2
EXPECTED SEGREGATION IN CROSSES OF AN INTEGRANT BY A $G^+ M^-$ STRAIN

Diploid derived from cross	Chromatid combination in tetrads	Type of tetrad	Phenotype of spores	Segregation	
				$G^+:G^-$	$M^+:M^-$
1. Integration of the plasmid (G^+M^+) ocurred at the G locus	A B	PD	G^+M^+	4: 0	2: 2
	A B		G^+M^+		
	A' B'		G^+M^-		
	A' B'		G^+M^-		
	A B'	NPD	G^+M^+	4: 0	2: 2
	A B'		G^+M^+		
	A' B		G^+M^-		
	A' B		G^+M^-		
	A B	T	G^+M^+	4: 0	2: 2
	A B'		G^+M^+		
	A' B		G^+M^-		
	A' B'		G^+M^-		
2. Integration of the plasmid (G^+M^+) occurred at the *M* locus	A B	PD	G^+M^+	4: 0	2: 2
	A B		G^+M^+		
	A' B'		G^+M^-		
	A' B'		G^+M^-		
	A B'	NPD	G^-M^-	2: 2	2: 2
	A B'		G^-M^-		
	A' B		$G'M^+$		
	A' B		G^+M^+		
	A B	T	G^+M^+	3: 1	2: 2
	A B'		G^-M^-		
	A' B		G^+M^+		
	A' B'		G^+M^-		
3. Integration of the plasmid (G^+M^+) ocurred elsewhere in the genome	A C	PD	G^+M^+	4: 0	2: 2
	A C		G^+M^+		
	A' C'		G^+M^-		
	A' C'		G^+M^-		
	A C'	NPD	G^-M^-	2: 2	2: 2
	A C'		G^-M^-		
	A' C		G^+M^+		
	A' C		G^+M^+		
	A C	T	G^+M^+	3: 1	2: 2
	A C'		G^-M^-		
	A' C		G^+M^+		
	A' C'		G^+M^-		

G and g, wild type and mutant allele of the cloned gene. M and m, wild type and mutant allele of the plasmid marker. X, chromosomal marker homologous to the cloned sequence. Plasmid DNA. Chromosomal DNA. ● Centromere. A/A', B/B' and C/C', Homologous chromosomes in parental strains. P, Parental Ditype; NPD, Non Parental Ditype and T, Tetratype.

TABLE 3

EXPECTED SEGREGATION IN CROSSES OF AN INTEGRANT BY A G^- M^+ STRAIN. SYMBOLS, AS IN TABLE 2

	Diploid derived from cross	Chromatid combination in tetrads	Type of tetrad	Phenotype of spores	Segregation	
					G^+:G^-	M^+:M^-
1.	Integration of the plasmid (G^+M^+) ocurred at the *M* locus	A B A B A' B' A' B'	PD	G^+M^+ G^+M^+ G^-M^+	2: 2	4: 0
		A B' A B' A' B A' B	NPD	G^-M^+ G^-M^+ G^+M^+ G^+M^+	2: 2	4: 0
		A B A B' A' B A' B'	T	G^+M^+ G^-M^+ G^+M^+ G^-M^+	2: 2	4: 0
2.	Integration of the plasmid (G^+M^+) ocurred elsewhere in the genome	B C B C B' C' B' C	PD	G^+M^+ G^+M^+ G^-M^+ G^-M^+	2: 2	4: 0
		B C' B C' B' C B' C	NPD	G^-M^- G^-M^- G^+M^+ G^+M^+	2: 2	2: 2
		B C B C' B' C B' C'	T	G^+M^+ G^-M^- G^+M^+ G^-M^+	2: 2	3: 1

ter cross because its transformability resembles that of the parental strain AJ483 (data not shown).

The *THR1* gene of *S. cerevisiae* was isolated from the genomic DNA library described in Materials and Methods, by complementation of the *thr1* mutation present in strain XMR9-9A. Spheroplasts of XMR9-9A ($Thr1^-$ $Leu2^-$) were prepared and transformed with 50 μg/ml of the library. Controls indicate that 99% of the spheroplasts were able to regenerate viable cells. The transformant colonies were selected in two successive steps: first, by plating the transformed spheroplasts on medium with the requirements of the strain, except leucine (SC-leu); second, by replica-plating on to the same medium, but lacking threonine (SC-thr). The transformation frequency was approximately 1.1×10^4 Leu^+ transformants/μg of DNA. Out of 2000 Leu^+ transformants tested, 4 presented the Leu^+ Thr^+ phenotype. According to the literature, these frequencies are to be expected, given the transformation method, the gene library used and the kind of gene cloned (5).

We next tested whether the Thr^+ phenotype of the transformant colonies was due to the presence in the cell of a plasmid complementing the *thr1* mutation, or to reversion of the *thr1* mutation present in the genome. These two circumstances can be distinguished using the method based

TABLE 4
ANALYSIS OF THE TETRADS DERIVED FROM THE CROSS OF THE INTEGRANT XMR24-3A::PMR10 AND MMY1

Markers	Segregation (n. tetrads)		
	PD	NPD	T
MAT-LEU	9	0	14
MAT-URA	5	3	15
LEU-URA	7	7	9
THR-URA	23	0	0

on the frequent mitotic loss of a replicating plasmid, when cells are grown on non-selective medium (1 % per generation for 2μ-based plasmids) (1). Isolated colonies of each Leu^+ Thr^+ transformant grown on YEPD plates were replica-plated onto SC-leu and SC-thr plates. In the four cases, all Leu^- colonies or colony sectors, which presumably have lost the plasmid, were also Thr^-. Conversely, all the Leu^+ colonies which retained the plasmid were also Thr^+. These results suggest that, in fact, both characters reside on a plasmid. The plasmids present in each of the transformants were called pMR1-1 to pMR1-4.

Plasmid characterization

To determine the size of the recombinant plasmids and carry out the restriction analysis of the insert, total DNA was prepared from the yeast transformants and used to transform *E. coli* DH5 α. Plasmid DNA was purified from the bacterial transformants. A rough restriction map of all four of them showed that pMR1-1 and pMR1-2 seem to be identical, with an insert of 4.5 kb, while pMR1-3 and pMR1-4 also seemed to contain an identical insert of 3.5 kb. A comparison between the restriction maps of both types of plasmid revealed a common region where the *thr1* complementing sequence could be contained (Fig. 1). Plasmid pMR1-4 was chosen for further analysis. Figure 1 also shows a more detailed restriction map of this plasmid. This map is in accordance with other authors publications (14, 22).

Integration of the cloned gene

As mentioned in the Introduction, in order to establish if the cloned gene is, in fact, *THR1*, we have taken advantage of the fact that in *S. cerevisiae*, the integration of a given DNA segment occurs mainly by homologous recombination (3). Therefore, integration of the *thr1* complementing fragment into the genome and statement that the integration had taken place at the *THR1* locus would prove its identity. The location of the integration site was made by classical genetic analysis.

In the first place it was considered convenient to subclone the presumptive *THR1* gene into an integrative plasmid (YIp). With this aim, a 1.7 kb *HindIII-HindIII* fragment of pMR1-4 was purified from an agarose gel, and ligated to a YIp5 vector previously cut with the same enzyme and dephosphorilated (Fig. 2). *E. coli* competent cells were transformed to ampicillin resistance with the ligation mixture. By restriction analysis, it was shown that the transformant colonies contained, in fact, the desired recombinant plasmid which was called pMR10 (Fig. 2).

TABLE 5
ASPARTATE KINASE AND HOMOSERINE KINASE ACTIVITIES OF STRAIN XMR9-9A, THE SAME STRAIN CONTAINING AN AMPLIFIED *THR1* GENE (IN pMR1-4) AND THE WILD TYPE STRAIN S288C

Strain	Specific activity (mU/mg protein)	
	Aspartate kinase	Homoserine kinase
XMR9-9A	243	0
XMR9-9A [pMR1-4]	141	4510
S288C	114	75

A suitable host for plasmid pMR10 was constructed by crossing strain XMR9-9A and the transformable DBY747 (see Table 1). Strain XMR24-3A ($Ura3^-$ $Thr1^-$), a transformable derivative of this cross, was chosen for further studies. The transformation frequency using an integrative plasmid increases if it is previously cleaved with a restriction enzyme because free DNA ends are extremely recombinogenic (17). If the cut is located within a yeast sequence, the integration is directed to the homologous locus. So, pMR10 was partially digested with *HindIII* and used to transform XMR24-3A spheroplasts. Yeast transformants were selected by plating on SC-ura medium. All the Ura^+ transformants selected were shown to be also Thr^+, indicating that the *HindIII-HindIII* fragment has *thr1* complementing activity. They were true integrants since they did not lose these phenotypes upon growth on a non-selective medium; one of them, TMR10, was selected for further studies.

Genetic analysis of the integrants

Theoretical basis: To check if the integration occurred at the desired locus, transformants should be crossed to strains carrying different combinations of wild type and mutant alleles of both the cloned gene, and the selectable marker. Analysis of the tetrads derived from these diploids will result in different segregation patterns, depending on the location of the integration site and its normal genetic locus. If the integration does take place at the genetic locus of the mutation, one can assume that the cloned gene truly corresponds to the mutated gene.

Table 2 illustrates the expected tetrad segregations in an hypothetical cross of an integrant strain (phenotype G^+ M^+; originally G^- M^-, transformed with the integrative plasmid), by a G^+ M^- strain; *G* and *M* represent the wild type cloned gene and plasmid marker, respectively; *g* and *m*, represent the recessive alleles; G^+ and M^+ represent the wild type phenotypes; G^+ and M^- represent the mutant phenotype; *X* represents a genomic locus which might be homologous to *G*, or to another sequence in the plasmid. In this Table, three possible cases of integration are described: 1) at the *G* locus; 2) at the *M* locus; and 3) at a locus *X*. In all three cases, the plasmid marker segregates in a 2+:2– fashion. It can also be stated that all M^+ are also G^+ reflecting the linkage of both markers. As for segregation of the cloned gene, two situations can be found:

I) it always segregates 4+:0–, meaning that integration has taken place at the *G* locus;
II) It segregates 2+:2– (Parental Ditype), 4+:0– (Non Parental Ditype) or 3+:1– (Tetratype) which means that the integration has taken place elsewhere in the genome. The relative

frequencies of these tetrads would indicate the distance of the integration site to the *G* locus; usually no linkage will be found, which means that a relative frequency would be 1PD : 1 NPD : 4T.

The latter result does not preclude that the plasmid does not contain the desired gene. It could also have happened that, despite having directed the integration by cleaving the gene, integration has taken place at the genomic locus of the plasmid marker. In order to test if this is the case, a second cross should be carried out between the integrant (G^+ M^+) and a G^- M^+ strain. Table 3 shows the expected segregation in two cases: 1) integration occurred at the *M* locus; and 2) integration occurred at a locus *X*. Here, the cloned gene (*G*) segregates always in a 2+:2– fashion but the plasmid marker can:

I) segregate always 4+:0–, meaning that integration has taken place at the *M* locus; or
II) segregate 2+:2–, 4+:0– or 3+:1–, which means that the integration has taken place elsewhere in the genome. In this case the relative frequencies of these tetrads indicate the distance of the integration site to the *M* locus; for the same reasons stated above, an excess of 3+:1– will be usually found.

If the integration has taken place at the *M* site, it is advisable to repeat the first cross but using a different integrant. If the integration has taken place outside *M* or *G*, most likely the cloned *g* complementing fragment does not correspond to the *G* gene. As stated before, complementation can occur because:

— the mutation to be complemented is of the non-sense type and the plasmid carries a tRNA gene that acts as a non-sense suppressor (4);
— the plasmid carries a gene that in high copy number is able to complement the *g* mutation; it can be either an otherwise silent copy of *G*, or an unrelated gene whose over-expression suppresses phenotypically the mutation.

Identification of the cloned gene:

We have used this kind of genetic analysis in order to locate the integration site of the pMR10 plasmid (Ura3$^+$ Thr1$^+$). The transformant was thus crossed to strain MMY1 (Ura3$^-$ Thr1$^+$). The diploid was sporulated and 23 complete tetrads were dissected and analyzed. In all of them the *THR1* marker segregated 4+:0–, indicating that the integration had taken place at the *THR1* locus. The other markers gave normal segregations given their respective chromosomal locations (Table 4); as expected, the *THR1* and *URA3* markers now appeared closely linked (the *THR1* gene is normally located at chromosome VIIIR; the *URA3* gene, at chromosome VL; 15). The *HIS* marker segregated 2+:2– (11 tetrads), 3+:1– (9 tetrads) or 4+:0– (3 tetrads), in consonance with the fact that more than one *HIS* gene, with two alleles each, is involved in the cross. The *HIS* marker is, thus, not appropriated for a simple genetic analysis.

The conclusion of this genetic analysis is that, in fact, we have cloned the *THR1* gene.

Biochemical characterization of the cloned gene

The amplification of a certain gene very often corresponds to an increase of the specific activity encoded by that gene. In order to confirm that, in fact, the *THR1* gene had been cloned, we determined the homoserine kinase activity of the transformant strain. For that purpose, strain XMR9-9A containing the pMR1-4 plasmid was grown on SC medium (containing the require-

ments of the strain); homoserine kinase was partially purified and its activity assayed. The wild type strain S288C and the original XMR9-9A strain, were used as a control. Also as a control, the aspartate kinase activity was measured in the same extract.

As shown in Table 5, the specific homoserine kinase activity is about 60 times higher in the transformant strain than in the wild type. This result is in accordance with the hypothesis that the strain contains many copies of the *THR1* gene.

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References

1. Armstrong, K. A., Som, T., Wolkert, F. C., Rose, A. and Broach, J. R. (1989). Propagation and expression of genes using 2μ circle vectors. *In:* P. J. Barr, A. J. Brake and P. Valenzuela (eds.). Yeast Genetic Engineering, pp. 165-192. Butterworths, UK.
2. Birnboim, H. C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7,** 1513-1523.
3. Calderón, I. L. and Ramos, C. (1990). Manipulación de genes en levaduras. *In:* Casadesús and Ruiz-Berraquero (eds.). Microbiología 1990, pp. 230-235. Secretariado de Publicaciones de la Universidad de Sevilla. Sevilla.
4. Calderón, I. L., Contopoulou, C. R. and Mortimer, R. K. (1984). Isolation of a DNA segment that is expressed as an amber suppressor when present in high copy number in yeast. Gene **29,** 69-76.
5. Clarke, L. and Carbon, J. (1976). A colony bank containing synthetic Col E1 hybrid plasmids representative of the entire *E. coli* genome. Cell **9,** 91-99.
6. Davis, R. W., Thomas, M., Cameron, J., St John, T. P., Scherer, S. and Padgett, R. A. (1980). Rapid DNA isolations for enzymatic and hybridization analysis. Meth. Enzymol. **65,** 404-411.
7. Gerring, S. L., Connelly, C. and Hieter, P. (1991). Positional mapping of genes by chromosome blotting and chromosome fragmentation. Meth. Enzymol. **194,** 57-77.
8. Hanahan, D. (1985). Techniques for transformation of *E. coli. In:* Clover (ed.). DNA cloning. A practical approach., vol. I, pp. 109-114. IRL Press, Oxford.
9. Hinnen, A., Hicks, J. B. and Fink, G. R. (1978). Transformation of yeast. Proc. Natl. Acad. Sci. USA **75,** 1929-1933.
10. Johnston, J. R., Hilger, F. and Mortimer, R. K. (1981). Variation in frequency of transformation by plasmid YRp7 in *Saccharomyces cerevisiae.* Gene **16,** 325-329.
11. Jones, E. W. and Fink, G. R. (1982). Regulation of amino acids and nucleotide biosynthesis in yeast. *In:* Strathern, Jones and Broach (eds.). Molecular Biology of the yeast *Saccharomyces.* Metabolism and gene expression, vol. 2, pp. 181-299. Cold Spring Harbor Laboratory, NY.
12. Lowry, O. H., Rosebrough, N. I., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.
13. Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
14. Mannhaupt, G., Pohlenz, H. D., Seefluth, A. K., Pilz, U. and Feldmann, H. (1990a). Yeast homoserine kinase: characteristics of the corresponding gene, *THR1,* and the purified enzyme, and evolutionary relationships with other enzymes of the threonine metabolism. Eur. J. Biochem. **102,** 1-8.
15. Mortimer, R. K., Schild, D., Contopoulou, C. R. and Kants, J. A. (1989). Genetic map of *Saccharomyces cerevisiae,* Edition 10. Yeast **5,** 321-403.
16. Nasmyth, K. and Reed, S. I. (1980). Isolation of genes by complementation in yeast: Molecular cloning of a cell-cycle gene. Proc. Natl. Acad. Sci. USA **77,** 2119-2123.
17. Orr-Weaver, T. L., Szostak, J. W. and Rothstein, R. (1983). Genetic applications of yeast transformations with linear and gapped plasmids. Meth. Enzymol. **101,** 228-245.
18. Ramos, C., Delgado, A. M. and Calderón, I. L. (1991). Inhibition by different amino acids of the aspartate kinase and homoserine kinase of the yeast *Saccharomyces cerevisiae.* FEBS Lett. **278,** 123-126.

19. Ratzkin, B. and Carbon, J. (1977). Functional expression of cloned yeast DNA in *E. coli.* Proc. Natl. Acad. Sci. USA **74,** 487-491.
20. Rose, M. D. and Broach, J. R. (1991). Cloning genes by complementation in yeast. Meth. Enzymol. **194,** 195-230.
21. Rothstein, R. (1991). Targeting, disruption, replacement and allele rescue: Integrative DNA transformation in yeast. Meth. Enzymol. **194,** 281-302.
22. Schultes, N. P., Ellington, A. D., Cherry, J. M. and Szostak, J. W. (1990). *Saccharomyces cerevisiae* homoserine kinase is homologous to prokaryotic homoserine kinases. Gene **96,** 177-180.
23. Sherman, F. and Hicks, J. (1991). Micromanipulation and dissection of asci. Meth. Enzymol. **194,** 21-38.
24. Sherman, F. and Wakem, P. (1991). Mapping yeast genes. Meth. Enzymol. **194,** 38-57.
25. Sherman, F., Fink, G. R. and Lawrence, C. W. (1986). Methods in yeast genetics. Cold Springer Harbor Laboratory, Cold Spring Harbor, New York.
26. Struhl, K., Stinchcomb, D. T., Scherer, S. and Davis, R. W. (1979). High frequency transformation of yeast, autonomous replication of hybrid molecules. Proc. Natl. Acad. Sci. USA **76,** 1035-1039.

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Biomasa de *Rhizopus oligosporus* como adsorbente de iones metálicos

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Summary

The effect of different parameters on the adsorption of metal ions by *Rhizopus oligosporus* has been studied. The uranium sorption by dried biomass was rapid, reaching in 5 min around 95% of the binding capacity. The uranium-binding capacity of the culture showed an inverse relation to the growth kinetic. The relationship between sorption and equilibrium concentration was similar to an adsorption isotherm. Using the Langmuir model, a maximum sorption capacity of 0.52 mmoles uranium/g dry biomass and an affinity constant of 101 l/mmol uranium at pH 4.15 were determined. The best capacity of the biomass to bind ions (UO_2^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+}) was best at pH 4.5-5.5. By using hydrochloric acid as eluant a 18% uranium removal of the biomass-bound ions was obtained at pH 1.0. The presence of other cations inhibited uranium-binding in the following order: $Cu^{2+} > Cd^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+}$.

Key words: Rhizopus oligosporus, *adsorption, metal ions.*

Resumen

Se estudió el efecto de diferentes parámetros sobre la adsorción de iones metálicos por *Rhizopus oligosporus*. La biosorción de uranio, mediante la biomasa seca, fue relativamente rápida, alcanzando a los 5 minutos cerca del 95% de la capacidad de captación en equilibrio. La capacidad de adsorción de uranio durante el cultivo presentó una tendencia inversa a la curva de crecimiento. La relación entre la capacidad de captación y la concentración en equilibrio fue semejante a una isoterma de adsorción. Utilizando el modelo de Langmuir se determinó que *R. oligosporus* presenta una capacidad máxima de adsorción de 0,52 mmoles de uranio/g biomasa seca y una constante de afinidad de 101 litros/mmol de uranio a pH 4,15.

La capacidad que tiene la biomasa de captar iones (UO_2^{2+}, Cu^{2+}, Cd^{2+} y Zn^{2+}) está relacionada con el pH de la solución, obteniéndose los mejores resultados de captación entre pH 4,5-5,5. Se realizó un estudio de reversibilidad de la unión biomasa-ión, obteniéndose un bajo porcentaje de liberación de uranilo, 18% a pH 1,0. La presencia de otros cationes inhibió la captación de uranio en el siguiente orden: $Cu^{2+} > Cd^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+}$.

(*) A quien debe dirigirse la correspondencia.

Introducción

Uno de los recursos naturales más afectados por la contaminación del medio ambiente es el agua. Este elemento vital cada vez recibe más contaminantes tanto orgánicos como inorgánicos producidos por la actividad industrial y doméstica del hombre. Entre los contaminantes inorgánicos hay que destacar a los metales pesados en solución, que causan problemas de toxicidad y bioacumulación en microorganismos, plantas y animales.

En Chile existe un alto grado de contaminación, especialmente por cobre, en la zona central, en los ríos Mapocho (3) y Aconcagua (González, S., 1983 III Simposio sobre Contaminación Ambiental. Tomo 1, p. 77-82) y en la laguna Caren (Reymond, A., 1984. Tesis de Título Químico-Farmacéutico, Universidad de Chile, Santiago), todos ellos cercanos a áreas con actividad minera propia de la zona. También se ha detectado la presencia de cobalto, cromo, manganeso, níquel, zinc y plomo en el río Mapocho (3) y de mercurio en el río Bío-Bío (Sanles, C., 1983. V Congreso Nacional de Ingeniería Sanitaria y Ambiental, Temuco), todos éstos provenientes de industrias. Este problema también preocupa a las autoridades de varias naciones, y es así que la Comunidad Económica Europea ha adoptado un acuerdo destinado al control de sustancias peligrosas (11), incluyendo los metales provenientes de efluentes industriales (11). Las técnicas convencionales de tratamiento de agua tales como: el control de pH, precipitación, intercambio iónico, electrólisis, extracción por solventes, etc., resultan poco eficientes o muy caras si se considera que las concentraciones iniciales de metales varían entre 10-100 ppm, requiriéndose una concentración final de 1 ppm después del tratamiento de aguas (8, 11). Los microorganismos tienen la capacidad de remover metales pesados desde soluciones muy diluidas, lo cual está ampliamente demostrado en *Zoogloea ramigera* (7), *Saccharomyces cerevisiae, Pseudomonas aeruginosa* (8, 9), *Bacillus subtilis* (1) y *Rhizopus arrhizus* (10, 12, 14). Esta propiedad puede ser de gran utilidad para el desarrollo de una tecnología alternativa, que sea de menor coste y de mayor eficiencia. A este fenómeno de captación de metales en solución por parte de microorganismos se le ha dado el nombre de biosorción (18).

Los hongos presentan en su pared celular una compleja estructura (4, 20) en la que existen múltiples sitios o grupos funcionales capaces de unirse por medio de diversos fenómenos a iones en solución (2, 4, 20). Debido a esto se han realizado numerosos estudios sobre la posibilidad de utilizar biomasas fúngicas para remover diferentes iones desde soluciones diluidas (2, 10, 11-17). Así , por ejemplo, Tsezos y Volesky (14) demostraron, mediante isotermas de adsorción, que *Rhizopus arrhizus* y *Penicillium chrysogenum* podían captar uranio o torio más eficientemente que una resina de intercambio iónico, usualmente empleada en la extracción de uranio y de otros iones metálicos.

En el presente estudio mostramos el efecto de algunos parámetros importantes que deben ser considerados en un posible uso de biomasa fúngica como adsorbente de iones metálicos provenientes de aguas contaminadas.

Materiales y métodos

Microorganismos y técnica de cultivo

Se utilizó la cepa del hongo *Rhizopus oligosporus* NRRL 2710 obtenida del Northern Regional Research Centre, USA. A partir de esta cepa se sembraron placas de Petri con medio agar patata dextrosa (Difco), incubándose a 28° C durante 4 días para obtener un buen crecimiento y esporulación. Posteriormente, se traspasó toda la biomasa obtenida a un matraz que contenía perlas de vidrio y una solución de cloruro de sodio 0,9% (25 ml). Enseguida se agitó vigorosamente el

matraz durante aproximadamente 10 minutos, filtrándose su contenido a través de gasas y algodón hidrófilo, obteniéndose de esta manera una suspensión de esporas. Se estandarizó el inóculo a una concentración de 1×10^8 esporas/ml. A partir de estas suspensiones se inocularon alícuotas de 1 ml en matraces Erlenmeyer que contenían 100 ml de caldo de triptona y soja (Difco), incubándose a 28° C, con agitación orbital a 160 rpm.

De esta forma se realizaron los estudios de capacidad específica de captación y el crecimiento de la biomasa con respecto al tiempo de cultivo. Para cada tiempo de cultivo se recogió toda la biomasa de un matraz. Para recoger la biomasa se procedió a filtrar al vacío con un filtro Whatman n.° 3, lavándose posteriormente la biomasa con una solución de cloruro de sodio al 0,9% (30 ml).

Las biomasas usadas en los experimentos de captación de metales fueron secadas a 85° C por 48 horas, después fueron molidas en un mortero, no determinándose el tamaño de partículas y finalmente guardadas en envases de polietileno en un ambiente sin humedad (desecador).

Técnicas para realizar captación de iones

En todos los experimentos de captación se utilizó una concentración inicial de 0,4 mM del metal en solución. Se mezclaron 50 ml de solución con 20 mg de biomasa y se colocaron en matraces en un baño termorregulado con agitación (160 rpm) a 28° C por 1 hora.

En los experimentos que miden la capacidad de captación con respecto a la concentración del ión en equilibrio, a una temperatura constante (isoterma de captación), se utilizaron alícuotas de 50 ml de nitrato de uranilo. La cantidad de biomasa utilizada varió en un intervalo entre 5 a 100 mg. Una vez realizada la adsorción, la biomasa se separó por centrifugación a 15.600 xg por 10 minutos.

Para estudiar la cinética de captación se utilizaron simultáneamente un conjunto de 26 matraces, conteniendo cada uno 100 ml de nitrato de uranilo y 25 mg de biomasa. Una vez transcurrido el tiempo de reacción deseado se separó la biomasa del correspondiente matraz por filtración, mediante filtros de membrana de 0,45 µm de tamaño de poro (Millipore), guardándose posteriormente el sobrenadante para su análisis químico.

Experimento de captación de iones metálicos a distintos pH

Se prepararon diferentes soluciones de igual concentración inicial (0,4 mM) de: $UO_2(NO_3)_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ y $3CdSO_4 \cdot 8H_2O$. Estas soluciones fueron equilibradas a distintos pH en soluciones diluidas de ácido clorhídrico o hidróxido de sodio. Alícuotas de 40 ml se hicieron reaccionar con una cantidad constante de biomasa (20 mg), midiéndose el pH antes y después de la reacción.

Inhibición de la unión del ión uranil por diferentes cationes

Las sales empleadas en la preparación de estas soluciones fueron: $UO_2(NO_3)_2 \cdot 6H_2O$, K_2SO_4, $Ca(NO_3)_2 \cdot 4H_2O$, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $3CdSO_4 \cdot 8H_2O$. Se utilizaron soluciones binarias, cada una de ellas constituidas por UO_2^{2+} (0,2 mM) y uno de los otros cationes (15 mM). El volumen de solución necesario para llevar a cabo la reacción de acumulación fue tal, que la concentración de uranio en el sobrenadante después de la reacción fuera menor de 0,1 mM (3, 5, 6, 12). Se pesaron 10 mg de biomasa y se agregó un volumen conocido de solución del ión metálico, se llevó a baño termorregulado a 28° C con agitación orbital (160 rpm) por 30 minutos.

TABLA 1
ADSORCION DE URANIO POR BIOMASA DE *RHIZOPUS OLIGOSPORUS* SECADAS A DISTINTAS TEMPERATURAS

Tiempo de cultivo (horas)	Captación (mmoles uranio/g biomasa seca)*		
	Sin secar	Secado a 85° C	Secado a 105° C
20	0,55	0,54	0,54
65	0,62	0,54	0,56

* Se utilizaron 50 ml de nitrato de uranilo y 20 mg de biomasa.

La biomasa se separó por centrifugación a 20.800 xg por 10 minutos. El sobrenadante se guardó en envases de polietileno para la cuantificación de los cationes presentes.

Experimento de elución del ión uranilo desde la biomasa

Se preparó una solución de uranio 0,4 mM a pH 5,2. Los matraces conteniendo 40 ml de solución de uranio y 20 mg de biomasa se mantuvieron a 28° C por una hora. Posteriormente la biomasa se separó y se lavó con una solución de NaCl 0,9%. Cada una de estas biomasas lavadas se resuspendieron en un volumen de 20 ml de agua destilada, ajustándose el pH con HCl hasta lograr el valor deseado y dejándose en contacto durante 20 minutos a temperatura ambiente. Posteriormente se separó nuevamente la biomasa, guardándose el sobrenadante para su análisis.

Análisis de los iones metálicos en solución

La determinación de uranio se realizó por el método del arsenazo III (8), y la medición de los otros cationes, por medio de un espectrofotómetro de absorción atómica Perkin Elmer, modelo 303.

Resultados

Efecto del secado sobre la capacidad de acumulación de uranio por la biomasa fúngica

En la Tabla 1 se visualiza que para *R. oligosporus* no existe un cambio apreciable en los valores de capacidad específica de captación de uranio, q, tanto en la biomasa húmeda como en la biomasa secada a dos temperaturas diferentes. Se puede decir que la capacidad específica de acumulación no cambia con la temperatura de secado. En los experimentos posteriores se utilizó micelio secado a 85° C en vez de biomasa húmeda debido a la facilidad de almacenamiento y manipulación de la biomasa en polvo.

Efecto del tiempo de cultivo sobre la capacidad de captación de uranio

El objetivo de ese experimento fue determinar la cantidad de biomasa que se obtiene a diferentes tiempos de cultivo y su capacidad específica de adsorión de iones.

En la Figura 1 se observa que *R. oligosporus* posee un crecimiento rápido, alcanzando su má-

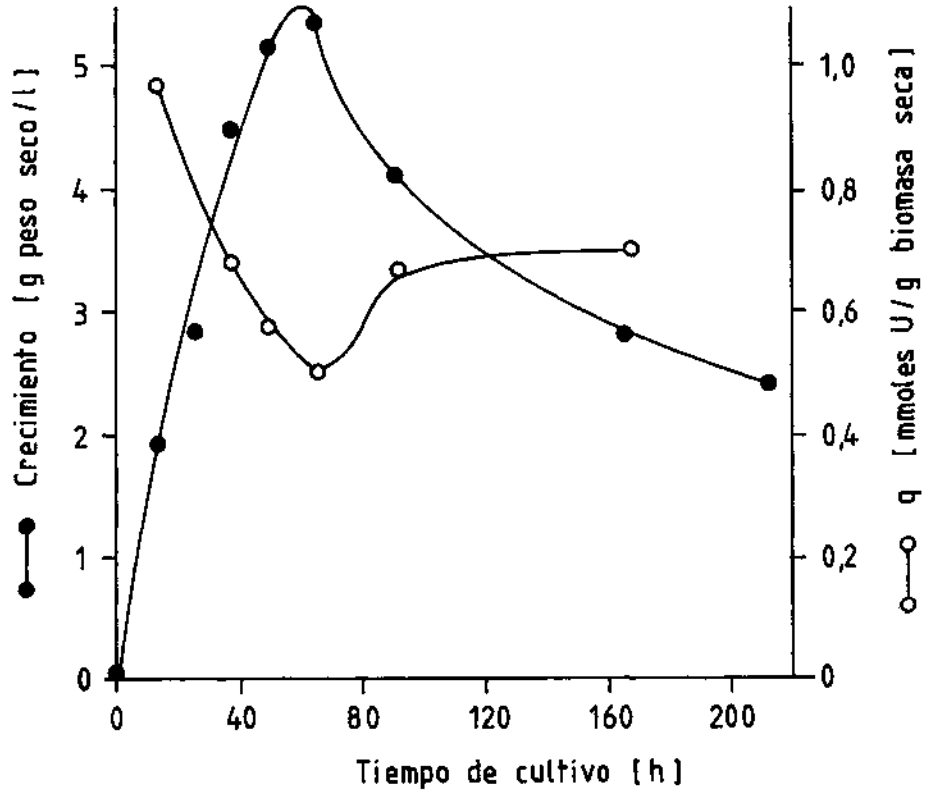

Fig. 1. Efecto del tiempo de cultivo sobre el crecimiento y la capacidad de captar uranio de *Rhizopus oligosporus* (50 ml de nitrato de uranilo; 20 mg de biomasa seca).

ximo desarrollo a las 70 horas de cultivo. Se observa también que el tiempo de cultivo afecta a la capacidad específica de captación de uranio. La curva de biosorción es inversa a la curva de crecimiento fúngico. Esto significa que la biomasa obtenida en el momento de mayor crecimiento tiene la menor capacidad para unir iones uranilo.

Cinética de captación de uranio por la biomasa fúngica

Es conocido que todo el fenómeno físico-químico o enzimático está influenciado por el tiempo de reacción. Por esto se consideró necesario relacionar la capacidad de acumulación con el tiempo de reacción. La biomasa fúngica se mantuvo durante tiempos variables en presencia de una concentración constante de nitrato de uranilo. Los resultados indicaron que la cinética de captación de uranio por parte de *R. oligosporus* es rápida (Fig. 2), alcanzando dentro de los 5 minutos iniciales aproximadamente el 95% del valor de capacidad específica de acumulación obtenida en el equilibrio. Entre los 5 y los 40 minutos el valor de q continuó aumentando hasta llegar al equilibrio, donde la capacidad específica de acumulación es independiente del tiempo de reacción. El pH de la solución disminuyó levemente respecto al pH de tiempo cero (pH de la solución sin biomasa), alcanzando dentro de los 5 minutos iniciales un valor constante (pH 4). Este experimento permitió determinar que el empleo de una hora de reacción entre la biomasa y la solución de metal pesado es un tiempo adecuado para asegurar que el sistema ha logrado un estado de equilibrio.

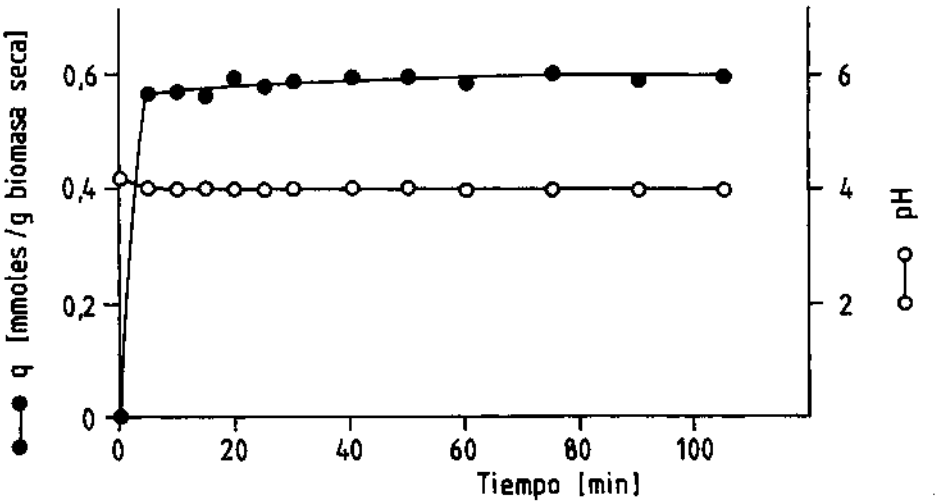

Fig. 2. Cinética de captación de uranio por *Rhizopus oligosporus* (100 ml de nitrato de uranilo; 25 mg de biomasa seca).

Isoterma de acumulación del ión uranilo

Se estudió a continuación la relación existente entre la capacidad de captación de la biomasa y la concentración del ión dentro del sistema. En la Figura 3 se observa que al aumentar la concentración de uranio en equilibrio (C_{eq}), aumenta el valor de la capacidad de acumulación, ten-

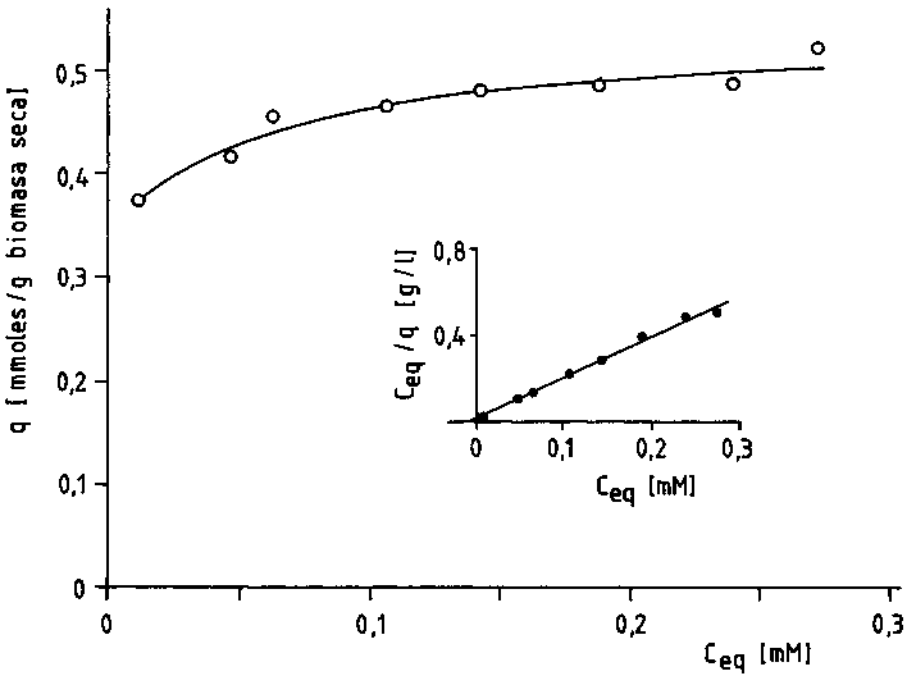

Fig. 3. Isoterma de captación de uranio por *Rhizopus oligosporus* (pH inicial = 4,15). Inserto: gráfico de Langmuir.

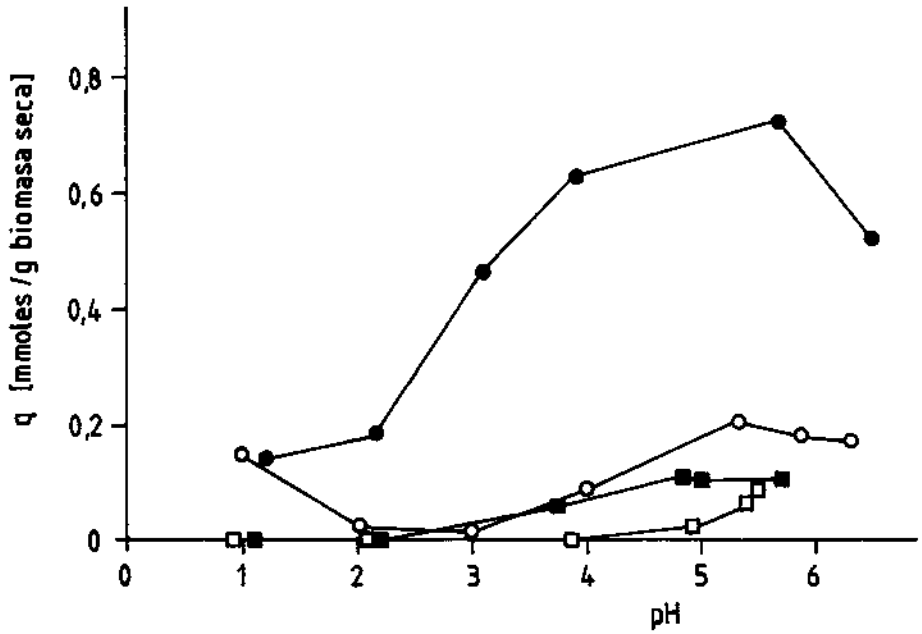

Fig. 4. Efecto del pH sobre la captación de diversos cationes por Rhizopus oligosporus. (•): Uranio. (o): Zinc. (■): Cobre. (□) Cadmio.

diendo esta última a un valor límite. Este valor límite, capacidad de captación máxima (q_m), representa la cantidad límite de metal que puede ser captado cuando se han ocupado todos los sitios de unión de la biomasa. También se puede observar que para bajas concentraciones de uranio en equilibrio, pequeños cambios en la concentración implican un significativo cambio en la biosorción. Pero la captación se hace casi independiente a altos valores de concentración de uranio en equilibrio.

Para poder cuantificar los parámetros de captación máxima (q_m) y constante de afinidad biomasa-ión (k) se procesaron los datos experimentales de las isotermas por medio del modelo de adsorción de Langmuir linearizado según la siguiente ecuación:

$$\frac{C_{eq}}{q} = \frac{C_{eq}}{q_m} + \frac{1}{K\,q_m}$$

Con esto fue posible determinar las constantes q_m= 0,52 mmoles/g de biomasa seca y K= 101 l/mmol.

Influencia del pH en la capacidad de captación

En la Figura 4 se observa que al aumentar el pH, también aumenta la capacidad de captación de la biomasa, excepto en el caso del zinc, en que disminuyó levemente el valor de q al aumentar el pH entre 1 a 2,5. A cualquier valor de pH la biomasa tiene la mejor capacidad de captación para uranio, comparado con los otros iones. El menor valor q correspondió siempre a cadmio, presentando una capacidad de captación nula a valores de pH inferiores a 4.

A pH superiores a 5,5 disminuyó el valor de q para uranio y zinc, siendo más notorio en el

TABLA 2
INHIBICION DE LA CAPTACION DE URANIO POR DIFERENTES CATIONES EN *RHIZOPUS OLIGOSPORUS*

Cationes añadidos (15 mM)	Inhibición*
Ninguno	0%
Cu^{2+}	43%
Cd^{2+}	24%
Zn^{2+}	20%
Mg^{2+}	15%
Ca^{2+}	5%
K^{+}	0%

* La concentración inicial de uranio fue de 0,2 mM. Se utilizó una concentración de biomasa de 0,17 g/l.

caso de uranio. No se pudo experimentar a pH mayores a 6 con cobre y cadmio por problemas de formación de precipitados.

Inhibición de la acumulación de uranio por diferentes cationes

El objetivo de este experimento fue establecer si la unión de uranio por el hongo se ve afectada por la presencia de otro catión en la solución. En la Tabla 2 se observa que la mayoría de los cationes utilizados fueron capaces de inhibir la captación de uranio en *R. oligosporus*. La capacidad inhibitoria de los cationes se ordenó así: $Cu^{2+} > Cd^{2+} > Zn^{2+} > Mg^{2+} > Ca^{2+}$; K^{+} no inhibió la acumulación de uranio. Cu^{2+} fue el catión que presentó el mayor porcentaje de inhbición de la captación. También se observa que Cd^{2+} y Zn^{2+} inhibieron en forma importante la unión de UO_2^{2+}. La adsorción del ión adicional añadido no fue determinada.

Elución de uranio desde la biomasa

Al comprobar que la biomasa fúngica seca es capaz de captar iones metálicos, lo cual depende del pH de la solución, se quiso evaluar la posibilidad de revertir el fenómeno de captación a través de la modificación del pH de la solución. La biomasa de *R. oligosporus*, previamente tratada con nitrato de uranilo al pH de máxima captación (pH 5,3), se trató con HCl a distintas concentraciones. Se eligió este ácido por la alta solubilidad que presenta el ión uranilo en él. En la Figura 5 se observa que a medida que disminuye el pH aumenta el porcentaje de liberación del ión uranilo, obteniéndose el 18% de liberación a pH 1, mientras que a pH mayores de 2 el porcentaje de liberación es muy bajo, no superando el 3%.

Discusión

El presente estudio da a conocer la influencia de algunos parámetros importantes a considerar en el hongo *R. oligosporus*, que puede ser utilizado como un biosorbente biológico de metales contaminantes.

El hongo se hizo crecer primero en un medio de cultivo apropiado en ausencia de iones me-

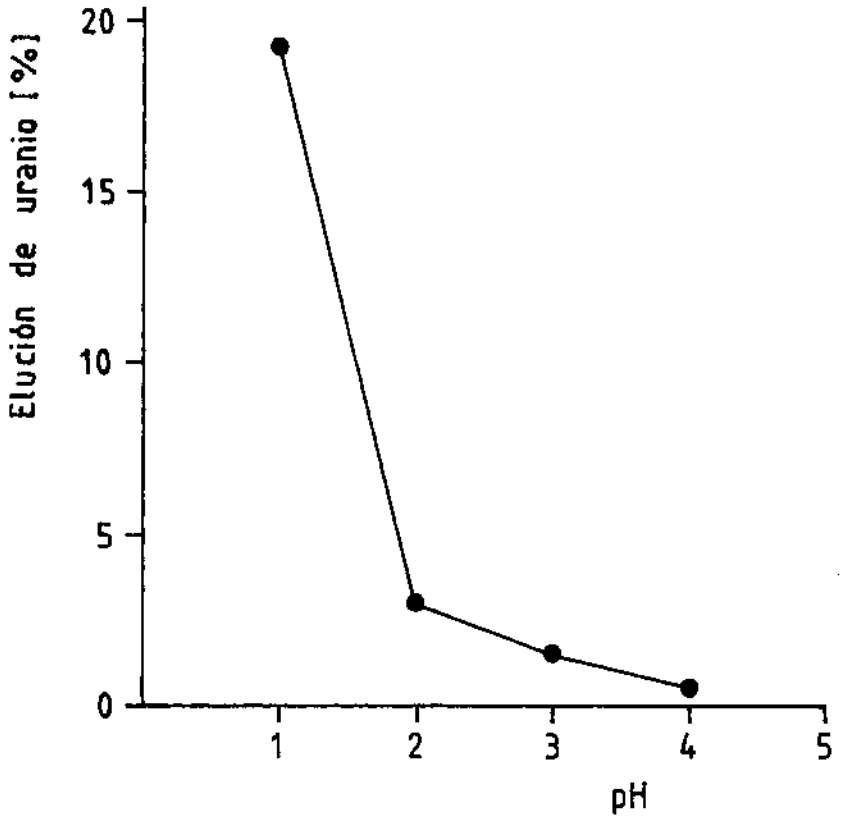

Fig. 5. Elución del uranio captado por *Rhizopus oligosporus* a distintos valores de pH.

tálicos. En una segunda etapa se realizó la reacción de captación de iones metálicos con biomasa lavada (tanto húmeda como seca). Al separar la fase de crecimiento de la etapa de biosorción de iones metálicos se evita el posible efecto tóxico de los metales pesados u otros elementos en solución podrían ejercer sobre el microorganismo en desarrollo.

En la reacción de captación de metales podrían participar tanto mecanismos de biosorción pasiva como mecanisms activos de bioacumulación (dependiente del metabolismo del hongo). Esto nos llevó a comparar la capacidad de captación de iones de biomasa húmedas (vivas) con biomasas secadas a distintas tempaturas. Dichos experimentos dejaron como conclusión que la captación de uranio por *R. oligosporus* es de carácter eminentemente físico-químico, no necesitando de un proceso metabólico activo para explicar la acumulación de uranio observada. Esto confirma lo descrito por varios investigadores (2, 18, 19).

El fenómeno físico-químico de biosorción es relativamente rápido, siendo esto una ventaja desde el punto de vista tecnológico, ya que se necesita poco tiempo de contacto entre la solución y la biomasa para que ésta alcance un alto porcentaje de captación. Treen y col (12) señalan que *Rhizopus arrhizus* presenta una tendencia de capacidad específica de captación de uranio, con respecto al tiempo de cultivo, semejante a lo observado en las primeras 70 horas de cultivo de *R. oligosporus*. La tendencia descrita en estos dos hongos puede significar que ésta sea una característica común dentro del género *Rhizopus*.

El siguiente paso de la investigación fue estudiar la isoterma de captación de uranio. El hecho de adoptar el modelo teórico de Langmuir no significa que este fenómeno se deba sólo a la

TABLA 3
RELACION ENTRE LA CONCENTRACION DE BIOMASA DE *R. OLIGOSPORUS* CON LA FRACCION q_m (LANGMUIR), LA CONCENTRACION EN EQUILIBRIO Y EL % DE REMOCION DE URANIO DE LA SOLUCION

Concentración de la biomasa (g/l)	q experimental/q_m	C_{eq} (mM)	% eliminación de uranio
0,4	0,94	0,19	50
1,0	0,72	0,01	97

adsorción o a la interacción entre un ión y un grupo químico de la estructura fúngica que actúa como sitio de unión, sino que permite evaluar la cepa de hongo en cuanto a su afinidad y capacidad de captación. Esta información permite tener un criterio para elegir hongos, dependiendo de la aplicación tecnológica que se desee.

Tsezos y Volesky (14) demostraron que una biomasa seca de *Rhizopus arrhizus*, obtenida de desechos industriales, es capaz de captar aproximadamente 0,76 mmoles de uranio/g biomasa seca a concentraciones en equilibrio mayores a 0,42 mM de uranio. En cambio Treen y col. (12) encontraron en otras condiciones experimentales y de cultivo que la biosorción de *Rhizopus arrhizus* es aproximadamente 0,46 mmoles de uranio/g biomasa seca a concentraciones en equilibrio de 0,42 mM. En este trabajo se determinó para *Rhizopus oligosporus*, según el modelo de Langmuir, una biosorción máxima (q_m) de 0,52 mmoles uranio/g biomasa seca, la cual es semejante a la observada en *Rhizopus arrhizus*, el que es considerado un buen biosorbente (10, 13, 14, 19). Esto podría significar que la alta capacidad de captación es una propiedad del género *Rhizopus* comparado con otros microorganismos tales como *Aspergillus niger, Penicillium chrysogenum, Pseudomonas fluorescens* (14).

Es necesario destacar que la concentración de biomasa utilizada en los experimentos de captación influye en parámetros importantes, tales como concentración del ión en solución cuando el sistema biomasa-solución alcanza un estado en equilibrio, fracción o porcentaje de la q_m teórica de la biomasa que realmente está siendo ocupado por los iones acumulados (q experimental/q_m) y el porcentaje de iones que han sido removidos de la solución con respecto a la cantidad de iones presentes en la solución inicial. Estas relaciones están ejemplificadas en la Tabla 3, en la cual se muestran los valores que adoptan estos parámetros a 2 concentraciones distintas de biomasa. Estos valores fueron obtenidos a partir de las respectivas isotermas de acumulación (Fig. 3). Se puede concluir que cuando aumenta la concentración de biomasa disminuye la concentración de uranio en equilibrio, aumenta el porcentaje de eliminación de uranio de la solución y disminuye la fracción q_m ocupada por los iones de uranilo que están acumulados en la biomasa. Es por esto que se debe estimar la concentración de biomasa más conveniente, según la concentración del ión en equilibrio que se desee lograr. Es así que para descontaminar eficientemente una solución diluida es preferible utilizar una alta concentración de biomasa para que el porcentaje de eliminación del ión contaminante sea alto. En cambio, si se desea aprovechar al máximo la capacidad de captación de la biomasa se debe usar una concentración tal que el valor de q obtenido sea semejante al valor de q_m teórico. Esta última consideración se tuvo en cuenta en el experimento de biosorción de cationes a diferentes pH, ya que a valores cercanos a q_m la captación se hace casi independiente de la concentración del ión en equilibrio.

La tendencia observada en dichos experimentos (aumento de q al aumentar el pH) es semejante a la obtenida con otros biosorbentes (9, 14, 15, 21) tales como *Saccharomyces cerevisiae, Streptomyces niveus* y *Rhizopus arrhizus*. El hecho de que a bajos valores de pH se presente una

baja capacidad específica de captación sugiere que existe una competencia por los sitios activos de unión de la biomasa entre los iones metálicos y los protones. Es así que Tsezos y Volesky (15) proponen que algunos grupos químicos (aminas, hidroxilo) presentes en la pared celular actúan como bases de Lewis, siendo capaces de coordinar especies ácidas (ácidos de Lewis) como son los iones metálicos y los protones. Esto puede ser una limitación para el uso tecnológico de estos hongos, ya que serían poco eficientes en presencia de efluentes muy ácidos. También hay que tener en cuenta que a medida que aumenta el pH comienzan a aparecer otras especies iónicas, como hidróxidos solubles, las que pueden precipitar en ciertas condiciones. Esto indica que al aumentar el pH, la química de la solución se hace más compleja, estableciéndose nuevos equilibrios entre las distintas especies iónicas (9, 10, 14).

En el estudio de liberación de uranio captado en la biomasa de *Rhizopus oligosporus*, usando distintas concentraciones de ácido clorhídrico para la elución, se obtuvieron bajos valores de liberación, no superando el 18% a pH 1. Esto puede deberse a una unión muy fuerte del catión con la pared del hongo o también al poco tiempo de contacto (20 minutos) entre la biomasa y el eluyente. En cambio, hay trabajos con bacterias (7) donde en condiciones similares se obtiene una eliminación del 100%. Tsezos (17) sugiere la conveniencia de usar agentes eluyentes, como por ejemplo bicarbonato de sodio, que no alteren o dañen la estructura de la biomasa para que no cambie la capacidad de captación que ésta posee.

Por último fue de interés estudiar la competitividad del ión UO_2^{2+} con otros cationes. Se sabe que una gran variedad de microorganismos presenta esta capacidad de captar diversos iones metálicos y que el ión uranilo es preferentemente unido por la mayoría de ellos (5, 6, 8, 10, 16). Se demostró que la mayoría de los iones inhibieron, en distintas proporciones, la acumulación del ión uranilo. Esto sugiere que dichos cationes se habrían unido a la biomasa, compitiendo con el ión uranilo por los sitios de unión. A pesar de que la concentración del ión inhibitorio fue 75 veces superior a la de uranio, se produjo una alta captación de este último ión, lo que indica que la biomasa presenta mayor selectividad por uranio. De igual manera, Nakajima y Sakaguchi (6) estudiaron en 83 especies de microorganismos (que incluían hongos, levaduras, actinomicetes y bacterias), la acumulación de UO_2^{2+} de Co^{2+} y de una mezcla equimolar de UO_2^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Mn^{2+}, Pb^{2+} y Hg^{2+}. Ellos encontraron que la acumulación de UO_2^{2+} y Co^{2+} en la mezcla fue siempre menor a la biosorción obtenida cuando estos iones estaban solos en solución y que la acumulación selectiva de iones de metales pesados por microorganismos está determinada por una competencia interiónica.

Abreviaturas:

q: Captación de metal.
q_m: Captación máxima de metal.
C_{eq}: Concentración del ión metálico en equilibrio.
K: Constante de afinidad.

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Bibliografía

1. Beveridge, T. J. and Murray, R. G. E. (1980). Sites of metal deposition in the cell wall of *Bacillus subtilis*. J. Bacteriol. **41**, 876-887.

2. Galun, M., Keller, P., Malki, D., Feldestein, H., Galun, E., Siegel, S. M. and Siegel, B. Z. (1982). Removal of uranium (VI) from solution by fungal biomass and fungal wall-related biopolymers Science **219,** 285-286.
3. Gaslaschi, G., Schalscha, E., Vergara, I. and Chang, A. C.. (1985). Trace metals in sediments of the Mapocho river, Chile, Environ. Technol. Lett **6,** 405-414.
4. Müller, E. and Loeffler, W. (1982). Mykologie: Grundiss für Naturwissenschaftler und Mediziner, pp. 81-85. Thieme, Stuttgart.
5. Nakajima, A., Horikoshi, T. and Sakaguchi, T. (1981). Studies on the accumulation of heavy metal elements in biologycal system XVII. Selective accumulation of heavy metal ions by *Chlorella regularis.* Eur. J. Appl. Microbiol. Biotechnol. **12,** 76-63.
6. Nakajima, A. and Sakaguchi, T. (1986). Selective accumulation of heavy metals by microorganisms. Appl. Microbiol. Biotechnol. **24,** 59-64.
7. Norberg, A. B. and Persson, H. (1984). Accumulation of heavy metal ions by *Zoogloea ramigera.* Biotechnol. Bioeng. **26,** 239-246.
8. Schumate II, S. E., Strandberg, G. W. and Parrot, Jr. (1978). Biological removal of metal ions from aqueous process streams. Biotechnol. Bioeng. Symps n.° **8,** 13-20.
9. Strandberg, G. W.,, Schumate II, S. E. and Parrot, Jr. (1981). Microbial cells as biosorbents for heavy metals: Accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa.* Appl. Environ. Microbiol. **41,** 237-245.
10. Tobin, J. M., Cooper, D. G. and Neufeld, R. J. (1984). Uptake of metal ions by *Rhizopus arrhizus* biomass. Appl. Environ. Microbiol. **47,** 821-824.
11. Townsley, C, C., Ross, I. S. and Atkins, A. S. (1986). Biorecovery of metallic residues from various industrial effluents using filamentous fungi. *In:* R. W. Lawrence, R. M. R. Branion and H. G. Ebner (eds.). Fundamental and applied biohydrometallurgy, pp. 279-289. Elsevier, Amsterdan.
12. Treeeen-Sears, M. E., Volesky, B., Neufeld, R. J. (1984). Ion exchange/complexation of the uranyl ion by *Rhizopus* biosorbent. Biotechnol. Bioeng. **26,** 1323-1329.
13. Treen-Sears, M. E., Martín, S. M. and Volesky, B. (1986). Control of *Rhizopus* biosorbents quality during propagation *In:* R. W. Lawrence, R. M. R. Branoin and H. G. Ebner (eds.). Fundamental and applied biohydrometallurgy, pp. 305-308. Elsevier, Amsterdan.
14. Tsezos, M. and Volesky, B. (1981). Biosorption of uranium and thorium. Biotechnol. Bioeng. **23,** 583-604.
15. Tsezos, M. and Volesky, B. (1982). The mechanism of uranium biosorption by *Rhizopus arrhizus.* Biotechnol. Bioeng. **24,** 385-401.
16. Tsezos, M. (1983). The role of chitin in uranium adsorption by *Rhizopus arrhizus.* Biotechnol. Bioeng. **25,** 2025-2040.
17. Tsezos, M. (1984). Recovery of uranium from biological adsorbents-desorption equilibrium. Biotechnol. Bioeng. **26,** 973-981.
18. Volesky, B. (1986). Biosorbent materials. Biotechnol. Bioeng. Symp. N° **16,** 121-125.
19. Volesky, B. (1987). Biosorbents for metal recovery. Trends Biotechnol. **5,** 96-101.
20. Wessels, J. G. H. (1986). Cell wall synthesis in apical hyphal growth. Internat. J. Cytol. **104,** 37-79.
21. Wnorowski, A. U. (1991). Selection of bacterial and fungal strains for bioaccumulation of heavy metals from aqueous solutions. Wat. Sci. Tech. **23,** 309-318.

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Comparative study of some factors affecting enumeration of moulds using dilution plate techniques

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Summary

The influence of dilution plating technique, nature of diluent, culture media and incubation period on the enumeration of moulds have been studied. Three new culture media containing Auramine, Gentian Violet and Malachite Green respectivelly have been induced in this study. No significant differences were observed between results obtained after 3, 5 and 7 days of incubation. Significantly higher recoveries were obtained using the surface-spread method than pour plate method. Using the first technique no effect of diluent was observed, and among the different culture media studied higher counts were obtained with medium containing Auramine.

Key words: Culture media, diluents, dyes, fungal enumeration, incubation period.

Resumen

Se ha analizado la influencia de la técnica de siembra en placa, la naturaleza del diluyente, el medio de cultivo y el tiempo de incubación sobre el recuento de hongos miceliares. Se han incluido tres nuevos medios de cultivo conteniendo Auramina, Violeta de Genciana y Verde de Malaquita, respectivamente. No han aparecido diferencias significativas entre los resultados obtenidos después de 3, 5 y 7 días de incubación. Los recuentos obtenidos mediante la técnica de siembra en superficie de agar han resultado significativamente superiores que los de la técnica por inclusión en agar. Utilizando la primera de estas técnicas no se ha observado efecto del diluyente empleado y mediante el medio de cultivo adicionado de Auramina se han obtenido recuentos fúngicos más altos.

Introduction

Nature and extent of fungal contamination are important criteria in judging hygiene and storage practices during the manufacture and distribution of food. The detection and quantification of fungi is therefore an essential part of the microbiological examination of foods. Many different methods are available: cultural, direct microscopic and indirect methods (8). When only one metho-

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dology is followed, every attempt should be made to ensure the maximum recovery of organisms under investigation.

The cultural methods allow detection of viable fungus material, even at relatively low contamination levels and among them, the most used are the colony count techniques. As in bacteriological analysis, the principle behind the propagule count is the preparation of sample homogenate, followed by serial dilution in an appropriate diluent and plating onto or into appropriate culture media. There are several factors which can affect results of propagule counts such as plating technique, nature of diluent, culture media and incubation period. To evaluate the influence of these factors on the enumeration of moulds, an experimental study has been designed to compare the recovery of the tested strains by the surface and pour-plate methods seven culture media, including three new media containing auramine, gentian violet and malachite green, respectivelly. The effect of incubation time has been evaluated comparing the results obtained after 3, 5 and 7 days of incubation.

Strains

The moulds used in this study were: *Aspergillus parasiticus* NRRL 2999, *Penicillium verrucosum* var. *cyclopium* CCFVB 417 (CCFVB denotes the Culture Collection of the Faculty of Veterinary of Barcelona), *Fusarium oxysporum* CCFVB 300 and *Alternaria alternata* CCFVB 252. These strains have been selected because of their high frequency of isolation in substrata such as foodstuffs and/or their ability to produce mycotoxins. The mould suspensions were prepared from cultures grown separately on 2 % Malt Extract Agar for seven days at 28° C. After incubation, spores were harvested by adding 10 ml of distilled water to the culture vessels and gently dislodging the spores with a flamed wire loop. The spore suspensions were then aseptically filtered through sterile gauze to remove mycelial debris, and the volumes adjusted so that the suspensions contained approximately 10^5 spores/ml as determined by a counting chamber. A mixed-spore suspension was prepared taking equal volumes of each individual spore suspension.

Diluents

A serial dilution of the mixed spore-suspension was made in the following five diluents: Tryptone water (TW), Distilled water (DW), Phosphate buffered saline (PBS), Saline water (0.90 % v/v) (SW), and Saline water + 0.05 % Tween 80 (SWT).

Culture media

Seven media (final pH, 5.6 ± 0.2) were tested for recovery of moulds from the mixed-spore suspension: Malt extract agar (MEA), containing (g/l): malt extract, 13; Dextrin, 2.5; Peptone, 5 and agar, 15; Sabouraud Dextrose agar (S) containing (g/l): Peptone, 10; Dextrose, 40 and agar, 15; MEA + 50 ppm of Rose Bengal (RB); MEA + 2 ppm of Dichloran (D); MEA + 25 ppm of Auramine (A); MEA + 5 ppm of Gentian Violet (GV) and MEA + 1 ppm of Malachite Green (MG).

Colony count techniques

Quantitative enumeration of fungal propagules was done on solid media using the pour plate and surface-spread method. Five plates were inoculated for each diluent and medium used.

TABLE 1

MEAN VALUES OF TOTAL COUNTS IN CFU/ml ($x10^{-4}$) OBTAINED BY THE SURFACE AND POUR-PLATE METHODS, USING DIFFERENT CULTURE MEDIA, DILUENTS AND INCUBATION TIMES

M	DIL	Surface-spread			Pour-plate		
		Incubation time (days)			Incubation time (days)		
		3	5	7	3	5	7
MEA	TW	46.0	46.0	46.0	41.0	41.0	41.0
	DW	50.4	50.6	50.6	36.4	36.4	36.4
	PBS	57.2	57.2	57.2	40.2	40.2	40.2
	SW	48.6	48.6	49.6	40.8	40.8	40.8
	SWT	55.8	56.2	56.2	46.6	46.6	46.6
S	TW	38.0	38.0	38.0	38.8	38.8	38.8
	DW	42.8	43.4	43.4	37.2	37.8	37.8
	PBS	46.2	46.2	46.2	42.8	43.6	43.6
	SW	58.0	58.4	58.4	38.8	39.4	39.4
	SWT	50.2	50.5	50.7	29.8	29.8	29.8
A	TW	56.6	56.8	56.8	37.8	37.8	37.8
	DW	55.0	55.0	55.0	47.6	48.0	48.0
	PBS	55.6	55.6	55.6	44.0	44.0	44.0
	SW	53.4	54.0	54.0	45.6	45.6	45.6
	SWT	62.8	62.8	62.8	53.4	53.4	53.4
D	TW	45.6	46.6	46.6	35.4	35.4	35.4
	DW	50.8	51.2	51.2	35.4	35.4	35.4
	PBS	47.8	47.8	47.8	35.2	35.2	35.2
	SW	55.4	55.8	55.8	38.5	38.5	38.5
	SWT	53.2	53.7	53.7	51.0	51.0	51.0
RB	TW	54.6	56.0	56.0	43.6	43.6	43.6
	DW	50.6	52.6	52.6	20.0	20.0	20.0
	PBS	50.2	56.2	56.2	44.7	44.7	44.7
	SW	48.8	51.8	51.8	42.4	42.6	42.6
	SWT	51.4	52.8	52.8	63.8	64.6	64.6
GV	TW	40.0	40.6	41.2	36.8	36.8	36.8
	DW	33.8	38.0	38.0	31.6	31.6	31.6
	PBS	33.0	34.4	34.4	33.0	33.0	33.0
	SW	38.8	41.4	42.4	38.8	38.8	38.8
	SWT	39.0	39.2	39.6	41.2	41.6	41.6
MG	TW	47.8	48.8	48.8	35.6	36.2	36.2
	DW	39.6	47.4	47.4	25.2	25.6	25.6
	PBS	44.0	47.6	47.6	39.6	40.0	40.0
	SW	42.0	44.6	46.2	33.0	33.2	33.2
	SWT	45.2	47.2	47.2	31.2	31.4	31.4
	x	48.3	49.5	49.6	39.3	39.5	39.5
	δ_{n-1}	11.1	10.8	10.7	9.5	9.5	9.5

M: culture media; DIL: diluents. MEA: Malt Extract Agar; S: Sabouraud Dextrose Agar; A: MEA + 25 ppm of Auramine; D: MEA + 2 ppm of Dichloran; RB: MEA + 50 ppm of Rose Bengal; GV: MEA + 5 ppm of Gentian Violet; MG: MEA + 1 ppm of Malachite Green. TW: Tryptone water; DW: Distilled water; PBS: Phosphate buffered saline; SW: Saline water; SWT: Saline water + 0.05% Tween 80.

TABLE 2
SUMMARY OF p VALUES FROM THE ANALYSIS OF VARIANCE OF TOTAL COUNT RESULTS

Main effects			Interactions			
Technique	(T)	**	T-M	**	T-M-D	**
Medium	(M)	**	T-D	**	T-M-t	n.s.
Diluent	(D)	**	T-t	n.s.	T-D-t	n.s.
Time	(t)	n.s.	M-D	**	M-D-t	n.s.
			M-t	n.s.		
			D-t	n.s.		

**: $p < 0.01$; n.s.: not significant.

a) *Surface-spread method:* Spread plates were prepared by spreading 0.1 ml aliquots of each dilution tested on surface-dried agar plates using a sterile, bent, glass rod. Disposable 9.0 cm Petri dishes containing 20 ml of media were used.
b) *Pour plate method:* Pour plates were prepared by delivering 1 ml aliquots of each dilution level tested into disposable 9.0 cm Petri dishes, adding 20 ml of the molten medium at 45° C and mixing at a room temperature.

Plates were incubated at 28° C for seven days. Only plates with 10-100 colony forming units (CFU) were used for counting. Total CFU/ml was determined after 3, 5 and 7 days of incubation. In the media inoculated using the surface-spread method, number of CFU/ml for each fungal species inoculated (partial counts) were also recorded after 5 and 7 days of incubation.

Results

Table 1 shows the mean value of total mould counts after 3, 5 and 7 days of incubation using the pour plate and the surface-spread methods, the five diluents and the seven culture media studied.

Data were subjected to analysis of variance, studying the main effects (method, culture medium, diluent and incubation time) and their interactions (2-way and 3-way interactions). The p value for each test and significant results are noted in Table 2. As no differences were observed between incubation time, the other three variables were studied using the results of mould counts obtained after three days of incubation.

The effect of method on recovery of moulds was determined by Student's t test. Significant

TABLE 3
SUMMARY OF p VALUES FOR THE ANALYSIS OF VARIANCE OF TOTAL COUNT RESULTS OBTAINED WITH SURFACE-SPREAD TECHNIQUE AND THE POUR PLATE TECHNIQUE

Technique	Medium	Diluent	Medium-diluent
Surface-spread	**	n.s.	n.s.
Pour-plate	**	**	**

**: $p < 0.01$; n.s.: not significant.

TABLE 4
EFFECT OF CULTURE MEDIUM ON RECOVERY OF MOULDS USING THE SURFACE SPREAD METHOD (NEWMAN-KEULS TEST)

x		GV	MG	S	D	RB	MEA	A
36.92	GV							
43.72	MG	*						
46.91	S	*						
50.45	D	*	*					
51.56	RB	*	*					
51.83	MEA	*	*					
56.86	A	*	*	*				

x: mean counts in CFU/ml (x 10^{-4}). *: $p < 0.05$.

differences were recorded ($p < 0.01$) due to the fact that the surface inoculated plates yield higher counts (x = 48.3) than the pour plates (x = 39.3).

As shown in Table 3, using the surface-spread technique, no statistical differences were recorded neither between diluents nor in the interaction medium-diluent. The significant differences observed between media ($p < 0.01$) were analysed by the Newman-Keuls test (Table 4).

Using the pour plate technique, significant differences ($p < 0.01$) were observed between media and diluents. It was not possible to determine which was the best medium or diluent due to a significant interaction ($p < 0.01$) between medium and diluent (Table 3). Taking in account the possible combinations medium-diluent, the Newman-Keuls test showed that RB with SWT achieved significantly higher counts (x = 63.8). Colony counts obtained with A medium and SWT were also high (x = 53.4), but no significantly different to those obtained with the combinations D-SWT, A-DW, MEA-SWT, A-SW, RB-PBS, RB-TW, S-PBS and RB-SW.

The partial counts for each inoculated species using the surface spread method are shown in Table 5. No differences were observed between results obtained after five and seven days of incubation, so the effect of culture medium and diluent were studied using results obtained after five days of incubation. The p value and significant results obtained by the analysis of variance are noted in Table 6. The significant differences were then analysed by the Newman-Keuls test except when there was not homogeneity of variance (*P. verrucosum* var. *cyclopium* and *A. alternata*). Results are shown in Table 7.

Discussion

Some Microbiological Manuals (12, 13, 15, 16) and several authors (10, 17) recommend five days minimum for the mould count. Zipkes *et al.* (17) reported that mould counts were significantly higher after 5 days than those achieved after 3 days, but they did not find significant changes in yeast counts between 3 and 5 days incubation data. From the results obtained in the present study, it may be deduced that mould counts are not affected by the incubation time. Therefore, it is possible to obtain a result after three days of incubation, according to the recommendation made by Janes and Tilbury (6).

The plating method may be also effect to the viable mould count. The pour-plate method is the technique recommended in the Microbiological Standard Methods for enumeration of fungi (12, 13, 15, 16). Zipkes *et al.* (17) made a comparison of yeast and mould counts by spiral, pour and

TABLE 5

PARTIAL COUNTS IN CFU/ml (x 10^{-4}) OF THE EACH INOCULATED STRAIN OBTAINED BY THE SURFACE-SPREAD METHOD USING DIFFERENT CULTURE MEDIA, DILUENTS AND INCUBATION TIME

M	DIL	Incubation time (days)							
		5	7	5	7	5	7	5	7
	TW	14.2	14.2	6.5	6.5	21.5	21.5	3.7	3.7
	DW	17.2	17.2	8.2	8.2	18.6	18.6	4.4	4.4
MEA	PBS	16.0	16.0	10.6	10.6	22.0	22.0	9.0	9.0
	SW	16.6	17.6	6.0	6.0	20.2	20.2	5.8	5.8
	SWT	18.0	18.0	10.8	10.8	20.2	20.2	7.4	7.4
	TW	14.2	14.2	8.6	8.6	13.0	13.0	2.2	2.2
	DW	13.6	13.6	10.6	10.6	15.6	15.6	3.2	3.2
S	PBS	19.8	19.8	9.0	9.0	14.0	14.0	5.4	5.4
	SW	27.4	27.4	9.2	9.2	14.4	14.4	7.2	7.2
	STW	14.5	14.7	8.0	8.0	20.7	20.7	7.2	7.2
	TW	18.8	18.8	7.8	7.8	25.6	25.6	6.6	6.6
	DW	21.4	21.4	8.0	8.0	20.6	20.6	5.0	5.0
A	PBS	17.8	17.8	8.4	8.4	23.4	23.4	6.0	6.0
	SW	16.0	16.0	6.8	6.8	22.2	22.2	9.0	9.0
	STW	19.4	19.4	9.4	9.4	27.4	27.4	6.6	6.6
	TW	18.8	18.8	4.6	4.6	18.6	18.6	4.4	4.4
	DW	18.4	18.4	13.8	13.8	13.8	13.8	5.0	5.0
D	PBS	15.8	15.8	6.8	6.8	19.2	19.2	6.0	6.0
	SW	17.6	17.6	7.4	7.4	23.2	23.2	7.4	7.4
	STW	19.4	19.4	6.6	6.6	20.0	20.0	7.6	7.6
	TW	21.8	21.8	7.0	7.0	23.2	23.2	4.2	4.2
	DW	23.0	23.0	7.4	7.4	18.6	18.6	4.2	4.2
RB	PBS	24.0	24.0	6.0	6.0	20.4	20.4	5.8	5.8
	SW	20.0	20.0	6.2	6.2	23.0	23.0	5.0	5.0
	STW	15.0	15.0	9.2	9.2	21.0	21.0	6.4	6.4
	TW	0.0	0.0	5.8	5.8	29.6	29.6	5.2	5.8
	DW	0.0	0.0	8.2	8.2	25.0	25.0	4.8	4.8
GV	PBS	0.0	0.0	2.4	2.4	25.4	25.4	6.6	6.6
	SW	0.0	0.0	3.6	3.6	27.8	27.8	10.0	10.4
	STW	0.0	0.0	4.8	4.8	28.6	28.6	5.8	6.0
	TW	11.2	11.2	9.4	9.4	26.2	26.2	2.0	2.0
	DW	7.6	7.6	11.6	11.6	22.4	22.4	2.4	2.4
MG	PBS	15.4	15.4	4.4	4.4	21.8	21.8	6.0	6.0
	SW	8.4	8.4	4.6	4.6	28.8	28.8	2.8	4.4
	STW	12.8	12.8	7.6	7.6	24.4	24.4	3.8	3.8

M: culture media; DIL: diluents. MEA: Malt Extract Agar; S: Sabouraud Dextrose Agar; A: MEA + 25 ppm of Auramine; D: MEA + 2 ppm of Dichloran; RB: MEA + 50 ppm of Rose Bengal; GV: MEA + 5 ppm of Gentian Violet; MG: MEA + 1 ppm of Malachite Green. TW: Tryptone water; DW: Distilled water; PBS: Phosphate buffered saline; SW: Saline water; SWT: Saline water + 0.05% Tween 80.

TABLE 6
ANALYSIS OF VARIANCE OF PARTIAL COUNTS RESULTS

	Medium	Diluent	Medium-diluent
P. verrucosum	**	n.s.	n.s.
A. parasiticus	**	**	n.s.
F. oxysporum	**	n.s.	n.s.
A. alternata	n.s.	*	n.s.

**: $p < 0.01$; *: $p < 0.05$; n.s.: not significant.

TABLE 7
EFFECT OF CULTURE MEDIUM ON RECOVERY OF *A. PARASITICUS* AND *F. OXYSPORUM*, AND EFFECT OF DILUENT ON RECOVERY OF *A. PARASITICUS* (NEWMAN-KEULS TEST)

A. parasiticus

x		GV	RB	MG	D	A	ME	S
4.96	GV							
7.16	RB							
7.76	MG	*						
7.95	D	*						
8.08	A	*						
8.50	ME	*						
9.12	S	*						

F. oxysporum

x		S	D	ME	RB	A	MG	GV
15.46	S							
18.95	D	*						
20.87	ME	*						
21.28	RB	*	*					
23.84	A	*	*					
25.32	MG	*	*	*	*			
27.28	GV	*	*	*	*			

A. parasiticus

x		SW	PBS	TW	SWT	DW
6.25	SW					
6.80	PBS					
7.11	TW					
8.24	SWT					
9.77	DW	*	*	*		

x: Mean partial counts in CFU/ml (x 10^{-4}). *: $p < 0.05$.

spread plate methods, obtaining higher recoveries by the spiral and spread methods. Pitt and Hocking (14) reported that spread plating is generally considered to be a more suitable technique for dilution plating than the pour plate method. Although other authors (8, 10) are in disagreement with this opinion. In our case, the spread plate technique gave significantly higher counts than the pour plate method. Although choice of method is related to a particular set of circumstances and may not exist a method which satisfies all the requirements, the pour plate method may present some disadvantages as the difficulty of isolation of colonies and their identification to at least the genus level. Moreover, the termal sensitivity of mould propagules can lead to a reduction in colony counts (3) and the volume of agar also can reduce pour plate propagule counts, presumably by reducing the oxygen tension (8).

The third factor analysed has been the nature of diluent. Although no differences have been obtained with the five diluents studied using the surface spread method, higher recoveries were obtained with SWT, followed by SW and PBS. Taking in account the effect due to diluent in the pour plate method, higher counts were obtained with SWT, PBS and SW. In the bibliographical review carried out no systematic investigation has been found of the effect of diluent on propagule counts, but in accordance with our results, some authors (8) mentioned that the presence of a wetting agent, such as Tween 80, can be beneficial to increase recoveries of moulds.

The last factor studied has been the culture media. Ideally, culture media for isolating and enumerating moulds should support recovery of all viable propagules. In addition, media should restrict the growth of spreading moulds, inhibit the development of bacterial colonies, and aid in identification of fungi to at least genus level. The effect of some substances such as acids, antibiotics, fungicides and dyes on the growth and counts of moulds have been studied by several authors (1, 2, 5, 7, 9, 11). In the present study, the suitability of two general purpose media as Malt Extract agar and Sabouraud Dextrose agar, as well as five media containing dyes were compared. The use of media containing Rose Bengal and Dichloran have been reported by many authors (1, 4, 5, 7). In preliminary studies (2), the effect of several dyes on colony diameter and enumeration of various fungi (Deuteromycetes and Zygomycetes) were investigated. From the results obtained we selected Auramine, Gentian Violet and Malachite Green because it was observed that Malt Extract agar containing 25 ppm of Auramine, 5 ppm of Gentian Violet and 1 ppm of Malachite Green respectively performed similarly as media containing 50 ppm of Rose Bengal (7) or 2 ppm of Dichloran (9). So, we decided to include these three new culture media in the comparative study reported here. Using the surface spread method, media containing 25 ppm of Auramine yield higher counts although they were no signifficant differences from those obtained using MEA, RB and D. Using the pour plate method, higher recoveries were obtained with A, RB and MEA media.

The colony-forming units of each inoculated species by the surface spread method were determined after 5 and 7 days of incubation and no significant differences were observed. Partial counts were not made after 3 days because growth and sporulation of fungal strains were very scarce, so it was necessary to increase the incubation time to minimum five days. This study was not performed using the pour plate method because it was very difficult to distinguish the different genera growing into the culture media. As shown in Table 5, significant differences were observed between culture media in partial counts of *P. verrucosum* var. *cyclopium*, *A. parasiticus* and *F. oxysporum*. There was no homogeneity of variance in counts of *P. verrucosum* var. *cyclopium*, but higher counts were obtained using RB, A, S, D and MEA without statistical differences between them. This species was inhibited in media containing Gentian Violet and the lowest recovery of *A. parasiticus* was obtained in this media (GV). In contrast, GV, MG and A media yield higher counts of *F. oxysporum*. The fact that Gentian Violet increased counts of this *Fusarium* species was observed in previous studies in our laboratory. Colony counts of *A. alternata* were not affected by the media used, but higher recovery was obtained with A, followed by GV, D, MEA, RB, S and MG, in decreasing order.

The effect of diluent was observed in partial counts of *A. parasiticus* and *A. alternata.* For the first strain, higher counts were recorded using DW and SWT. Although there was not homogeneity of variance in the case of *A. alternata,* higher recoveries were obtained in decreasing order with SW, SWT, PBS, DW and TW.

After consideration of all media developed for enumerating fungi, one has to conclude that one ideal medium does not exist because it is difficult that it contain all the attributes necessary to become the one general purpose medium for fungi. From the results obtained in the present work, it seems that medium containing Auramine is satisfactory for growth and enumeration of mould propagules because it allows equivalent or overall recovery of mould counts, especially those of slower growing fungi.

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References

1. Bothast, R. J. and Fennell, D. I. (1974). A medium for rapid identification and enumeration of *Aspergillus flavus* and related organisms. Mycologia **66,** 365-369.
2. Bragulat, M. R., Abarca, M. L., Bruguera, M. T. and Cabañes, F. J. (1991). Dyes as fungal inhibitors: Effect on colony diameter. Appl. Environ. Microbiol. **57,** 2777-2780.
3. Flanningan, B. (1973). An evaluation of dilution plate methods for enumerating fungi Lab. Pract. **22,** 530-531.
4. Henson, O. E. (1981). Dichloran as an inhibitor of mold spreading in fungal plating media: Effects on colony diameter and enumeration. Appl. Environ. Microbiol. **42,** 656-660.
5. Hocking, A. D. and Pitt, J. I. (1980). Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. Appl. Environ. Microbiol. **39,** 488-492.
6. Janes, L. and Tilbury, R. H. (1981). A comparison of media and methods for the enumeration of yeasts and moulds in refined sugar products. J. Appl. Bacteriol. **51,** p. XIV.
7. Jarvis, B. (1973). Comparison of an improved rose bengal-chlortetracycline agar with other media for the selective isolation and enumeration of moulds and yeasts in foods. J. Appl. Bacteriol. **36,** 723-727.
8. Jarvis, B., Seiler, D. A. L., Ould, A. J. L. and Williams, A. P. (1983). Observations on the enumeration of moulds in food and feedingstuffs. J. Appl. Bacteriol. **55,** 325-336.
9. King, A. D., Hocking, A. D. and Pitt, J. I. (1979). Dichloran-rose bengal medium for enumeration and isolation of molds from foods. Appl. Environ. Microbiol. **37,** 959-964.
10. Koburger, J. A. and Norden, A. R. (1975). Fungi in foods. VII. A comparison of the surface, pour plate, and most probable number methods for enumeration of yeasts and molds. J. Milk Food Techol. **38,** 745-746.
11. Mossel, D. A. A., Visser, M. and Mengerink, W. H. J. (1962). A comparison of media for the enumeration of moulds and yeasts in foods and beverages. Lab. Pract. **11,** 109-112.
12. Official Methods of Analysis (1984). 14th Ed., AOAC, sec. 46.011, p. 941. Arlington.
13. Pascual, M. R. (1982). Técnicas para el análisis microbiológico de alimentos y bebidas. Ministerio de Sanidad y Consumo. Centro Nacional de Alimentación y Nutrición. Servicio de Microbiología (CNAN). Madrid.
14. Pitt, J. I. and Hocking, A. D. (1985). Fungi and food spoilage. Academic Press. Sidney.
15. Saco, M., Díaz, M. A. and San Gabriel, A. (1986). Microbiología de materias primas para piensos compuestos y otros alimentos animales. Ministerio de Agricultura, Pesca y Alimentación. Madrid.
16. Standard Methods for the examination of Dairy Products (1985). 15th Ed. Richardson, G. H. Chap. 9.8, p. 197. Washington.
17. Zipkes, M. R., Gilchrist, J. E. and Peeler, J. T. (1981). Comparison of yeast and mold counts by spiral, pour, and streak plate methods. J. Assoc. Off. Anal. Chem. **64,** 1465-1469.

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Detección de *Bacillus larvae* en poblaciones mixtas de esporas bacterianas a partir de restos larvales

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Summary

An accurate laboratory technique for the detection of *Bacillus larvae* from larval remains of *Apis mellifera* with mixed bacterial spore populations was developed. The incorporation of nalidixic acid to the culture medium (3 µg/ml) was a satisfactory procedure for the separation of *Bacillus larvae* strains from *Bacillus alvei* motile colonies.

Key words: Bacillus larvae, Bacillus alvei, Melissococcus pluton, *American fouldbrood, European foulbrood,* Apis mellifera.

Resumen

Se ha desarrollado una técnica de laboratorio para identificar a *Bacillus larvae* en poblaciones mixtas de esporas bacterianas a partir de restos larvales de *Apis mellifera.* La adición de ácido nalidíxico al medio de cultivo (3 µg/ml) permitió el desarrollo de *Bacillus larvae* evitando la difusión de las colonias móviles de *Bacillus alvei.*

La loque americana es la enfermedad más grave de origen bacteriano que afecta a las larvas de las abejas (*Apis mellifera* L.). Desde su detección en la Argentina (1, 9) comenzaron a manifestarse casos de patología confusa que enmascaraban la sintomatología clínica de la enfermedad (2). Dichos síntomas aparecían en los marcos de cría, dando a la misma un aspecto salteado, con opérculos hundidos y perforaciones irregulares. Los restos larvales, de consistencia entre acuosa y viscosa, formaban hilos de no más de 2,5 cm de largo al introducir una aguja a través del opérculo, a diferencia de la masa viscosa que se estiraba en forma elástica en los casos de loque americana «típica». Las escamas, de color castaño oscuro, adheridas a la cara inferior de las celdas, resultaban propias de loque americana.

Al efectuar la prueba de la gota pendiente modificada (1, 11) sobre restos larvales y escamas se observaron esporas con movimiento browniano pertenecientes a *Bacillus larvae* White, agente causal de la loque americana, y en la misma muestra, esporas de *Bacillus alvei* Chesire and Cheine, microorganismo asociado con la loque europea. Ocasionalmente aparecieron las formas vegetativas de *Melissococcus pluton* (White) Bailey, agente causal de la loque europea y esporas de otras

especies de *Bacillus*. El diagnóstico correcto de loque americana y europea es muy importante dado que ambas enfermedades se controlan de distinta forma, desde el empleo de diferentes antibióticos a distintas dosis hasta la quema total de las colmenas afectadas o irradiación con rayos gamma del material contaminado.

El propósito de este estudio fue desarrollar una técnica de laboratorio eficaz para el aislamiento y diferenciación de *B. larvae* a partir de muestras con poblaciones mixtas de esporas bacterianas.

Los aislamientos se efectuaron a partir de restos de larvas que contenían esporas de *B. larvae* asociadas con otras especies de *Bacillus*, en su mayoría *B. alvei*. Cada muestra se suspendió en 5 ml de agua destilada estéril y después de permanecer 15 minutos a temperatura ambiente se sometieron a alguno de los siguientes tratamientos:

a) *Calor a baño maría:* Los tubos se calentaron a 85-90° C en baño maría durante 30 minutos y un inóculo, con asa de platino, de la suspensión se sembró en medio J sólido (6) o en agar cerebro corazón enriquecido con vitamina B_1 (CCB_1) (11).
b) *Proceso de calor-incubación en caldo nutritivo (CN):* Se empleó una modificación de la técnica de Rose (10), calentando las muestras en baño maría a 70° C durante 15 minutos para inactivar las formas no esporuladas. Los restos de larvas se tomaron de forma aséptica y las esporas se concentraron por centrifugación a 3.000 rpm durante 30 minutos descartando el sobrenadante. A cada tubo se le añadieron 5 ml de CN estéril y se incubaron durante 12 horas a 37° C; posteriormente se calentaron en baño maría a 70° C durante 15 minutos. Este proceso de calor-incubación en CN se repitió 2 veces más a intervalos de 12 horas; después se calentaron a 70° C durante 15 minutos y se centrifugaron a 3.000 rpm durante 30 minutos eliminándose el sobrenadante. El precipitado se resuspendió en agua destilada estéril y se sembró en medio J semisólido (6) o en medio de germinación de Bailey y Lee (4). Se incubaron a 37° C y el cultivo se transfirió a medio sólido (J o CCB_1).
c) *Adición de ácido nalidíxico:* Las muestras se calentaron en baño maría a 70° C durante 10 minutos para destruir las formas no esporuladas y activar la germinación de las esporas de *B. larvae*. Posteriormente se sembraron en estrías sobre medio J o CCB_1 adicionado de una solución estéril de ácido nalidíxico (concentración final de 3 µg/ml) (Hornitzky M. A. Z., 1990, *comunicación personal*) y se mantuvieron en una atmósfera de 10% de CO_2 en aire.

En todos casos, las siembras se incubaron a 36-37° C hasta la aparición del desarrollo bacteriano.

Los cultivos de *B. larvae* se identificaron mediante: reacción de Gram, producción de catalasa; supervivencia y crecimiento en CN, reacción de Voges-Proskauer, hidrólisis del almidón, hidrólisis de gelatina, producción de ácido a partir de d-manitol y utilización de citrato de sodio (1, 6, 12).

Sólo el proceso de calor-incubación en CN (b) y la adición de 3 µg/ml de ácido nalidíxico al medio de cultivo (c) resultaron eficaces para la obtención de colonias puras de *B. larvae* evitando la difusión de las colonias móviles de *B. alvei* en la superficie del medio. Las esporas de *B. alvei* soportaron tratamientos térmicos superiores a 90° C durante 30 minutos.

Sobre un total de 144 muestras analizadas se identificó a *B. alvei* en el 93,75% de las mismas y a *B. larvae* junto con *B. alvei* en un 45,93%. Las cepas de *B. larvae* resultaron Gram (+), catalasa (–), V-P (–), no hidrolizaron el almidón ni utilizaron citrato de sodio, hidrolizaron la gelatina, no fueron capaces de sobrevivir a numerosas resiembras en CN y la producción de ácido a partir de manitol proporcionó resultados variables. Las cepas de *B. alvei* presentaron reacción de Gram (+) (–) o variable, catalasa (+), V-P (+), hidrolizaron el almidón y la gelatina, no utilizaron citrato de sodio, no acidificaron el manitol y sobrevivieron a más de 10 subcultivos en CN. La adición de

ácido nalidíxico al medio de cultivo permitió obtener colonias puras de *B. larvae* y al mismo tiempo inhibió el desarrollo de *B. alvei.* La técnica de calor-incubación en CN también resultó efectiva para separar ambas especies, pero presenta el inconveniente de una mayor demanda de tiempo. Si se emplea cualquier otro método de aislamiento, el correcto diagnóstico de loque americana se ve dificultado por la presencia de esporas de *B. alvei,* las cuales germinan más rápidamente originando colonias móviles que en 24-48 horas cubren toda la placa de cultivo e imposibilitan la visualización de las colonias de *B. larvae.*

Más del 90% de las muestras analizadas que presentaban sintomatología sospechosa (loque americana, loque europea o confusa) contenía esporas viables de *B. alvei,* microorganismo asociado a casos de loque europea (11), cuya presencia se considera evidencia de una infección previa de esta enfermedad (8). Las colmenas afectadas por loque europea son fácilmente invadidas por microorganismos secundarios como *B. alvei* y *B. laterosporus* (3) cuando estas larvas son operculadas, no forman pupas y *B. alvei* se multiplica en sus restos, los cuales se estiran en forma de hilos, confundiéndose con la sintomatología producida por loque americana (5, 8). La presencia de esporas de *B. alvei* junto con *B. larvae* en larvas muertas por loque americana estaría contradiciendo la creencia generalizada de que *B. larvae* sólo aparece en cultivo puro (3, 5). Los resultados aquí expuestos demostraron la existencia de poblaciones mixtas de esporas bacterianas de *B. larvae* y *B. alvei* en material viscoso y escamas procedentes de marcos de cría de panales afectados por loque americana. Casos similares se han observado en Australia (7, 8). La incidencia tan alta de *B. alvei* en las muestras analizadas (93,75%) indicaría que esta especie saprofita sería un colonizador común del apiario y se multiplicaría y esporularía sobre larvas muertas por loque europea, loque americana o sacbrood. La gran cantidad de esporas de *B. alvei* estaría en relación directa con casos de loque europea endémica originando cuadros de sintomatología confusa agravados por el alto grado de resistencia de esta bacteria a la oxitetraciclina, antibiótico ampliamente difundido en la Argentina para el control de ambas loques.

La existencia de estos cuadros clínicos atípicos exige una técnica de laboratorio que permita detectar con certeza la presencia de *B. larvae* en los mismos. La incorporación de ácido nalidíxico al medio de cultivo y la técnica de calor-incubación en CN resultaron satisfactorias para alcanzar este objetivo

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Bibliografía

1. Alippi, A. M. (1990). Técnicas de laboratorio para el aislamiento e identificación de *Bacillus larvae* White, agente causal de la loque americana. Serie Difusión CIC **2** (6), 1-19.
2. Alippi, A. M. y Núñez, L. (1991). La loque americana en Argentina. Vida Apícola **49,** 20-24.
3. Bailey, L. (1984). Patología de las abejas. pp. 28-43. Ed. Acribia, Zaragoza.
4. Bailey, L. and Lee, D. C. (1962). *Bacillus larvae:* Its cultivation «in vitro» and its growth «in vivo». J. Gen. Microbiol. **29,** 711-717.
5. Gochnauer, T. A., Furgala, B. y Shimanuki, H. (1975). Enfermedades y enemigos de la abeja melífera. *En:* Dadant e hijos (eds.). La colmena y la abeja melífera. pp. 791-848. Hemisferio Sur. Uruguay.
6. Gordon, R, Haynes, W. C. and Pang, C. H-N. (1973). The genus *Bacillus.* Agriculture Handbook No. **427.** Agricultural Research Service, USDA, Washington DC, USA.
7. Hornitzky, M. A. Z. and Karlovskis, S. (1989). A culture technique for the detection of *Bacillus larvae* in honey bees. J. Apic Res. **28,** 118-120.

8. Hornitzky, M. A. Z. and Wilson, S. C. (1989). A system for the diagnosis of the major bacterial brood diseases of honeybees. J. Apic. Res. **28,** 191-195.
9. Núñez L. y Alippi, A. M. (1990). Diagnóstico, caracterización y control de *Bacillus larvae* White. Gaceta del Colmenar **576,** 13-15.
10. Rose, R. I. (1969). *Bacillus larvae* isolation, culturing and vegetative thermal death point. *J. Inverter. Pathol.* **14,** 411-414.
11. Shimanuki, H. and Cantwell, G. E. (1978). Handbook for the diagnosis of honey bee diseases, parasites and pests. Plant Protection Institute. Agricultural Research Service. Beltsville, Maryland.
12. Sneath, P. H. A. (1984). Section 13: Endospore-forming Gram-positive rods and cocci. *In:* Krieg, N. R. and Holt, J. G. (eds.). Bergey's Manual of Systematic Bacteriology. Vol. **2,** pp. 1104-1125. The Williams and Wilkins Co. Baltimore.