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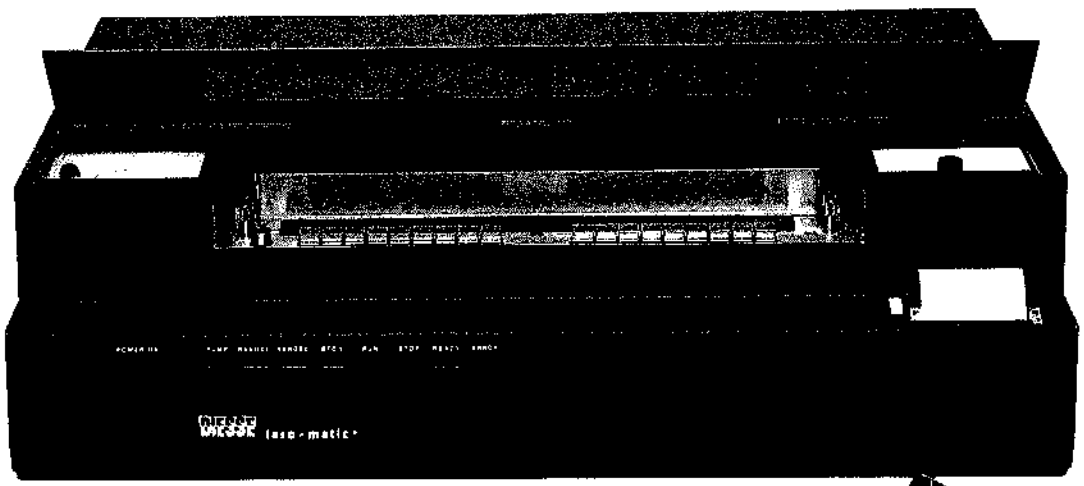

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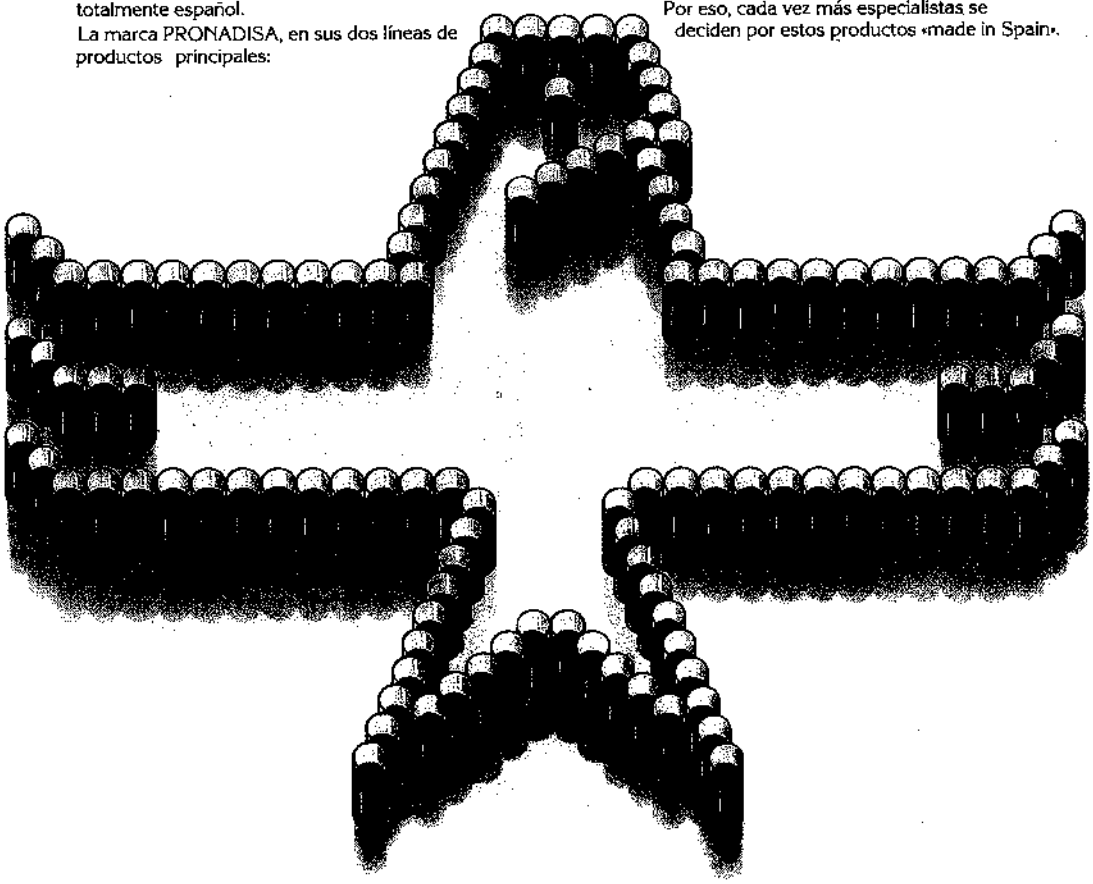

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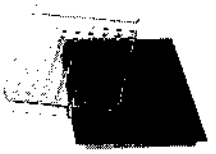

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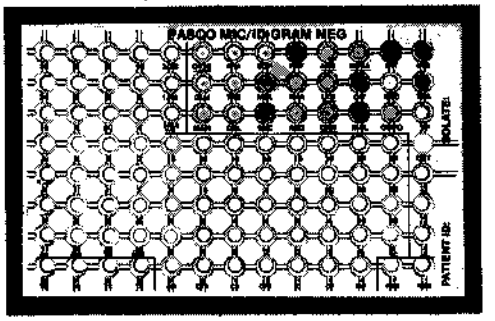


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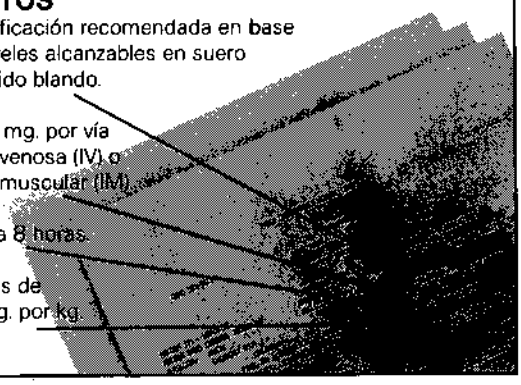

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MICROBIOLOGIA SEM 3 (1987), 149 - 162 **MINIREVIEW**

# Molecular and ecological aspects of antibiotic resistance in the *Bacteroides fragilis* group.

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*(Received August 25, 1987)*

**Summary**

Current problems of antibiotic resistance in the *Bacteroides fragilis* group are reviewed. The original susceptibility (before 1976) of this group of strict anaerobic microorganisms to some beta-lactams, tetracyclines and lincosamides is presently severely damaged, and new mechanisms of resistance, such the enzymatic inactivation of chloramphenicol, seem to arise. Conjugative transfer of chromosomal resistance genes appears to be the main strategy for the spread of resistance and in various cases plasmids are involved. The origin of such resistance genes remains obscure. There is a possibility of gene exchange between *Bacteroides* and *Enterobacteriaceae*, but there are problems of plasmid maintenance and/or gene expression. In some cases a striking homology of the *Bacteroides* resistance determinants with those of Gram-positive organisms can be documented.

*Key words:* Bacteroides fragilis, *antibiotic resistance.*

**Resumen**

En este trabajo se revisan los problemas actuales de la resistencia a antibióticos en *Bacteroides* del grupo *fragilis*. Antes de 1976, la mayor parte de estos microorganismos anaerobios mostraban una alta sensibilidad a varios beta-lactámicos, así como a tetraciclinas y lincosamidas. Actualmente los niveles de resistencia a estos antibióticos son alarmantes, y parecen surgir otros tipos de resistencia, como la inactivación enzimática del cloranfenicol. La diseminación de esta resistencia se hace sobre todo por conjugación de genes cromosómicos, aunque en algunos casos hay plásmidos implicados. El origen de estas resistencias es oscuro. Aunque hay posibilidad de intercambio génico entre *Bacteroides* y *Enterobacteriaceae*, existen problemas de mantenimiento de plásmidos y/o expresión. Curiosamente se observa homología entre genes de resistencia de *Bacteroides* y de Gram-positivos.

(*) Corresponding author.

## Introduction

A new insight into the physiological, ecological an clinical importance of the anaerobic bacterial microflora associated to man and animals has been possible by the recent advancement of the techniques for isolation and identification of such group of organisms (34). Strict anaerobic bacteria are by far our most abundant colonizers, and particularly those belonging to the family *Bacteroidaceae* (66). *Bacteroides fragilis* is indeed the most isolated anaerobic organism in clinical infections. In fact, among the 13 bacterial genera included in the family *Bacteroidaceae* according to Bergey's Manual (35), only *Bacteroides*, *Fusobacterium* and *Wolinella* are encountered with a significant frequency in pathological samples (75). The other genera, considered as normal colonizers of the mucosal epithelium of the digestive or genital tract, seem to be only rarely responsible for clinical infections, although we have isolated some of them as causative agents of severe bacteremia in immunosuppressed patients (60).

The first step in *Bacteroides* identification is to distinguish between bile-susceptible and bile-resistant species. Obviously this classification differentiates two ecologically distinct groups: those belonging to the normal intestinal microflora from those associated to other mucosal surfaces (66). Interestingly, extraintestinal *Bacteroides*, with the exception of *B. gracilis*, seem to present a much higher susceptibility to antibiotics, although a certain increase in beta-lactam resistance has been recently noted (87).

Among the intestinal group of *Bacteroides*, the asaccharolytic species *B. splanchnicus* is infrequently involved in clinical pathology (75), but the group of saccharolytic species (the *B. fragilis* group, BFG) constitute the most prevalent anaerobes found in human infections. The BFG was considered until 1976 as being composed of several subspecies of a single species, *B. fragilis* (35). Further DNA and rRNA homology studies have revealed that this saccharolytic, bile-resistant group of organisms is in fact composed of seven well-separated species (*B. fragilis, B. distasonis, B. vulgatus, B. ovatus, B. thetaiotaomicron, B. uniformis* and *B. eggerthii* –the last relatively asaccharolytic-) as well as some other homology groups recently denominated with the suggestive names of *B. caccae, B. merdae* and *B. stercoris* (Table 1) (38) (39).

Until recently, antibiotic susceptibility studies on anaerobic bacteria were rarely performed in clinical microbiology because of the apparent constant uniformity of results. Nevertheless, during the last ten years, the appearance of unexpected antibiotic resistance patterns, particularly among the members of the *B. fragilis* group, has forced the performance of susceptibility studies in each relevant isolate(75). Moreover, a continuous surveillance of the trends of antibiotic resistance is urgently needed in each hospital to assure good results in blind therapy of anaerobic infections when individual data of suceptibility are not immediately available.

TABLE 1
BACTERIAL SPECIES OF THE *BACTEROIDES FRAGILIS* GROUP

| | |
|---|---|
| *Bacteroides caccae* | *Bacteroides ovatus* |
| *Bacteroides distasonis* | *Bacteroides stercoris* |
| *Bacteroides eggerthii* | *Bacteroides thetaiotaomicron* |
| *Bacteroides fragilis* | *Bacteroides uniformis* |
| *Bacteroides merdae* | *Bacteroides vulgatus* |

*Natural insusceptibility of BFG to antimicrobial agents*

*Aminoglycosides.*–Most anaerobic bacteria are unsusceptible to aminoglycosides, just like facultative organisms when they are forced to grow under strict anaerobic conditions (except in some cases where an alternative electron acceptor, like nitrate, is present in the medium). As aminoglycosides penetrate into the cell through an energy-dependant system, it has been suggested that the drug entry is dependant on the electron-transport chain using oxygen –or nitrates– as the final acceptor (11). In fact *B. fragilis*, like *Clostridium perfringens*, does not transport either streptomycin or gentamycin inside the cell. That may explain the natural unsusceptibility to these agents, as isolated ribosomes of *B. fragilis*, in the presence of aminoglycosides, failed to incorporate aminoacids; moreover, cellular extracts of *B. fragilis* do not inactivate aminoglycosides (11). Paradoxically, *B. fragilis* may be susceptible, for unknown reasons, to spectinomycin and its derivative trospectinomycin (6'-propylspectinomycin sulfate) under special conditions(52).

*Antifolate agents.*–*B. fragilis* is naturally unsusceptible to trimethoprim because its dihydrofolate reductase cannot be inhibited by this drug (83). Sulfonamides are practically non-acting on normal or heavy *B. fragilis* inocula, and they are not recommended for treatment; nevertheless, minute incoula may present a certain susceptibility (37).

*Glycopeptide antibiotics*, like vancomycin or teicoplanin are non active against *Bacteroides*, as in the case of other Gram-negative organisms (20) *Fosfomycin* has no activity on BFG, probably because of the lack of an effective mechanism of transport (27). *Nalidixic acid* has no effect on *B. fragilis*, although other *4-quinolones* like ciprofloxacin or difloxacin may have some activity at intermediate level (4) (26).

*Rifamycins* (20) (41) and *fusidic acid* (74) present a good *in vitro* activity on BFG, although the *in vivo* effect has never been demonstrated. The easy appearance of resistant mutants to these agents, which represent a positive advantage for *in vitro* selection of selective markers in genetical work (19) (45), may also occur under *in vivo* conditions, particularly in long-term treatments.

*Tetracyclines*

Until the mid of the 60's, tetracyclines were considered the first choice antibiotics for blind treatment of *B. fragilis* infections (25). Nowadays the prevalence of resistant strains is very high, 79 % of the strains are tetracycline resistant in our Hospital, and figures around 80-90 % have been published elsewhere (Table 2) (17) (76) (54). This rapidly-growing curve of tetracycline resistance in *Bacteroides* is an excellent example of the high efficiency of acquisition of resistance to antibiotics in some bacterial organisms.

Like in *E. coli*, there is a biphasic tetracycline transport in *B. fragilis* (23). Nevertheless in BFG the fast initial energy-independent phase seems to be present in both susceptible and resistant strains. Only the second phase (active transport) appears to be negatively controlled by the tetracycline resistance genes. The acquisition of tetracycline resistance has been associated to the appearance of a new 35 kD membrane protein probably related with the antibiotic transport (54). The original MIC values of resistant strains can be enhanced by a factor of 10 by a previous culture on subinhibitory concentrations of tetracycline. This observation leads to the conclusion that tetracycline resistance is inducible in *Bacteroides*, as it is in most facultative pathogens. From these inducible strains

TABLE 2
ANTIBIOTIC RESISTANCE IN THE *BACTEROIDES FRAGILIS* GROUP (a)

| Antibacterial agent | Critical concentration mg/l | % of resistant strains |
|---|---|---|
| Benzylpenicillin | 10 | 91 - 100 |
| Ampicillin + Sulbactam or Clavulanic Acid | 16 | < 1 |
| Piperacillin or Carboxypenicillins | 128 | 5 - 15 |
| Cefoxitin | 32 | 2 - 25 |
| Cefoperazone | 32 | 32 - 49 |
| Cefotaxime | 32 | 27 - 50 |
| Latamoxef | 32 | 7 - 45 |
| Imipenem | 8 | < 1 |
| Clindamycin | 4 | 3 - 23 |
| Tetracyclines | 4 | 59 - 81 |
| Chloramphenicol | 16 | < 1 |
| Metronidazole | 16 | < 1 |

(a) compilation of (3) (56) (61) (75) (76) and (87)
(b) ampicillin concentration

constitutive mutants can be isolated, both in the laboratory or even in clinical settin (56) (57). In most cases, tetracycline resistance can be transferred by conjugation to susceptible strains of the BFG (57). This conjugative transfer is also inducible: incubation of donor strains in the presence of subinhibitory tetracycline concentrations enhances very significantly the conjugation rate. The presence of plasmids mediating such conjugative events has never been documented (43), although the acquisition of tetracycline resistance may be occassionally linked to the mobilization of non-conjugative plasmids (44). On many occasions, other antibiotic resistance markers are co-transferred together with tetracycline resistance. The non-plasmidic genetic elements mediating transfer may be similar to the conjugative transposons usually found in Gram-positive cocci (14). In that case, probably transfer genes and resistance genes should be associated in the same mobile genetic element being able to jump from a chromosome to another without intermediate replicative forms.

Both the induction of the expression and the induction of transfer have been documented under *in vivo* conditions, in the intestinal tract of gnotoxenic mice (56) or in animal experimental abscesses (12). Certainly those bacterial features, allied with the overuse of tetracyclines in humans and the heavy consumption of these drugs as feed additives (50), may explain the rapid rise of tetracycline resistant *Bacteroides* around the world.

*Beta-lactams*

A classic rule of the treatment of human anaerobic infections has been: "use penicillin if *B. fragilis* can be excluded". Organisms of the BFG are generally fully resistant or moderately susceptible to penicillins (76)(87). Only some carboxy- or ureido-penicillins,

like carbenicillin or piperacillin, have been used in the treatment of some clinical infections. All monobactams and most cephalosporins are also inactive on the BFG, with the exceptions of cefoxitin, cefotetan, cefmetazol and moxalactam, all of them supplied with a methoxy group in the position 7 of the bicyclic nucleus of the molecule. Anaerobic *Bacteroides* infections –and particularly mixed aerobic-anaerobic infections– have been succesfully treated with all these agents. The new class of beta-lactams, the carbapenems, and particularly imipenem, seem to be the most promising drugs of this family in the treatment of BFG infections(46) (Table 2).

Beta-lactam resistance in *Bacteroides* may be the consequence of several different mechanisms, sometimes collaborating in a single isolate. The most prevalent mechanism is the presence of a constitutive, generally membrane-bound beta-lactamase, which is detected in 75-100 % of the strains. This beta-lactamase has particularly cephalosporinase activity, being less active on penicillins. The amount of enzyme in a given isolate correlates rather well with the level of antibiotic resistance. Isoelectric focusing studies show that there are several different BFG beta-lactamases, but all of them run into the acidic part of the gel (IP 4.6 to 5.3) (47) (51). These enzymes are susceptible to the inhibition of clavulanic acid or sulbactam (3) (24), and combinations of ticarcillin + clavulanic acid may be of interest in the near future for treatment.

Apart from these ubiquitous cephalosporinases, some other beta-lactamases with different substrate profiles have been recently recognized. In 1982 a *B. fragilis* beta-lactamase was isolated with a strong penicillinase activity, presenting an OXA-like inactivation profile, and capable of inactivating huge amounts of carbenicillin, ticarcillin or piperacillin (68). In 1983 the first cefoxitin-hidrolysing strains of the BFG were detected (18) (88). In most cases cefoxitin-resistance is due to the presence of a broad-spectrum beta-lactamase, inactivating all beta-lactams, including imipenem (2) (16). In some other cases, cefoxitin resistance is related with the production of a substantial amount of a less active beta-lactamase (19). Therer is also the possibility of strains lacking those beta-lactamases but resistant to cefoxitin. In some of these strains resistance seems to be related with changes in PBP1 or PBP2 (equivalents to PBP1 and PBP3 in *E. coli*), linked to the loss of a 49-50 kD membrane protein (53).

The ability to produce beta-lactamase is generally transferable by conjugation from resistant to susceptible strains: that is the case for the ubiquitous cephalosporinase (44), for the penicillinase described in 1982 (68), and also for the cefoxitin-inactivating enzymes (19). Just as in the case of tetracycline, no plasmids seem to be involved in the conjugative process.

### *Chloramphenicol*

Chloramphenicol is the only antimicrobial agent used in clinical practice which is active without exception on all anaerobic bacteria (75). Because of this, chloramphenicol has been considered as the drug of choice for "blind treatments" of anaerobic infections, particularly if the central nervous system is affected. Although acquired resistance to this antibiotic remains very rare (76) (87) (Table 2), some failures during treatment of BFG infections have been detected (67). Unfortunately, the chloramphenicol susceptibility of the involved strains was not studied.

*Bacteroides*, like other microorganisms, possess nitroreductases which are able to inactivate chloramphenicol to a certain extent (40). Reduction of the nitro group of chloramphenicol has been in fact documented incubating the drug in the presence of heavy inocula of *B. fragilis* (48). With the normal cell density used in standard susceptibility testing procedures such isolates appears to be fully susceptible. The relationship of this nitroreductase-mediated mechanism of resistance with treatment failures remains to be explored.

The first documented case of acquired chloramphenicol resistance in *Bacteroides* was reported in 1978 from Australia (9). Two faecal strains of *B. fragilis* presenting a moderate level of resistance (MIC = 12.5 mg/l) were isolated, which showed a constitutive production of a chloramphenicol acetyltransferase (CAT) (69). The enzyme, which was only partially characterized, presented a 10 times lower activity than the CAT II enzyme of *E. coli*, although it was similarly susceptible to DTNB (5,5' dithiobis-2-nitrobenzoic acid, a specific thiol group inhibitor). The genetic bases of CAT production in *Bacteroides* remaine obscure as experiments of conjugation and transformation were inconclusive, and no plasmids were detected (8).

In 1985 the frist evidence in anaerobic Gram-negative organisms of the presence of a genetic element encoding CAT synthesis was documented in our laboratory in a *B. uniformis* isolate. The strain was obtained and identified by M. Reig from the culture of a pelvic abscess, and presented a chloramphenicol MIC of 128 mg/l. This phenotype was associated with the presence of a conjugative plasmid of 39,5 kb (pRYC3373), determining the constitutive production of a DTNB-susceptible CAT (45). AGain the enzyme appeared to be similar to the enterobacterial CAT II, but the specific activity of the enzyme in the Spanish *B. uniformis* isolate was about ten times higher than those of the Australian isolates, which may explain the higher level of antibiotic resistance.

The *cat* gene *(catF)* was localized in the restriction map of the plasmid pRYC3373 by hybridization using as a probe a synthetic oligonucleotide corresponding to the active site of the CAT enzyme, a highly conserved region in all previously sequenced CATs (6) (69). Different DNA fragments carrying the *cat* gene from *Bacteroides* were cloned into *E. coli*, which remained suceptible to chloramphenicol. Nevertheless, there was a partial expression of the *cat* gene in *E. coli*, as acetylated products of ($^{14}$C)-Chloramphenicol were detected in thin layer chromatography when bacterial extracts of *E. coli* were incubated with the antibiotic. A restriction target inside the *cat* gene was localized after an internal deletion suppressing the CAT phenotype. Subcloning from this restriction site in suitable vectors enabled the studies on the DNA gene sequence. Analysis of the *cat* sequence of the *Bacteroides* plasmid pRYC3373 showed a great homology with those obtained for other *cat* genes. Unexpectedly, the closest homology was found with the *cat* sequence of chloramphenicol-resistant Gram-positive organisms. *E. coli* transcriptional initiation consensus sequences (33) are present in the upstream non-coding sequence of *catF*. It was impossible to find in the 5' end of the structural gene possible binding sequences to the *E. coli* ribosomes. This fact may explain the low expression of the *Bacteroides cat* in *E. coli* as a result of an inefficient translational initiation (28) (Martínez-Suárez, J. V., *et al.* 1987, in preparation). Recent analysis in our laboratory of the Australian CAT-producing *Bacteroides* strain isolated in 1978 (kindly provided by M.Britz) showed the presence of several plasmids, one of them of about 40 kb, a size very similar to our plasmid pRYC3373. Using as a probe a small fragment of the Spanish plasmid, including the

DNA sequence corresponding to the CAT active site, the presence of homologous sequeces to our *cat* gene was detected in the Australian plasmid, now denominated pBF47A. No regions of homology with *cat* was found in the other plasmids of the 1978 isolate nor in the chromosome of the same strain (Martínez-Suárez, J. V. *et al.* 1987 in preparation). These results strongly suggest that the plasmids pRYC3373 and pBF47A, both determining CAT production, may share a similar or identical *cat* gene. The different levels of resistance that they produce in the host strains could be explained by minor differences in regulatory regions perhaps related with the different G + C % of *B. uniformis* (45-48 %) and *B. fragilis* (41-44 %).

TABLE 3
MECHANISMS OF ANTIBIOTIC RESISTANCE IN THE *BACTEROIDES FRAGILIS* GROUP [a]

| Antimicrobial agent | Mechanism of resistance | Expression [b] | Conjugative transfer | Genetic determination [f] |
|---|---|---|---|---|
| Tetracyclines | new membrane protein (impaired transport?) | I / C | + / – | chromosomal? |
| Benzylpenicillin, Cephaloridin [d] | ubiquitous cephalosporinase | C | + / – | chromosomal? |
| Carboxy, ureido-penicillins | OXA-type beta-lactamase | C | + | non studied |
| Cefoxitin and Imipenem | broad spectrum beta-lactamases, changes in PBP1 or PBP2 and loss of a membrane protein [e]; other mechanisms [f] | C | + / – | chromosomal? |
| Chloramphenicol | chloramphenicol acetyl-transferase | C | + / – | plasmidic |
| Clindamycin | rRNA methylation | I / C | + / – | chromosomal or plasmidic |
| Metronidazole | impaired transport; diminished nitroreductase activity | C | – | chromosomal? |

(a) references in the text (b) I: inducible; C: constitutive (c) chromosomal? when no plasmids have been found encoding for this character (d) and other beta-lactams susceptible to this cephalosporinase (e) only in the case of cefixitin (f) in cefoxitin resistant strains where no other mechanisms have been detected.

*Nitroimidazoles*

Nitroheterocyclic compounds are usually very active on anaerobic bacteria (10) (58). The introduction in medical practice of metronidazole, and subsequently, of other 5-nitroimidazoles, can be considered as one of the major recent advances in antianaerobic

specific therapy. These drugs act as strong oxidant agents. Their reduction to intermediate active molecules appears to be linked to the presence of functional electron-transport proteins, like ferredoxine, actin at the same Eh (42), which explains the selective action on anaerobes. Reduced metronidazole has a bactericidal effect on *B. fragilis*, probably inhibiting DNA replication (58) (71).

In most surveys of susceptibility of anaerobic bacteria (17) (76) (87) no metronidazole resistance has been detected (Table 2). In rare occassions the isolation of resistant *B. fragilis* strains has been reported after long nitroimidazole treatments (22) (36). The mechanism of resistance seems to be related with an impaired transport rate or a diminished reduction of the molecule to its active form (77). No plasmids or transfer of metronidazole resistance has been detected in these strains. As the same type of resistance is obtained by *in vitro* mutagenesis, it can be suggested that the natural resistant strains are the result of one or more chromosomal mutations.

*Macrolides and lincosamides*

Clindamycin is considered as one of the most effective antibiotics in the treatment of BFG infections. Because of the negligible rate of bacterial resistance to this drug, it constituted the treatment of choice for many years. Although in most cases clindamycin remains in a privileged position in the antianaerobic armamentary, resistance to this drug began to grow after 1976 (65). The proportion of resistant strains is considerably variable between different countries and hospitals, ranging from 3 to 23 % in the case of the BFG (Table 2), which makes a continuous surveillance of the rate of resistant strains at local level essential (61) (76) (87).

Clindamycin-resistant strains do not modify or inactivate the drug, and there is no difference in clindamycin transport in susceptible strains *versus* resistant ones (82). Although direct proof has never been presented, it is quite possible that clindamycin resistance may be due, like in Gram-positive organisms, to the methylation of adenine residues in the 23S rRNA, reducing the clindamycin affinity for its target in the 50S ribosomal unit. This statement has been recently supported by some observations on *Bacteroides* obtained in part in our laboratory (62). First of all, the presence of a cross-resistance phenotype including macrolides, lincosamides and streptogramins (MLS antibiotics) was documented. Moreover, in 1987 the first evidence of the inducibility of such a phenotype was obtained (62), suggesting –like in the case of Gram-positive organisms– the presence of a post-transcriptional mechanism of activation of the 23S rRNA methylase synthesis (21) (84). This mechanism would explain the selection of clindamycin resistant strains as a result of the overuse of macrolides or streptogramins, used not only in human and veterinary medicine, but also as animal feed additives (50). Finally, a plasmid gene *(ermF)* determining MLS constitutive resistance in *B. fragilis* has been recently sequenced (59); the study of the sequence revealed a great similarity with those obtained from the *erm* genes of Gram-positive organisms, coding for the synthesis of the 23S rRNA methylases.

Three different conjugative plasmids carrying constitutive resistance genes for MLS antibiotics have been described in BFG. The plasmid pIP410 (54) (55) –also known as pBF4 (86)–, was isolated in France from a *B. fragilis* strain; its size is 41 kb and contains

the sequenced *ermF* gene (59). The plasmids pCP1 (30), obtained in California from a *B. thetaiotaomicron* strain, and pBFTM10 (80) (81), isolated from *B. fragilis* in Boston, have only a 15 kb size (the smallest conjugative plasmids?), and are probably identical. Finally, the plasmid pBI136 (73), of 82 kb, was detected from a *B. ovatus* isolated in France. Although these three MLS-resistance plasmids present a different restriction pattern, all of them contain an identical homologous sequence, probably the MLS-resistance exhibiting a high homology with the *ermF* gene. Although there is no definite evidence of the existance of conjugative transposons in *Bacteorides*, these results strongly suggest that there is evidence of transposition events involving both the resistance determinant and the direct repeated sequences in *Bacteroides* or in *E. coli*, that has defined the transposons Tn*4351* and Tn*4400* respectively (64) (70) (72).

In most cases, constitutive resistance to MLS antibiotics cannot be associated with the presence of plasmids. Nevertheless, isolated MLS resistance remains transferable by conjugation and can be also mobilized during the transfer of tetracycline resistance determinants (43) (44) (45). The chromosome of these resistant strains harbour DNA sequences exhibiting a high homology with the *ermF* gene. Although there is no definite evidence of the existence of conjugative transposons in *Bacteroides*, these results strongly suggest that transposable elements involved in MLS resistance may reside either in plasmids or in the bacterial chromosome (13) (79).

### *On the origin and fate of resistance genes in* Bacteroides *of the* fragilis *group*

In the case of tetracycline, metronidazole or beta-lactam reistance there are no available data indicating the possible external origin of the resistance determinants, although the mechanisms involved are similar to those found in other microorganisms. Specific BFG enzymatic activities (nitroreductases, beta-lactamases) or structural proteins (membrane proteins involved in antibiotic transport) may have evolved independently in this group of anaerobes forced by antibiotic selection pressure. It must be remembered that the BFG beta-lactamases appear to be unique, and constitute a separate group in all current classifications.

On the other hand, the tetracycline determinant *tetM*, characteristic of the *Streptococcus* conjugative transposons, which has been also found in *Neisseria*, has never been detected in *Bacteroides* (63). Further molecular studies on such *Bacteroides* resistance determinants will be needed to understand their possible origin.

In the cases of chloramphenicol or MLS resistance the best current knowledge of the involved genetical elements makes it possible to postulate some hypothesis on its origin. In both types of resistance the G + C content of the corresponding structural genes, *ermF* (59) and *catF* is around 34 %, a quite different proportion than those characteristic of the *B. fragilis* (41-44%) or *B. uniformis* (45-48 %) genome. Moreover, if the aminoacid sequence (deduced from DNA nucleotide sequence) of the *ermF* or *catF* products is compared with those obtained from other *erm* or *cat* genes, a higher homology was unexpectedly found with that corresponding to Gram-positive organisms, such as *Bacillus* or *Staphylococcus* (59) (Martínez-Suárez, J. V. *et al*, 1987, in preparation), and a lesser homology with those coming from Gram-negative species. In the case of the gene *ermC* of *Staphylococcus aureus* (G + C % = 30-35 %), homology with the *ermF* sequence cover particularly the 5'

region of the *Bacteroides* gene. Nevertheless, the insertion sequences flanking *ermF* seem to be typical of *Bacteroides,* and have been found in several copies in the bacterial chromosome (59). Curiously, another DNA hybridization study provided no conclusive data on the relationship between the MLS resistance determinants from BFG and from Gram-positive organisms (15).

An exogenous resistance determinant present in a plasmid or, more likely, in a conjugative transposon may have been introduced in *Bacteroides* and inserted by non-homologous recombination either on one of the numerous BFG cryptic plasmids (7), or in the chromosome. In the latter case, the fate of the exogenous resistance determinant may have been: 1) the permanence in the bacterial chromosome, with different possible expression levels depending on the site of insertion; 2) the building-up of a transposable element by integration in the vicinity of an insertion sequence subsequently duplicated; 3) the insertion of such a transposable element into cryptic plasmids: this may have been the case of the MLS resistance plasmids pCP1 or pBI136, as in the original wild strains it was possible to isolate the corresponding cryptic plasmids devoid of the resistance determinant (30) (73) and 4) the production of a conjugative transposon, by trapping of conjugative transfer genes during duplication of *Bacteroides* insertion sequences flanking the resistance genes (59).

Notwithstanding, BFG organisms present some significant differences in the usual genetical strategy for antibiotic resistance in aerobic or facultative Gram-negative bacteria, such as the apparent predominance of conjugative chromosomal resistance genes or the special small-size transfer region in conjugative plasmids –in pBFTM10 all replicative and transfer functions seem to be located in a 5 kb fragment (78)–. The closer homology of BFG chloramphenicol and MLS resistance genes with those of Gram-positive organisms may suggest that *Bacteroides* could have received such genes, presumptively originated in Gram-positive soil bacteria (some of them antibiotic producers) (5), earlier than Gram-negative aerobic or facultative bacteria. The evolution and taxonomic position of the genus *Bacteroides* is far from clear. Recent taxonomic studies based on 16S-rRNA sequences (49) (85) pointed out a striking relationship between the strict anaerobic *Bacteroides* and some strict aerobic soil bacteria like *Cytophaga, Flavobacterium* or *Flexibacterium.* The possibility of gene transfer from Gram-positive organisms to this group of organisms remains to be explored.

Obviously the BFG organisms share with potentially pathogenic *Enterobacteriaceae* a common habitat in the intestinal ecosystem. Transfer of plasmids between *B. fragilis* and *E. coli* is possible, but plasmid maintenance is limited by the existing replicative barrier. Natural plasmids of each organism are not able to replicate into the other, although hybrid shuttle plasmids have been constructed (31) (78) which replicate in both types of hosts. The conjugative *Bacteroides* transposons may be transferred into *E. coli* and the corresponding genes are inserted at random in the chromosome (78). When these transposons are reintroduced back into *Bacteroides* they can mobilize *E. coli* plasmids and even insert some of them in the chromosome of the recipient cell (70). Thus, there is a possibility of exchange of genes between aerobic-facultative and anaerobic organisms. Nevertheless, the acquired genes are frequently poorly expressed. Chloramphenicol and MLS resistance genes originating in *Bacteroides* does not confer resistance when cloned into *E. coli* (31) (78), and the opposite is also true. It can be suspected if these difficulties of expression are consequences from an impaired initiation of translation (see above), or

problems related to codon usage: codons recognized by the minor tRNAs of *E. coli* are found throughout *ermF* and *catF* (Martínez-Suárez, J. V. *et al.*, 1987, in preparation) (29). In any case, some non-resistance *Bacteroides* genes have been successfully expressed into *E. coli* (1) (32). Antibiotics exert impressive selective forces. It has been speculated that these natural compounds mediating interbacterial interactions positively stimulate interbacterial gene transfer and genetic evolution. Resistance genes may serve as leaders of such phenomena, eventually mobilizing other bacterial genes. Due to high production and use of antibiotics in human industry a true antibiotic pollution has occurred possibly causing an accelleration in bacterial evolution. The rapid rise and establishment of antibiotic resistance genes in the bacterial world forces us to continually survey for the presence of new mechanisms of resistance, particularly for organisms dangerous for mankind. The importance of this biological phenomenon needs a much more basic and multidisciplinary research.

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# Resistance of vaccinia virus to interferons: Modulation of the 2-5A system in interferon-treated, vaccinia virus infected cells

by

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**Summary**

In this study we have examined if resistance of vaccinia virus to interferon (IFN) correlates with virus-induced alterations of the 2-5A system. We have shown that in various IFN-treated vaccinia virus infected cells of mouse, monkey and human origins, the intracellular levels of 2-5A are low early in infection but exhibit a sharp rise late in infection. In spite of the presence of 2-5A, activation of the 2-5A dependent RNase, as measured by the rRNA cleavage assay, does not occur or is delayed in the course of virus infection. Howewer, when cycloheximide, an inhibitor of protein synthesis is added at the time of virus infection, extensive cleavage or rRNA is observed in IFN-treated, infected cells. If cycloheximide is added at various times after virus infection, rRNA cleavage is gradually prevented and a virus-induced inhibitor of the 2-5A system can be detected between 1-2 hr post infection. A function encoded by a ts 22 mutant of vaccinia virus blocked rRNA cleavage. Restriction of rRNA cleavage during virus infection correlated with dephosphorylation of 2-5A.

Our findings suggest that modulation of the 2-5A system by vaccinia virus involves the production of an activator and simultaneous synthesis of an inhibitor(s). Viral ds-RNA is likely to be the activator while a function encoded by ts 22 mutant is involved in inhibition of the 2-5A system. Other viral functions (ATPase and phosphatase) may also be involved in modifications of the 2-5A system by regulating 2-5A levels and altering the integrity of 2-5A. Modifications of the 2-5A system, during vaccinia virus infection might contribute to the resistance of this cytoplasmic DNA virus to IFN.

*Key words: vaccinia virus, interferon, 2-5A system.*

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**Resumen**

En este trabajo se ha estudiado si la resistencia al interferón (IFN) del virus vaccinia está relacionada con alteraciones del mecanismo de la 2-5A sintetasa/endoribonucleasa. Se ha observado que en diferentes líneas celulares tratadas con IFN e infectadas con el virus vaccinia se producen unos bajos niveles intracelulares de 2-5A a tiempos tempranos de la infección, pero que posteriormente sufren un fuerte incremento. A pesar de esta presencia de 2-5A, no se produce la activación de la endoribonucleasa (medida por el ensayo de la degradación del RNA ribosómico), o en el mejor de los casos, esta activación aparece retrasada durante la infección viral. Sin embargo, en presencia de cicloheximida (un inhibidor de la síntesis de proteínas), se produce una fuerte degradación del RNA ribosómico. Esta degradación es gradualmente prevenida cuando los cultivos celulares infectados son tratados con cicloheximida a diferentes tiempos después de la infección y se detecta la síntesis de un inhibidor del mecanismo del 2-5A entre las 1-2 horas postinfección. Un mutante ts22 del virus vaccinia codifica por una función que es capaz de bloquear la degradación del RNA ribosómico. Además, la prevención de la degradación del RNA ribosómico durante la infección viral podría estar también relacionada con una defosforilación del 2-5A.

Nuestros resultados sugieren que el virus vaccinia es capaz de modular el mecanismo del 2-5A mediante la producción de un activador y la síntesis simultánea de uno o varios inhibidores. Es muy probable que el activador sea RNA viral bicatenario, mientras que la función codificada por el mutante ts22 estaría involucrada en la inhibición del mecanismo del 2-5A. Otras funciones virales como la ATPasa y una fosfatasa podrían también modificar el mecanismo del 2-5A, alterando los niveles y la integridad del 2-5A. Estas modificaciones del mecanismo del 2-5A durante el proceso de infección viral podrían contribuir a la resistencia observada del virus vaccinia al IFN.

**Introduction**

Vaccinia virus replication is relatively resistant to IFN in some cell lines (23, 28). Resistance of vaccinia virus replication to IFN, as measured with cell extracts early in infection, has been correlated with two vaccinia virus induced enzymes: ATP-ase, causing degradation of ATP and phosphatase which dephosphorylates 2-5A (24). In intact cells and at late times post infection 2-5A is produced in high levels, but failed to activate the 2-5A dependent RNase (8, 28). A variable and complex mixture of authentic 2-5A, nonphosphorylated core 2-5A and numerous compounds of unknown structure have been found in some IFN-treated, vaccinia virus-cell systems (29). The complexity of the pattern and the levels of 2-5A varied with culture conditions (29). Resistance of vaccinia virus replication to IFN has also been correlated with a virus mediated inactivation of the ds-RNA dependent protein kinase (23, 27, 34). A specific inhibitory factor that interacts with ds-RNA and that appears within 90 min post infection has been associated with inhibition of the ds-RNA dependent protein kinase (35). An exception to this general phenomenon of vaccinia virus resitance to IFN was found in mouse L cells grown in suspension (20). In these cells IFN causes a drastic inhibition of both viral and cellular

protein synthesis soon after virus infection, and this correlates with activation of the 2-5A dependent RNase (9, 10). Based on these findings we postulated that vaccinia virus escapes an early IFN-mediated blockade, because of differences in viral gene expression between cells; this leads to newly synthesized viral products that interfere at some step with activation of the 2-5A synthetase/RNase system (10, 23). In this report we have characterized further the relationship of the 2-5A system to the phenomenon of resistance of vaccinia virus replication to IFN.

## Materials and methods

### *Cells and viruses*

The mouse L cell lines [Mill Hill (MH) and Rutgers (R)] used in this investigation were the same as previously described (7, 20). BSC-40 cells are a continuous line of African green monkey kidney cells. WI-18 and HeLa S3 cells are continuous lines of human cells. The origin of these cell lines have been described previously (23). Cells (L-MH and HeLa S3) were grown in suspension cultures in Eagle's medium supplemented with 10 % newborn calf serum (NCS). Cells (L-MH, L-R, BSC-40 and WI-18) were grown in monolayer cultures in Dulbecco's Minimal Essential (DME) medium supplemented with 10 % NCS (L-MH, L-R and BSC-40) or 10 % fetal calf serum (FCS) (WI-18). The plaque purified WR strain of vaccinia virus was propagated by infecting HeLa S3 spinner cells at a multiplicity of 0.1 pfu/cell and purified according to the method of Joklik (14), as previously described (6). The particle to pfu ratio was about 30 when titrated in BSC-40 cells. Vaccinia virus ts-22 mutant was provided by Dr. R. Condit (Buffalo, N. Y.).

### *Interferons*

Virus-induced mouse fibroblastoid interferon [containing 15 % (α) and 85 % (β) species] was prepared and partially purified on an antibody affinity column at a specific activity of $4\text{-}6 \times 10^7$ u/mg. Virus-induced human (α) lymphoblastoid interferon (sp. act., $1 \times 10^6$ u/mg) was provided by Wellcome Research Laboratories. Titrations were carried out either in mouse L-929 or human WI-18 cells with VSV as a challenge virus against a mouse IFN standard (G 002-90511) and human leukocyte standard (G 023-901-527) from the Antiviral Substances Program, NIH, and are given in reference units.

### *Radiobinding assay for 2-5A*

This competitive protein binding assay is based on the specificity of binding between 2-5A and the 2-5A dependent endoribonuclease (17, 36). Cells ($20\text{-}30 \times 10^6$) grown in suspension and in monolayers were pretreated with IFN for 20 hr. Thereafter, cells in suspension were infected with vaccinia virus at 500 particles/cell while cells in monolayers (10 cm dish) were infected with 1000 particles/cell. Infection conditions in suspension and monolayer cultures were selected in order to obtain the same amount of cell-as-

sociated virus for all cultures. Labeled 3H-thymidine vaccinia virus was used to determine the amount of cell-associated virus (6). At the end of the adsorption period, 30 min for cells in suspension and 60 min for cells in monolayers, virus inoculum was removed and cells were supplemented with medium containing 2 % NCS or FCS depending on the cell line. The end of the adsorption period is referred to as zero time. At various times after infection, cells were washed 3x with phosphate buffered saline (PBS), resuspended in 0.5 ml of 10 % trichloroacetic acid (TCA) and incubated for 10 min at 4 °C. TCA-soluble extracts were obtained after 5 min of centrifugation in an Eppendorf microfuge. The supernatant was collected, extracted once with 0.5 ml mixture of Freon: tri-n-octylamine (3:1, vv), vortex, and spun for 1 min in microfuge. The upper phase was lyophilized, resuspended in half of the packed cell volume of buffer A (20 mM Tris-HCl, pH 7.6, 85 mM potassium chloride, 5 mM magnesium acetate, 1 mM ATP and 5 % glycerol) and radiobinding assays were carried out with this material (28). The ppp (A2'p)3A(32P)pCp (3000 Ci/mmol) was obtained from Amersham. Each assay (50 µl) contains 10 µl of TCA-soluble extract ($7 \times 10^6$ cell equivalents), 20 µl of rabbit reticulocyte lysate, 10 µl of [32P]2-5A at 4 nCi/10 µl in buffer A, and 10 µl of distilled water. A calibration curve was run in parallel with 2-5A tetramer 5'-triphosphate at various concentrations (0.1-5 nM). The mixtures were incubated at 4 °C for 90 min and the protein bound 2-5A was measured by filtration through nitrocellulose filters (Millipore, HAWP-024). Incubation mixtures were added to filters that were prewashed in buffer B (buffer A minus ATP), washed three times with one ml each of buffer B, then dried and radioactivity counted in toluene based scintillant.

*Covalent binding of 2-5A to the endoribonuclease*

The procedure developed for covalent linkage of labeled 2-5A to proteins in cell extracts was used (36). Labeled ppp(A2'p)3A(32P)pCp (3000 Ci/mmol, 100,000 cpm/assay) was preincubated at 4 °C for 60 min with 10 u/ml of bacterial alkaline phosphatase (BAP) from P-L Biochemicals, in 20 mM Hepes-KOH buffer pH 7.5. The phosphatase was inactivated with 2 mM EDTA and heating at 90 °C for 5 min. The oligonucleotide products were oxidized with 10 mM of freshly prepared sodium meta periodate and the solution incubated in the dark for 1 hr at room temperature. Ethylene glycol and glycerol were added to final concentrations of 60 mM and 5 % (v/v) respectively, to inactivate excess sodium metaperiodate and the solution incubated for a further 5 min. To the periodate-oxidized material (40 µl) was added 5 of $S_{10}$ cells extract (7.5 µg of protein) and 5 µl of 10x buffer A, followed by incubation at 4 °C for a further 2 hr. (and reduced with 10mM cyanoborohydride). The protein was precipated with acidified acetone (acetone/1 M HCl, 120:1), washed twice with acetone, dried, solubilized in sample buffer and electrophoresed on sodium dodecylsulphate/polyacrylamide gel (SDS-PAGE).

For the preparation of $S_{10}$, cells ($5 \times 10^6$ were washed three times with PBS and the packed cell pellet resuspended in 250 µl of buffer (20 mM Hepes pH 7.5, 120 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 10 % glycerol), containing 0.5 % NP-40. The cells were kept 10 min on ice and the cell homogenate was centrifuged at 10,000xg for 10 min and the supernatant cell extract removed ($S_{10}$).

*Cleavage of ribosomal RNA*

RNA was isolated from cell pellets ($50 \times 10^6$ cells). The isolation, denaturation, electrophoresis of RNA in 1.8 % agarose gels, and staining were carried out as described (9, 32).

*Stability and integrity of 2-5A in cell extracts*

HeLa $S_3$ cells were treated with 500 u/ml of human IFN for 18 hr, then infected with vaccinia virus (500 particles/cell), and at various times post infection $S_{10}$ cell extracts were prepared from $1 \times 10^7$ cells. Labeled 2-5A was synthesized from ($\alpha$-$^{32}$P)-ATP, with 2-5A synthetase enzyme fraction from IFN-treated, HeLa $S_3$ cells, that was bound to poly (I):(C) agarose and purified as previously described (23). Analysis of core 2-5A products by PEI was as follows. Phosphorylated 2-5A (20,000 cpm/20 µl reaction) in buffer B was incubated for 1 hr at 30 °C with extracts (12 µg of protein) from untreated and IFN-treated, infected cells. Reactions were centrifuged in Eppendorf for 5 min and 10 µl aliquots treated with 36 units of bacterial alkaline phosphatase for 1 hr at 37 °C. The enzyme was inactivated by heating at 90 °C for 5 min, proteins removed by centrifugation and 2 µl aliquots spotted on PEI-cellulose plates. Thin-layer chromatography (TLC) was in 1 M acetic acid and products visualized by autoradiography. Analysis of phosphorylated 2-5A products was as follows. Conditions for incubation of phosphorylated $\alpha$-$^{32}$P labeled 2-5A with extracts from uninfected and virus infected cells were as above, except that BAP treatment was omitted and 0.75 M potassium phosphate pH 3.4 was used as solvent.

TABLE 1
INTRACELLULAR LEVELS OF 2-5A IN UNTREATED AND IFN-TREATED VACCINIA VIRUS-INFECTED CELLS OF DIFFERENT ORIGINS

| | 2-5A (nM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Untreated | | | | IFN-treated | | | |
| Cells | L-R | BSC-40 | HeLa $S_3$ | WI-18 | L-R | BSC-40 | HeLa $S_3$ | WI-18 |
| Hr. pi. | | | | | | | | |
| 0 | < 1 | < 1 | < 1 | < 1 | < 1 | < 1 | < 1 | < 1 |
| 2 | < 1 | < 1 | 7.0 | < 1 | 2.0 | < 1 | 10 | < 1 |
| 4 | < 1 | < 1 | 8.5 | < 1 | 4.0 | < 1 | 11.2 | < 1 |
| 8 | 10.0 | 1.4 | 170.0 | 2.0 | 70.0 | 37.5 | 6225.0 | 2.5 |
| 16 | | | 30.0 | | | | 1225.0 | |
| 24 | 2.1 | 3.0 | 17.0 | 7.5 | 93.7 | 1500.0 | 1187.5 | 187.5 |

Cells ($1 \times 10^7$) of various origins growing in 10 cm dishes or in suspension were pretreated with IFN (500 u/ml) for 18 hr and infected with vaccinia virus. The growth conditions for each cell line were as described under Materials and Methods. TCA-soluble cell extracts were prepared at different times post infection and tested for levels of 2-5A by the radiobinding assay described under Materials and Methods.

## Results

*Kinetic of synthesis of 2-5A in untreated and IFN-treated, vaccinia virus infected cells of different origins.*

To examine if resistance of vaccinia virus to IFN correlates with virus-induced alterations of the 2-5A system, we first measured the intracellular levels, of 2-5A in the course of virus infection in cell lines of various origins where vaccinia virus replication is relatively resistant to IFN (23). Untreated and IFN-treated cells were infected with purified vaccinia virus and at various times post infection, TCA-soluble extracts were prepared and assayed for 2-5A (Table 1). In untreated, infected cells we found low levels of 2-5A at late times post infection (> 8 hr pi). These levels were higher in infected HeLa cells which contain elevated levels of 2-5A synthetase (32).

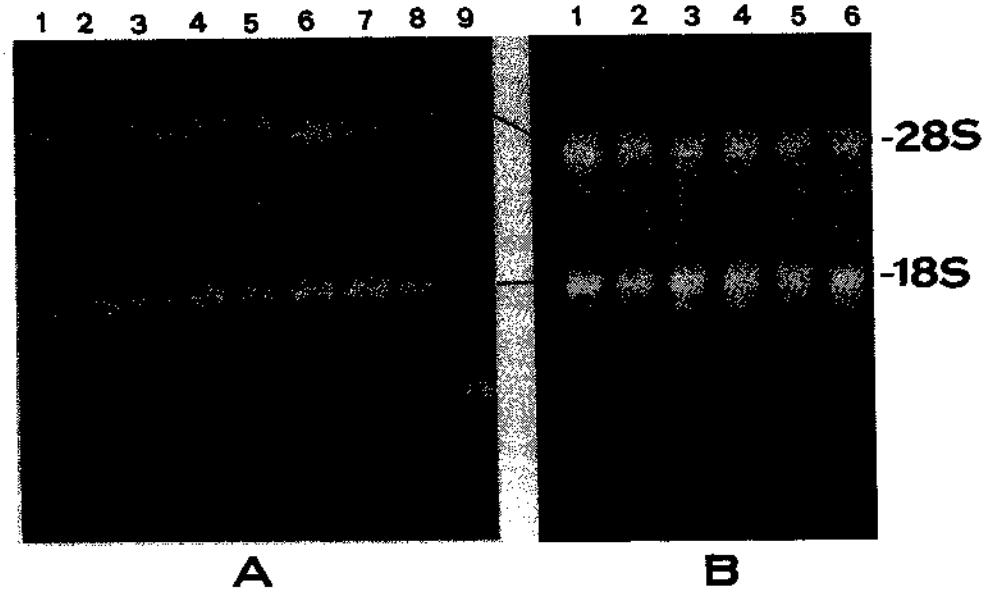


Fig. 1. Extent of rRNA cleavage in untreated and IFN-treated, vaccinia virus infected mouse cells. Treatment of the cells with IFN (500 u/ml), infection with vaccinia virus, isolation of RNAs, separation by agarose gel electrophoresis and staining with ethidium bromide were described under Materials and Methods. Panel A, represents RNAs from mouse L cells at 4 hr pi (Lanes 2-5) and 8 hr pi (lanes 6-9). Infected cells, untreated (lanes 2, 3, 6, 7), and IFN-treated (lanes 4, 5, 8, 9). Lanes 3, 5, 7, 9 represents RNAs from cells infected in the presence of 100 μg/ml of cycloheximide (CY). Panel B, represents RNAs from IFN-treated, infected mouse cells when CY was added at time zero (lane 2), 1 hr (lane 3), and 2 hr (lane 4); in lane 5, infected cells were not treated with CY, while in lane 6 infected cells were treated with 5 mM hydroxyurea. Lane 1, represents uninfected cells. RNAs were collected at 4 hr pi.

In IFN-treated, infected cells there is a slight increase in the production of 2-5A up to 4 hr pi, while at later times post infection (8 h pi), there is a marked increase in 2-5A for all of the cell lines. The extent of this increase differ among cell lines. By 24 hr pi, the levels of 2-5A declined in HeLa $S_3$ cells most likely as a result of virus-induced cell destruction. These findings established that 2-5A is produced in various amounts in vaccinia virus infected cells, that the levels of 2-5A are markedly enhanced by IFN, that high yields of 2-5A are found late in infection and that the levels of 2-5A differ between cell lines. These findings confirm and extend previous observations made by others (11, 28).

*2-5A activates the 2-5A dependent RNase in IFN-treated, vaccinia virus infected cells in the absence of protein synthesis.*

Because 2-5A is produced in various amounts in vaccinia virus, infected cells and because virus-induced proteins may influence the degree of activation of the 2-5A dependent RNase, we next examined the relationship between activation of the 2-5A dependent RNase during infection with virus induced proteins. Thus, we measured rRNA breakdown and levels of 2-5A in infected cells treated with an inhibitor of protein synthesis, such as cycloheximide (CY).

In Fig. 1 (A) CY was added to cells at the zero time of virus infection and an ethidium bromide-stained gel of RNAs from untreated and IFN-treated, infected L cells is shown.

TABLE 2
INTRACELLULAR LEVELS OF 2-5A IN UNTREATED AND IFN-TREATED VACCINI VIRUS-INFECTED CELLS IN THE ABSENCE OR PRESENCE OF CYCLOHEXIMIDE

| | 2-5A (nM) | | | |
|---|---|---|---|---|
| Cells | Untreated | | IFN-treated | |
| | (-) | (+) cycloheximide | (-) | (+)cycloheximide |
| L-MH | | | | |
| 4 hr pi | < 1 | < 1 | 2.5 | 4.2 |
| 8 hr pi | 1.1 | 2.5 | 20.0 | 35.0 |
| BSC-40 | | | | |
| 4 hr pi | 1.2 | 1.9 | 1.6 | 3.8 |
| 8 hr pi | 1.4 | 3.6 | 37.5 | 70.5 |
| HeLa $S_3$ | | | | |
| 4 hr pi | 5.6 | 9.4 | 11.8 | 22.5 |

Cells ($1 \times 10^7$) of various origins growing in 10 cm dishes or in suspension were pretreated with IFN (500 u/ml) for 18 hr and infected with vaccinia virus. The growth conditions for each cell line were as described under Materials and Methods. Cycloheximide (100 μg/ml) was added following virus infection. TCA-soluble extracts were prepared at different times post infection and tested for levels of 2-5A by the radiobinding assay.

Two time points are represented. In IFN-treated, infected L cells (Fig. 1A), only low levels of rRNA cleavage were observed by 4 hr pi (lane 4) with a slight increase at 8 hr pi (lane 8). Howewer, extensive cleavage of rRNA was observed at 4 hr pi when protein synthesis was blocked with cycloheximide (lane 5). In Fig. 1B CY was added to IFN-treated mouse L cells at 1 and 2 hr pi and rRNA cleavage was measured at 4 hr pi. The extent of rRNA cleavage that developed when CY was added at zero time of virus infection (lane 2) was partially prevented when CY was added at 1 hr (lane 3) and was completely blocked when CY was added at 2 hr (lane 4) or when CY was not added during infection (lane 5). Moreover, cleavage of rRNA was also prevented in the presence of an inhibitor of viral DNA synthesis (lane 6). Similar results to mouse L cells were obtained in BSC-40 and in Hela cells (not shown).

To determine if the rRNA cleavage seen with inhibition of protein synthesis correlated with 2-5A production we measured the intracellular levels of 2-5A. Table 2 shows that when protein synthesis was inhibited with cycloheximide, significant levels of 2-5A were observed during infection. In CY-treated cells we consistently found higher levels of 2-5A than in the absence of the drug. The results of Fig. 1 and Tables 1 and 2 established that 2-5A produced in IFN-treated, infected cells is capable, in the absence of protein synthesis, of activating the 2-5A dependent RNase. Howewer, if viral proteins are synthesized during infection, 2-5A failed to activate the RNase.

*A vaccinia virus gene product is required to restrict rRNA cleavage in IFN-treated infected cells.*

To show that failure of 2-5A to activate the 2-5A dependent RNase could be mediated by specific viral product, we used a temperature sensitive (ts) mutant of vaccinia virus (ts-22). This mutant is abortive at late times post infection and encodes a function that prevents specific rRNA breakdown at the non-permissive temperature of 40 °C in non-IFN-treated cells (5, 22). Because the effect of IFN on ts 22 has not been studied, we characterized viral protein synthesis and rRNA cleavage in IFN-treated, infected cells. As shown in Fig. 2 by SDS-PAGE analysis, untreated and IFN-treated BSC-40 cells infected with ts-22 at the non-permissive temperature synthesized the same spectrum of viral proteins at 5 hr pi but at late times post infection both viral and cellular proteins are inhibited regardless of IFN treatment. At the permissive temperature (35 °C) vaccinia virus proteins are synthesized, regardless of IFN treatment. The results of rRNA cleavage are presented in Fig. 3 at 4 hr and 8 hr pi, at the two temperatures. To assure a requirement for protein synthesis, the extent of rRNA cleavage was compared with cells treated with cycloheximide added at time zero of infection. As expected, extensive rRNA cleavage develops at the permissive temperature only in CY-treated cells (lanes 7 and 8). Howewer, at the non-permissive temperature extensive rRNA cleavage occurs in IFN-treated, infected cells (lane 14). When protein synthesis was blocked with cycloheximide, more rRNA cleavage occurred in IFN-treated, infected cells (lanes 12 and 16) than in non-IFN-treated, infected cells (lanes 11 and 15). The pattern of rRNA cleavage products was similar at the two temperatures.

The above findings strongly suggest that the function encoded by vaccinia virus ts 22 interferes with the 2-5A system.

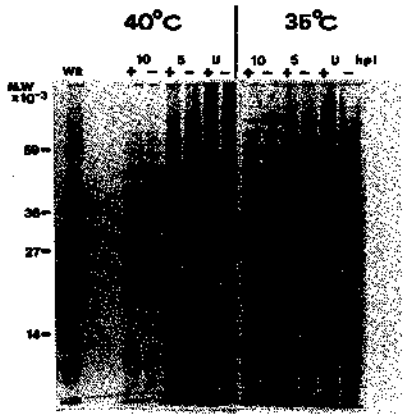


Fig. 2. Effect of IFN on vaccinia virus protein synthesis in cells infected with ts 22 mutant. BSC-40 cells grown at 37 °C in 24 Limbro plate were treated with 500 u/ml of human IFN for 18 hr, then infected at 35 °C and at 40 °C with 10 pfu/cell of purified ts-22 mutant of vaccinia virus and at various times post infection cells were labeled for 1 hr with 20 μCi/ml of $^{35}$S-methionine. Cells were washed, collected by cetrifugation, disrupted by freeze-thawing and equal amount of protein (10 μg) was loaded on 12 % SDS-PAGE gels. Untreated (-) and IFN-treated (+) cells. Numbers on top denote the times post infection in hours. (U), uninfected cells. (WR) $^{35}$S-labeled vaccinia virus structural proteins.

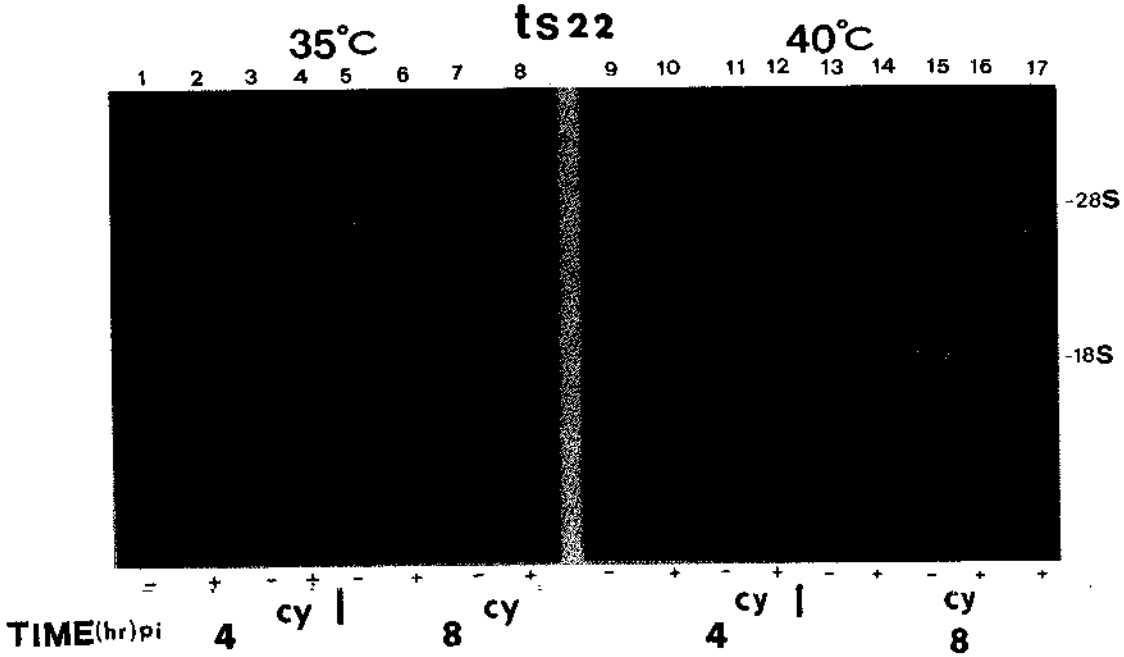


Fig. 3. Extent of rRNA cleavage in untreated and IFN-treated, monkey cells infected with an abortive late ts-mutant of vaccinia virus. BSC-40 cells grown at 37 °C in 60 mm dishes were treated with 500 u/ml of human IFN for 18 hr and then infected with 10 pfu/cell of ts-22 vaccinia virus mutant at 35 °C and at 40 °C in the absence or presence of 100 μg/ml of cycloheximide. RNAs were isolated at 4 hr and 8 hr pi, run on 1.8 % agarose gel and stained with ethidium bromide (9). Untreated (-); IFN-treated (+) cells; cycloheximide-treated (CY) cells. Uninfected cells (U).

*Extent of binding of 2-5A to viral proteins in the course of virus infection.*

Interference of vacinia virus with the 2-5A system may be the result of sequestration of 2-5A by viral proteins. This possibility was tested by SDS-PAGE after measuring the extent of covalent binding of labeled 2-5A to viral proteins in cell extracts (36). As indicated in Fig. 4, a band migrating with an apparent molecular weight of 77,000 was found to be radioactively labeled in extracts from uninfected L and BSC-40 cells. The 2-5A binding protein (77K) is thought to be the RNase (18, 36). The level of this protein was markedly stimulated by IFN as previously noted by other workers with different cell lines (13, 18, 32). Control experiments using cell extracts from IFN treated, uninfected cells and exogenously labeled 2-5A showed that labeling was prevented by cold competitor $ppp(A2'p)_3A$ (90 % competition was obtained with 0.5 nM). When extracts containing

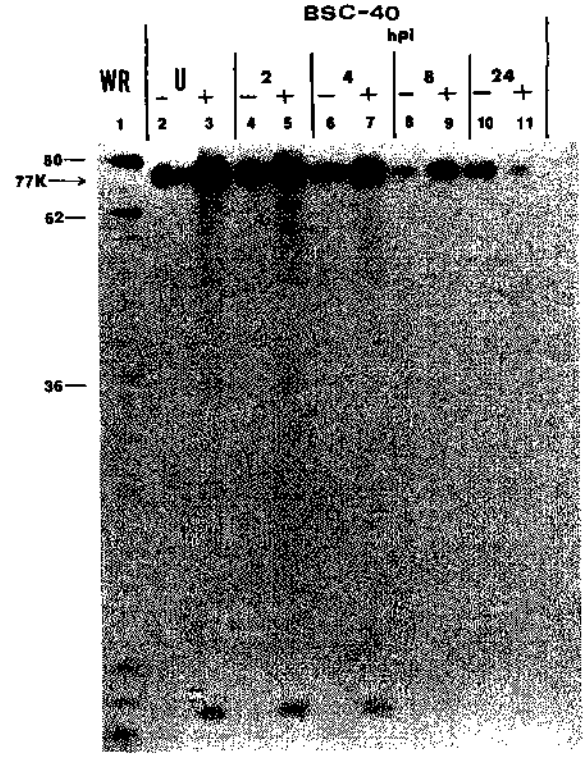


Fig. 4. Extent of covalent binding of labeled 2-5A to proteins in cell extracts from untreated and IFN-treated, vaccinia virus infected cells. Conditions for IFN-treatment, virus infection and measurements of covalent binding of labeled 2-5A to proteins were as described under Materials and Methods. Analysis of labeled 2-5A bound to proteins (lanes 2-11) in cell extracts from BSC-40 cells. The origin of the cell extracts was as follows. proteins from vaccinia virus infected cells at 4 hr pi, used as molecular weight marker (lane 1). Uninfected cells, untreated (lane 2) and IFN-treated (lane 3). Infected cells at 2 hr pi (lane 4), 4 hr pi (lane 6), 8 hr pi (lane 8) and 24 hr pi (lane 10). The reduced intensity observed in lane 8 is due to lesser amount of protein loaded in the gel. IFN-treated, infected cells at 2 hr pi (lane 5), 4 hr pi (lane 7), 8 hr pi (lane 9) and 24 hr pi (lane 11). SDS-PAGE analysis was in 12 % gels. The molecular weights of some vaccinia-induced polypeptides in infected cells are indicated.

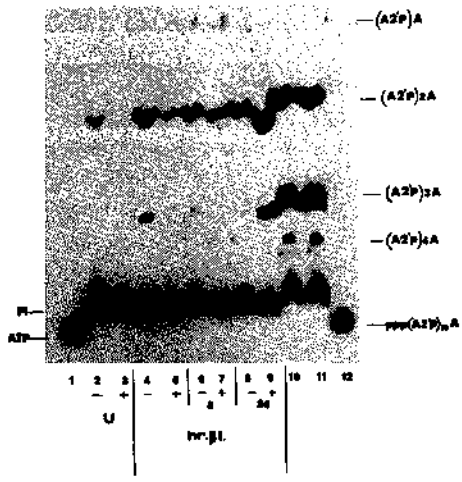

Fig. 5. Extent of degradation of labeled 2-5A in extracts from untreated and IFN-treated, vaccinia virus infected HeLa $S_3$ cells. Phosphorylated 2-5A (20,000 cpm/20 µl reaction) in buffer B was incubated for 1 hr at 30 °C with extracts (12 µg of protein) from untreated and IFN-treated, infected cells. Reactions were centrifuged in Eppendorf for 5 min. and 10 µl aliquots treated with 36 units/ml of bacterial alkaline phosphatase (BAP) for 1 hr at 37 °C. Thin-layer chromatography (TLC) was in 1M acetic acid and products visualized by autoradiography. The origin of the samples is: ($\alpha$-$^{32}$P)ATP (lane 1); 2-5A incubated with extracts from uninfected, untreated (lane 2) and IFN-treated (lane 3) cells; 2-5A incubated with extracts from virus infected cells prepared at 4 hr pi (lane 4), 8 hr pi (lane 6) and 24 hr pi (lane 8); 2-5A incubated with extracts from IFN-treated, virus infected cells prepared at 4 hr pi (lane 5), 8 hr pi (lane 7) and 24 hr pi (lane 9); 2-5A without incubation (lane 10) and incubated only with buffer B (lane 11); phosphorylated 2-5A non-treated with BAP (lane 12).

equal amounts of protein from infected cells, with or without IFN treatment, were incubated with labeled 2-5A, labeling of the 77K protein decreased as infection progressed and no viral proteins were observed on the gels. Because of the level of the 2-5A dependent RNase is not affected during infection (1, 28), the reduced binding of labeled 2-5A to the RNase is most likely the result of competition by endogenous 2-5A. This competition was observed in extracts from infected L and BSC-40 cells treated with inhibitors of protein synthesis (not shown). These results provided further evidence that authentic 2-5A is produced in vaccinia virus infected cells and that 2-5A accumulates at late times post infection. We did not observe the presence of 2-5A bound to viral proteins, suggesting that linkage of 2-5A to viral proteins is not the mechanism for blocking 2-5A action.

*Stability and integrity of 2-5A in extracts from IFN-treated vaccinia virus infected cells.*

Since naturally occurring 2-5A is rapidly degraded *in vivo* by a 2-5A phosphodiesterase (31), it was of interest to determine the stability and integrity of 2-5A in vaccinia virus, infected cells. To investigate stability, $^{32}$P labeled 2-5A (trimer, tetramer and pentamer) was mixed with ($S_{10}$) extracts from uninfected and virus infected cells. TLC was used to examine the degree of $^{32}$P 2-5A degradation using as a solvent 1M acetic acid. There was

a difference in the extent of degradation of 2-5A in extracts from uninfected and virus infected. As shown in Fig. 5, extracts from uninfected cells, untreated (lane 2) or IFN-treated (lane 3), caused degradation of 2-5A, since after BAP treatment most of the labeled 2-5A was converted into Pi, with small amount of dimer and trimer. And enhanced degradation of 2-5A was observed in extracts from IFN-treated cells, most likely as a result of an IFN-mediated induction of 2-5A phosphodiesterase. In extracts from infected cells, untreated or IFN-treated, the tetramer and trimer were mor treated cells (not shown). Control experiments, using extracts from uninfected cells, labeled 2-5A and cold competitor 2-5A tetramer (up to 250 nM), showed that the difference in degradation of 2-5A in

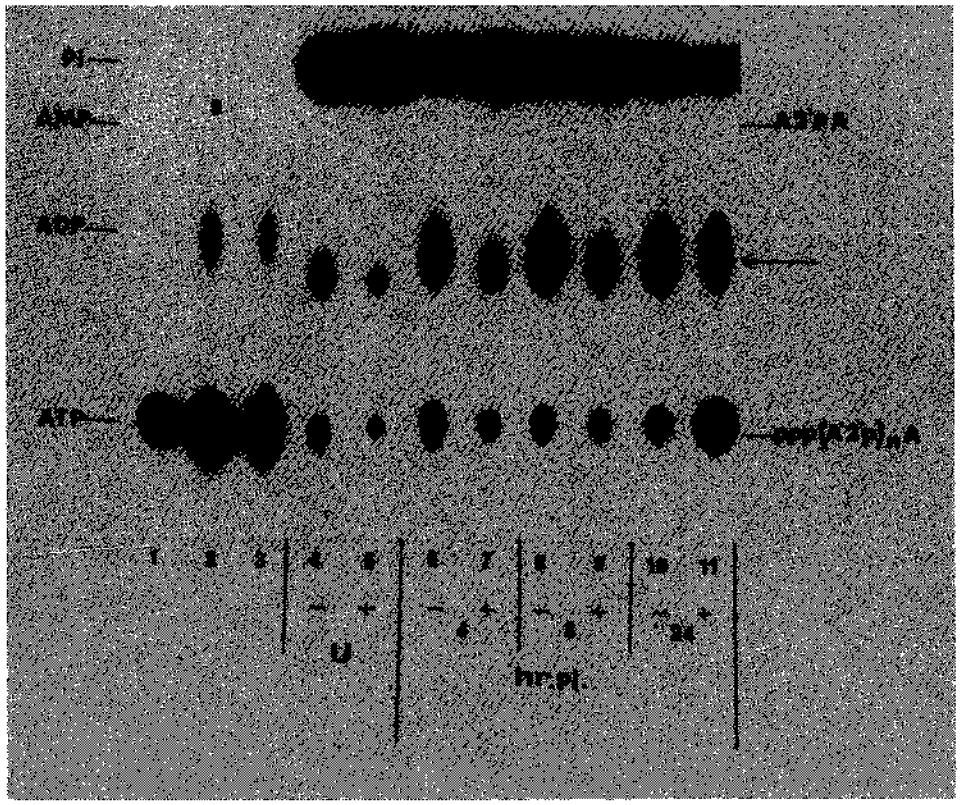


Fig. 6. PEI-cellulose analysis of phosphorylated 2-5A products. Conditions for incubation of phosphorylated α-$^{32}$P labeled 2-5A with cell extracts from uninfected and virus infected cells were as for Fig. 5 except that BAP treatment was omitted and 0.75 M potassium phosphate pH 3.4 was used as a solvent. The origin of the samples is: (α-$^{32}$P)ATP, (lane 1); 2-5A without incubation (lane 2) and incubated only buffer B (lane 3); 2-5A incubated with extracts from uninfected, untreated (lane 4) and IFN-treated (lane 5) cells; 2-5A incubated with extracts from virus infected cells prepared at 4 hr pi (lane 6), 8 hr pi (lane 8), and 24 hr pi (lane 10); 2-5A incubated with extracts from IFN-treated, virus infected cells prepared at 4 hr pi (lane 7), 8 hr pi (lane 9), and 24 hr pi (lane 11). The optical density markers are indicated. Similar results were obtained with 30 min. and 90 min. incubations.

extracts from virus infected cells could not be explained by the levels of intracellular 2-5A. Thus, it appears that in vaccinia virus, infected cells 2-5A is not rapidly degraded possibly because of a decrease in the activity of 2-5A phosphodiesterase.

To determine if some changes in phosphorylation of 2-5A occurred during infection, we measured the extent of dephosphorylation of $^{32}$P-labeled 2-5A after incubation with $S_{10}$ cell extracts. Phosphorylated 2-5A derivatives were examined by TLC using as solvent 0.75 M potassium phosphate buffer, pH 3.4 (Fig. 6). Under these chromatographic conditions a time-dependent accumulation of incomplet phosphorylated 2-5A (denoted by arrow) was observed with extracts from virus infected cells. These are similar findings to those observed previously with poly (I):(C) agarose bound enzyme fractions from vaccinia virus infected cells (23, 24). Accumulation of dephosphorylated 2-5A in cell extracts was prevented if protein synthesis was inhibited with cycloheximide (not shown).

The findings of Figs. 5 and 6 provided additional evidence that in the course of vaccinia virus infection of cells there are modifications of the 2-5A system.

## Discussion

The aim of this study was to establish if vaccinia virus acts as a biological modifier of the 2-5A system, in an attempt to correlate virus-induced modifications of the 2-5A system with the known resistance of this cytoplasmic DNA virus to inhibition by IFN. We have shown that the 2-5A system is modulated by vaccinia virus.This modulation occurs throμh production of an activator and simultaneous production of inhibitor(s) of the 2-5A system. The activator is viral RNA. Evidence for RNA as an activator of the 2-5A system is because extensive rRNA cleavage is observed in IFN-treated, infected cells in the presence of cycloheximide (Figs. 1 and 2), under conditions where virus transcription is not altered by IFN (9, 10, 23). Since the only known activator of the 2-5A system is ds-RNA (19) and vaccinia virus is known to produce significant amounts of ds-RNA during infection as a result of symmetrical transcription (2, 4, 33) it is reasonable to conclude that vaccinia ds-RNA acts as activator of the 2-5A system.

Evidence for an inhibitor of the 2-5A system in IFN-treated, vaccinia virus infected cells is as follows. First, rRNA cleavage is not observed or is greatly reduced during the course of infection (Figs. 1 and 3). Second, the occurrence of an inhibitor of rRNA cleavage is observed between 1-2 hr pi (Fig. 1B). Third, the function encoded by ts 22 mutant of vaccinia virus is required to restrict rRNA cleavage during infection (Fig. 3). It is of interest that the function encoded by ts 22 is expressed at both early and late times post infection (Condit, personal communication, VI Poxvirus-Iridovirus meeting, Sept. 24-28, 1986 Cold Spring Harbor, N. Y.).

Vaccinia virus synthesizes more than 200 polypeptides during infection and several of these proteins are enzymes that use ATP (21). It is possible that besides the ts 22 mutation there are other vaccinia products that interact, directly or indirectly, with the 2-5A system. Interaction of viral enzymes with the 2-5A system could lead to alterations in 2-5A levels and to modifications in the integrity of 2-5A or both. As shown in Table 1, the intracellular levels of 2-5A are low at 4 hr pi and exhibit a sharp rise at 8 hr pi. Under the multiplicity of infection used here, viral DNA synthesis begins at about 1.5 hr and reaches a peak between 3-4 hr pi (7). One would expect that by 4 hr pi during the normal

course of virus infection more ds-RNA and 2-5A should be produced than in infected cells treated with cycloheximide. Howewer, rRNA cleavage is more severe in the latter system and twice as much 2-5A is produced when protein synthesis is inhibited (Table 2). We have previously postulated that degradation of ATP by vaccinia virus ATPases, which are nucleic acid dependent enzymes, could contribute to limit the levels of 2-5A by 4 hr pi (24). We have recently cloned and mapped a vaccinia virus ATPase gene which will provide the means to study the role of this enzyme on the 2-5A system (30). At 8 hr pi, the virus replication cycle is nearly completed and there is a high production of 2-5A (Table 1). This rise in 2-5A levels late in infection is characteristic of IFN-treated, vaccinia virus infected tissue culture cells (Table 1; 28), and is likely to be the result of accumulation of large amounts of viral ds-RNA.

Modifications in the integrity of 2-5A during infection is another level of modulation of the 2-5A system by vaccinia virus and could be carried out by virus-induced phosphatases. Rice et al (1985) have provided evidence for the occurrence of non-phosphorylated core 2-5A in trichloroacetic acid-soluble material from IFN-treated, vaccinia virus infected cells. We have also obtained biochemical evidence that vaccinia virus can induce dephosphorylation of 2-5A following incubation of labeled 2-5A with cell extracts (Fig. 6). Alterations in 2-5A integrity by vaccinia virus-induced phosphatase would be in line with our previous observations that dephosphorylated 2-5A is produced in poly (I):(C) agarose-bound enzyme fractions from IFN-treated, vaccinia virus infected cells (24).

Modifications of the IFN system by vaccinia virus serves a selective advantage for this cytoplasmic virus to escape blockade by IFN. Indeed, vaccinia virus can be maintained persistently in cells continuously treated with IFN (25). There is increasing evidence that animal viruses have developed strategies to overcome the blockade of IFN. In the case of IFN-treated cells infected with SV-40 (12) and herpes (3) viruses, it has been observed the occurrence of 2-5A products that do not activate cleavage of rRNA. In the case of adenovirus, it has been observed that one viral product VAI-RNA, prevents activation of the IFN-induced eIF-2 alpha kinase (15).

In conclusion, the observations reported in this study provided evidence that vaccinia virus products act as biological modifiers of the 2-5A system. This modulation appears to involve the production of an activator and inhibitor(s) of the 2-5A system. Viral ds-RNA is likely to be the activator of the 2-5A system while a function encoded by ts 22 mutant is involved in inhibition of the 2-5A system. Other viral functions (ATPase and phosphatase) might also be involved in modifications of the 2-5A system. The use of vaccinia virus mutants will undoubtedly provide the means to identify viral functions that play a role in the phenomenon of the resistance of vaccinia virus to IFN. In this regard, temperature sensitive and deletion mutants of vaccinia virus have been isolated (5, 26) and some of these mutants, like ts 22, elicit modification of the 2-5A system while others have shown different degrees of sensitivity to IFN (Páez and Esteban 1987; in preparation).

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# Development of precipiting antibodies in rabbits inoculated with *Streptococcus pneumoniae* by crossed immunoelectrophoresis

by

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**Summary**

The antibody response of rabbits immunized with a strain of *Streptococcus pneumoniae* serotype 1 was studied by crossed immunoelectrophoresis. Sera taken every 4 weeks up to eight months post inoculation were tested in order to observe the development of precipiting antibodies, a complete response being encountered on week ten.

*Key words: Crossed immunoelectrophoresis, pneumococcal precipiting antibodies.*

**Resumen**

Se estudió la respuesta de anticuerpos en conejos inmunizados con una cepa de *Streptococcus pneumoniae* serotipo 1 utilizando la técnica de inmunoelectroforesis cruzada. Sueros tomados cada cuatro semanas hasta el octavo mes de la inoculación fueron estudiados con objeto de observar la aparición de anticuerpos precipitantes. A partir de la semana 10 se encontró una respuesta completa.

## Introduction

Immunoelectrophoresis has been widely used method for the antigenic analysis of microorganisms (1, 4, 6, 7) as well as for the detection of antigens in clinical samples (2, 9).

For a good performance of immunoelectrophoresis and especially for crossed immunoelectrophoresis (CIE) it is neccessary to use antisera-containing precipiting antibodies. Large inoculation schemes (3) are required for these antibodies to appear. In this work we describe the appearance of precipiting antibodies in rabbits inoculated with a pneumococcal antigen detecting them by CIE method.

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## Material and methods

*Antigen preparation:*

*Streptococcus pneumoniae* number 75,862 (serotype 1) from our collection, was used through out this study. Pneumococci were grown in liquid medium (Todd-Hewitt) with 1 % horse serum. Cells were centrifuged at 10,000 g for 20 min, washed six times in PBS 0.01M (pH 7.2), suspended in distilled water and disrupted by sonication at 20,000 Hz/sec for three cicles of 5 minutes. Sonicate was then centrifuged at 48,000 g for 20 min at 4 °C and the supernatant was harvested and filtered through a 0.22 µm filter (Millipore). This filtrate was considered to be the antigen. The protein content of this antigen was determined according to the method of Lowry (5), using bovine albumine as a reference. Protein content was estimated to be 4 mg/ml.

TABLE 1
APPEARANCE OF PRECIPITING ANTIBODIES

| Week post-inoculation | Number of precipiting lines in: | |
|---|---|---|
| | Intermediate gel | Reference gel |
| 0 | 0 | All |
| 5 | 3 | All but 3 |
| 8 | All but 4 | 4 |
| 10 | All | All * |
| 12 | All | All * |
| 16 | All | All * |

* Precipiting lines in reference gel were a continuation of those iniciated in intermediate gel.

*Antisera production:*

New Zeland rabbits were inoculated, by following the method of Harboe and Ingild (3) slightly modified. Briefly, rabbits were inoculated intracutaneously by inyecting 500 µl of antigen (2 mg protein) together with the same volume of Freunds incomplete adjuvant. Rabbits were immunized once a month during a period of seven months, a sample of serum being extracted previous to every immunization.

Rabbits were finally exanguinated on the eighth month (final antiserum).

*Crossed immunoelectrophoresis (CIE)*

CIE with intermediate gel was used. The method was performed as described by Svendsen *et al* (8), by using 1.1 % agarose (Behring) in sodium barbital buffer, pH 8.6,

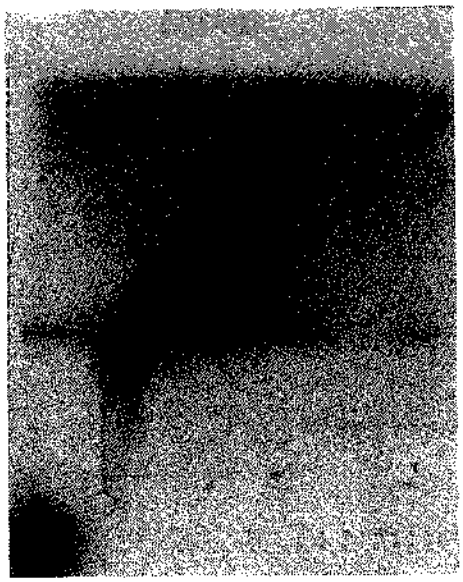

Fig. 1.–Intermediate gel contains the pre-immune serum. Reference gel contains the final antiserum.

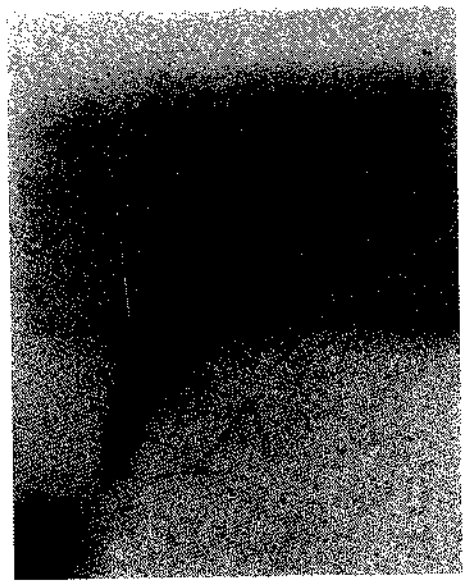

Fig. 2.–Intermediate gel contains the antiserum obtained after 5 weeks post inoculation. Reference gel contains the final antiserum.

Fig. 3.–Intermediate gel contains the antiserum obtained after 8 weeks post inoculation. Reference gel contains the final antiserum.

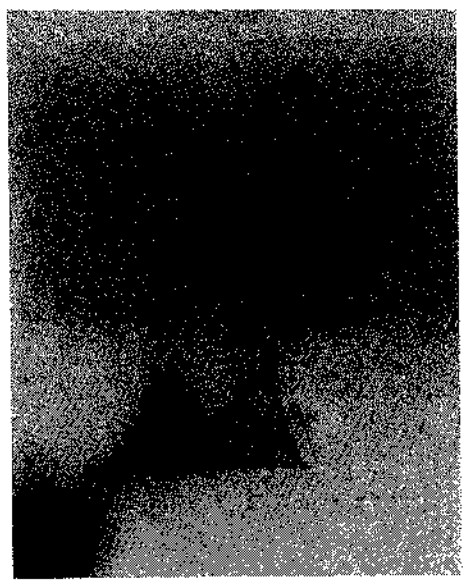

Fig. 4.–Intermediate gel contains the antiserum obtained after 10 week post inoculation. Reference gel contains the final antiserum.

and ionic strength 0.075. For every assay the amount of protein used was 80 µg. Antisera incorporated into the reference gel or the intermediate gel was diluted 20 µl of sera per $cm^2$.

First dimension electrophoresis was performed at 10 °C by applying 10 volts/cm for 3 hours. Bromophenol blue was simultaneously run, and first dimension electrophoresis was stopped when the marker had migrated 4 cm. Second dimension was run at 1-2 volts/cm for 20 hours.

Washing and drying procedures were also performed according to Svendsen *et al* (8). Gels were stained with Coomassie blue.

## Results

Table 1 shows the results obtained when the final antiserum was included in the reference gel and the succesive antisera (from pre-immune serum to 16 weeks post-inoculation) were included in the intermediate gels. These results are documented in Figs. 1 to 4. In Fig. 1, intermediate gel contains the pre-immune serum, all precipiting lines appear in the reference gel. In Fig. 2, the intermediate gel contains the antiserum obtained after 5 weeks post inoculation and three precipiting lines are visible in the intermediate gel. Fig. 3 shows that in week 8, all but 4 of the final precipiting lines can be appreciated. Fig. 4 demonstrates that a total response in precipiting antibodies is evidenced on week 10.

## Discussion

Harboe *et al* (3) described that for the production of high titered precipiting antibodies against bacterial antigens, a large schedule of immunization –eight months to several years– was necessary (3). In the present work, the antibody response of rabbits immuniz-J ed with a strain of *S. pneumoniae* was studied observing that ten weeks after the first inoculation a complete response was achieved, since patterns obtained in week 10 and in month 8 could be considered identical as seen in fig. 4.

However, not all pneumococcal antigens were able to generate the antibody response at the same time; there were early antibodies detected on week 5 and late antibodies that appeared on week 10.

These results must be interpreted cautiously, since the different immunitary capacity of individuals must be taken into account. But it seems likely, that in some cases –as the one described here– a complete response of antibodies can be achieved with relatively short schedules of immunization.

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# Chromosomal origin of acetyltransferase AAC(6') specifying amikacin resistance in *Serratia marcescens*

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**Summary**

Two clinical isolates of *Serratia marcescens* resistant to aminoglycoside-aminocyclitols and other antibiotics have been examined for aminoglycoside-modifying enzymes. Both strains were amikacin-resistant, and this resistance was mediated by an acetyltransferase AAC(6'). *S. marcescens* 737 contains a single conjugative plasmid, pUZ 737, of 135 kilobases, which confers resistance to gentamicin and tobramycin by a nucleotidyltransferase, ANT(2"), and to kanamycin, neomycin, butirosin and lividomycin by a phosphotransferase, APH(3'). *S. marcescens* 1830 does not contain extrachromosomal DNA, and it produced only the above mentioned AAC(6'). The presence of AAC(6') and associated aminoglycoside resistance are not dependent on the presence of a detectable plasmid, not transferred by conjugation, and not cured. Therefore, this enzyme is probably encoded by a chromosomal gene.

*Key words: Amikacin resistance, aminoglycoside-modifying enzymes,* Serratia marcescens.

**Resumen**

Se ha estudiado la presencia de enzimas modificantes de aminoglicósidos en dos aislamientos clínicos de *Serratia marcescens* resistentes a aminoglicósidos-aminociclitoles y otros antibióticos. Ambas cepas eran resistentes a amikacina y esta resistencia era debida a una acetiltransferasa AAC(6'). *S. marcescens* 737 era portadora de un plásmido conjugativo, pUZ 737, de 135 kilobases, que confería resistencia a gentamicina y tobramicina mediante una nucleotidiltransferasa ANT(2"), y resistencia a kanamicina, neomicina, butirosina y lividomicina por una fosfotransferasa APH(3'). *S. marcescens* 1830 carecía de DNA extracromosómico y producía únicamente la mencionada AAC(6'). La presencia del enzima AAC(6') y la resistencia a aminoglicósidos debida a este enzima no

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está asociada a ningún plásmido detectable, y no puede ser transferida por conjugación ni curada. Por ello, este enzima está probablemente codificado por un gen de localización cromosómica.

## Introduction

Plasmid-determined modification of antibiotics is the most common type of resistance to aminoglycosides encountered in both gram-negative and gram-positive organisms. A series of modifying enzymes have been isolated from bacterial strains resistant to this large group of antimicrobial agents which includes a number of clinically important compounds.

The semisynthetic aminoglycoside amikacin is effective against many gentamicin or kanamycin-resistant strains of *Enterobacteriaceae, Pseudomonas* and *Staphylococcus*. However, there have recently been reports of chromosome and plasmid-mediated resistance to amikacin (13, 16, 17, 23, 26).

Resistance to amikacin is associated with the three known enzymatic modifications, N-acetylation, 0-phosphorylation, or 0-nucleotidylation. Although the 6'-N-acetyltransferase [AAC(6')] is the enzyme most commonly found, other enzumes for which amikacin serves as a substrate include 2"-0-phosphotransferase [APH(2")] and 3'-0-phosphotransferase [APH(3')] and 4'-0-nucleotidyltransferase [ANT(4')] (4, 7, 20).

In this paper we present a study of two *S. marcescens* strains resistant to amikacin and other aminoglycosides. Resistance to amikacin is mediated by a 6'-acetyltransferase encoded by a gene located on the chromosome.

## Materials and methods

### *Bacterial strains and plasmids*

*S. marcescens* 737 was isolated from wound exudate of an ambulatory patient. *S. marcescens* 1830 was isolated from sputum of a patient hospitalized in the intensive care unit. *S. marcescens* 18 $A^+$ (pigmented, bacteriocin type 883-653) (24) was used for bacteriocin typing. *S. marcescens* 18 A-UZ ($Nal^r$, mutant of 18 $A^+$, bacteriocin type 883-653) (Navarro, M. 1981. M. D. thesis, University of Zaragoza) and *Escherichia coli* K-12 J62 ($F^-$, $Nal^r$, pro, his, trp, lac) (1) were used as recipient strains in mating experiments. The clinical strains *E. coli* 15159, *E. coli* 6055, and *Salmonella enteritidis* 7277, and their *E. coli* K-12 transconjugants, each of them produce the APH(3')-II enzyme, were used for protein blotting and enzyme immunoassay. The following plasmids were used for molecular weight determination: R40 a (Inc C; $Ap^r$, $Km^r$, $Su^r$; 144 Kb), R144 (Inc Iα; $Km^r$, $Tc^r$; 93 Kb), N3 (Inc N; $Sm^r$, $Su^r$, $Tc^r$; 50 Kb), and S-a (Inc W; $Cm^r$, $Km^r$, $Sm^r$, $Su^r$; 45 Kb) (21).

### *Antibiotics*

The antibiotics were provided by the manufacturers: kanamycins and amikacin, by Bristol Myers; gentamicins, netilmicin and sisomicin, by Schering; neomycin by Upjohn;

paromomycin and butirosin, by Parke Davis; tobramycin and apramycin, by Eli Lilly; lividomycin by Roger Bellon; ribostamycin by Morrith; dibekacin by Lefa; tetracycline by Pfizer; ampicillin by Normon, and nalidixic acid by Sigma.

*Determination of drug resistance*

Antibiotic susceptibility was determined by disc diffusion on Mueller-Hinton agar (2). Minimal inhibitory concentrations were determined by serial two-fold dilutions of antibiotic in Mueller-Hinton agar.

*Conjugation and curing procedures*

Transfer of antibiotic resistance from wild strains to *E. coli* K-12 took place either in liquid medium (6, 25) or on membrane filters (10). Transconjugants were selected on MacConkey agar plates containing antibiotics at the following concentrations: ampicillin, 50 μg/ml; tetracycline, 10 μg/ml; kanamycin, 25 μg/ml; gentamicin, 10 μg/ml; tobramycin 10 μg/ml; nalidixic acid, 250 μg/ml.

Plasmid-curing experiments were performed by incubation of bacteria for 24 hr in LB medium containing acriflavine (12) or ethidium bromide (3). Serial dilutions were plated onto MacConkey agar and individual colonies were screened for antibiotic-susceptibility by replica plating (15).

*Typing by bacteriocins*

*S. marcescens* isolates were typed by bacteriocin production (8) and bacteriocin sensitivity (9).

*Assay for aminoglycoside-modifying enzymes*

To assay for aminglycoside-modifying enzymes crude extracts of *S. marcescens* strains and *E. coli* K-12 transconjugants were prepared by ultrasonic disruption. Enzymatic activities were measured by the cellulose phosphate paper binding assay (11). The reaction mixture for phosphorylation consisted of 10 μl of phosphotransferase buffer, 10 μl (10 μCi/μmole per ml) of ($\gamma$-$^{32}$P) ATP, (Amersham), 10 μl of enzyme preparation, and 2 μl of antibiotic (1mg/ml). Assays were incubated at 30 °C for 30 min, and 20 μl samples were counted. For acetylation or adenylylation, 10 μl (8 μCi/μmole per ml) of (1-$^{14}$C) acetyl coenzyme A (Amersham), or 10 μl (10 μCi/μmole per ml) of ($^{14}$C) ATP respectively were used instead of ($\gamma$-$^{32}$P) ATP.

*Isolation and characterization of plasmid DNA*

Plasmid DNA was isolated according to the method described by Crosa and Falkow (5). Bacteria were treated with lysozyme and lysed by the nonionic detergent Triton

X-100 at 65 °C. Plasmid DNA was precipitated by polyethylene glycol 6000, resuspended and precipitated by ethanol.

Plasmid DNA was analyzed by electrophoresis in 0.7 % agarose slab gels (18) using Tris-borate buffer (89mM Tris, 2.5mM EDTA, and 8.9mM boric acid).

*Western blotting and enzyme immunoassay*

Crude extracts of *S. marcescens* 737, *S. marcescens* 18A-UZ (pUZ 737) and *E. coli* K-12 (pUZ 737) were separated on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This was performed on 12.5 % slab gels with the buffer system of Laemmli (14). Proteins were electrophoretically transferred to nitrocellulose sheets by the method of Towbin *et al.* (22) and reacted overnight at 45 °C with APH(3')-II- specific antiserum (J. Davies - BIOGEN-Geneva) in TBT buffer (10mM Tris.HCl, pH 7.5, 150mM NaCl, 0.77mM sodium azide, 3 % bovine serum albumin, 0.1 % Triton X-100). After washing with TBS-BSA buffer (as above without Triton X-100) the blot was incubated in fresh TBT buffer containing ($^{125}$I)-protein A (0.5-2μCi) (Amersham) which bound to the antibody already bound to the immobilized APH(3')-II enzyme. The blot was then washed again in TBS-BSA buffer, dried and exposed to X-Ray film (Kodak RP/R54) for 18-24 hr at -70 °C.

## Results

*S. marcescens* 737, bacteriocin type 888-633, was resistant *in vitro* to ampicillin, carbenicillin, chloramphenicol, sulfonamide, gentamicin, kanamycin, sisomicin, tobramycin, and amikacin. The minimal inhibitory concentrations of the most representative aminoglycosides are listed in Table 1.

TABLE I
SUSCEPTIBILITY TO REPRESENTATIVE AMINOGLYCOSIDE ANTIBIOTICS, PRODUCTION OF MODIFYING ENZYMES, AND PLASMID CONTENT.

| STRAIN | Minimal Inhibitory Concentration [a] (mg/l) | | | | Aminoglycoside-modifying enzyme | | | Plasmid size (Kb) |
|---|---|---|---|---|---|---|---|---|
| | Km | Gm | Tm | An | AAC | ANT | APH | |
| *S. marcescens* 737 (pUZ 737) | >128 | 64 | 128 | >16 | (6') | (2") | (3')-III | 135 |
| *S. marcescens* 737 cured | 32 | 0.5 | 64 | >16 | (6') | | | |
| *E. coli* K-12 (pUZ 737) | 128 | 32 | 32 | 1 | | (2") | (3')-III | 135 |
| *S. marcescens* 1830 | 32 | 1 | 32 | >16 | (6') | | | |

A Km, kanamycin; Gm, gentamicin; Tm, tobramycin; An, amikacin.

The phosphocellulose paper binding assay demonstrated the existence of acetyltransferase, nucleotidyltransferase, and phosphotrasferase activities in the crude extract of *S. marcescens* 737. The acetylating activity was due to the presence of an AAC(6') with affinity for kanamycin, neomycin, gentamicin $C_{1a}$, dibekacin, and amikacin. The nucleotidylating activity was due to an ANT(2") with affinity for gentamicin, tobramycin, sisomicin, a kanamycin. The capacity to phosphorylate kanamycin, neomycin, ribostamycin, and paromomycin indicated the presence of an APH(3'). Since the aminoglycosides lividomycin and butirosin were also substrates for this enzyme it could be identified as APH(3')-III, or APH(3')I plus APH(3')-II. The results of the enzyme immunoassay demonstrate that anti- APH(3')-II did not cross-react with APH(3') enzymes from *S. marcescens* 737 or from *S. marcescens* 18A-UZ and *E. coli* K-12 transconjugants but it cross-reacted well with all APH(3')-II tested (Fig. 1). This result suggest that the pUZ737 plasmid coded for an APH(3')-III that phosphorylate both butirosin and lividomycin.

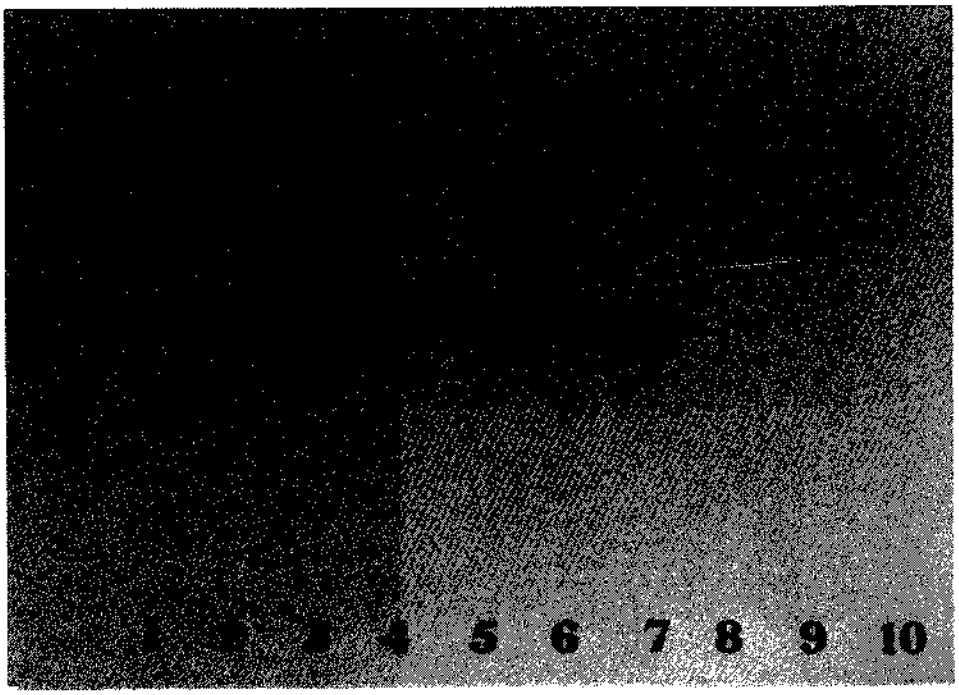


Fig. 1. Autoradiogram of protein blot. Samples of ten crude extract preparations were separated on 12.5 % SDS-PAGE, and electroblotted onto NC filter as described. The filter was incubated o/n with APH(3')-II specific anti-serum, and then incubated in TBT buffer containing ($^{125}$I)-protein A. After washing the filter was autoradiographed. (1) *E. coli* K-12 J62. (2) *E. coli* 15159 (pUZ62). (3) *E. coli* K-12 J62 (pUZ62). (4) *E. coli* K-12 6055 (pUZ638). (5) *E. coli* K-12 J62 (pUZ638). (6) *S. enteritidis* 7277 (pUZ655). (7) *E. coli* K-12 J62 (pUZ655). (8) *S. marcescens* 737 (pUZ737). (9) *S. marcescens* 18A-UZ (pUZ737). (10) *E. coli* K-12 J62 (pUZ737). The arrow indicates the position of the APH(3')-II enzyme.

*S. marcescens* 737 harboured a single plasmid of 135 kilobases, pUZ737, as shown by agarose gel electrophoresis of isolated plasmid DNA (Fig. 2). The pUZ737 plasmid was transferred to *E. coli* K-12 and to *S. marcescens* 18A-UZ by conjugation at a frequency of $10^{-2}$ to $10^{-4}$ at 24 hr. Transconjugants were resistant to ampicillin, carbenicillin, chloramphenicol, sulfonamide, gentamicin, kanamycin, and tobramyci produced two classes of aminoglycoside-modifying enzymes, the phosphotransferase APH(3')-III and the nucleotidyltransferase ANT(2''). *S. marcescens* 737 was cured by exposure either to ethidium bromide or to acriflavine. The cured strain, bacteriocin type 888-633, was resistant to kanamycin, neomycin, dibekacin and amikacin, and elaborated a single enzyme, AAC(6'). (Table 1).

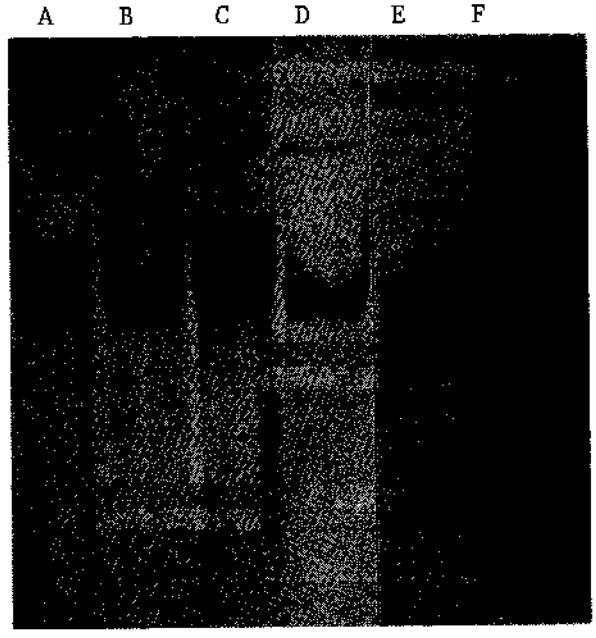


Fig. 2. Analysis by agarose gel electrophoresis of the plasmid DNA content. Electrophoresis was carried out in 0.7 % agarose slab gel for 3 hr at 120 V. (A) Plasmid N3. (B) *S. marcescens* 737. (C) *E. coli* K-12 (pUZ737). (D) *S. marcescens* 737 cured. (E) *S. marcescens* 1830. (F) *S. marcescens* 737 (pUZ737).

*S. marcescens* 1830, bacteriocin type 888-838, was resistant *in vitro* to ampicillin, carbenicillin, tetracycline, sulfonamide, kanamycin, tobramycin, and amikacin. The minimal inhibitory concentrations of several aminoglycosides are listed in Table I. The resistance of this strain to aminoglycosides was mediated by a single enzyme, AA(6'), with a substrate profile similar to those of the 6'-N-acetyltransferases found in *S.marcescens* 737 (pUZ737) and *S. marcescens* 737 cured. (Table 2).

TABLE 2
SUBSTRATE RANGES OF 6'-N-ACETYLTRANSFERASE FROM *S. marcescens* STRAINS.

| SUBSTRATE | Antibiotic acetylated[a] % | | |
|---|---|---|---|
| | *S. marcescens 737* (pUZ737) | *S. marcescens* 757 (cured) | *S. marcescens 1830* |
| Kanamycin A | 100 | 100 | 100 |
| Gentamicin $C_{1a}$ | 74 | 98 | 52 |
| Gentamicin $C_1$ | 0 | 0 | 1 |
| Sisomicin | 107 | 94 | 96 |
| Tobramycin | 137 | 144 | 80 |
| Dibekacin | 107 | 110 | 93 |
| Netilmicin | 93 | 96 | 52 |
| Neomycin B | 88 | 66 | 74 |
| Butirosin | 43 | 50 | 62 |
| Amikacin | 31 | 26 | 54 |
| Ribostamycin | 162 | 183 | 58 |
| Lividomycin | 0 | 0 | 0 |
| Paromomycin | 0 | 0 | 0 |

[a] Acetylation is expressed relative to that of kanamycin A as 100 %.

*S. marcescens* 1830 contained no plasmid DNA (Fig. 2). The antibiotic resistance could be transferred neither by conjugation nor by filter mating technique. The resistance pattern of the strain was not affected by curing agents.

## Discussion

Resistance to amikacin among gram-negative bacilli isolated at the University Hospital of Zaragoza is not usual despite the fact that enzyme-mediated resistance may be transferable by conjugation (23, 26) and encoded by the multiresistance transposon Tn 2424 (17). The isolation of two *S. marcescens* strains resistant to multiple antibiotics, including amikacin, lead us to study the mechanism of aminoglycoside resistance and the location of these antibiotic resistance genes.

Analysis of the enzymatic activities in these two strains demonstrate that both of them possesed acetylating activity when measured against the aminoglycosides kanamycin, neomycin gentamicin $C_{1a}$, dibekacin, and amikacin. The substrate profiles of these acetyltransferases resembled each other closely, and they are indicative of an AAC(6') of type I (19). Neither phosphorylating nor nucleotidylating activity for amikacin was detected in the studied strains. This result demonstrates that the enzyme responsible for resistance to amikacin in both strains is the AAC(6'), the enzyme most frequently associated to amikacin-resistance in gram-negative clinical isolates. Finally it should be noted that one of the strains, *S. marcescens* 737, showed significant nucleotidylating and phosphory-

lating activities that indicate the presence of two different enzymes. The nucleotidyltransferase correspond to an ANT(2") that modify gentamicin, and tobramycin, whereas the phosphotransferase correspond to an APH(3') with affinity for kanamycin, neomycin, butirosin, and lividomycin. Although we have not adscribed the APH(3') to one particular type, the results of the enzyme immunoassay strongly suggest that *S. marcescens* 737 synthesized an APH (3')-III, enzyme not yet found among *Enterobacteriaceae* (19). It is interesting to mention that the protein blotting experiments using specific anti-sera may be useful to identify and to compare the phosphotransferases of different types and from different sources. A more detailed study might be carried out to asses whether the APH(3') from *S. marcescens* 737 is similar to that of described APH(3')-III from *P. aeruginosa* and from gram-positive organisms (19).

To study the location of these aminoglycoside resistance genes we performed conjugation and curing experiments followed by plasmid DNA isolation.

Conjugational transfer of antibiotic resistance either to *E. coli* K-12 or to *S. marcescens* 18A-UZ was obtained with *S. marcescens* 737 as donor strain. The transconjugant strains were resistant to the aminoglycosides kanamycin, gentamicin, and tobramycin, but not to amikacin, and presented similar nucleotidylating and phosphorylating activities to those found in the donor strain.These data and the identification of a single plasmid, pUZ737, of 135 kb in all the three strains suggest that the genes for both aminoglycoside-modifying enzymes were located on it. Curing experiments confirmed that this plasmid carried the genes for these enzymes since cured *S. marcescens* 737 lacked plasmid DNA and enzyme synthesis. Focusing the attention on the amikacin-resistance, it should be mentioned that this resistance, mediated by the AAC(6'), could not be transferred by mating experiments and not cured. These findings suggest that the AAC(6') is coded by a gene located on the chromosome.

Similar experiments were performed with *S. marcescens* 1830. Attempts either to transfer or to cure aminoglycoside-resistance determinants were unsuccessful. This fact and the failure to detect extrachromosomal DNA by the commonly used plasmid isolation procedures indicate that the enzyme AAC(6') seems to be encoded by a chromosomal gene.

The results of this investigation demonstrate the presence of chromosomal amikacin-resistance in two multiresistant strains of *S. marcescens*. Two aspects are interesting to mention. First, the similar findings obtained by John *et al* (13), for clinical isolates from three USA hospitals. They reported six different representative amikacin-resistant *S. marcescens* with chromosomal production of AAC(6'). Five of them contained a single conjugative plasmid that coded for ANT(2") and/or APH(3') enzymes, and another was plasmid-free and produced only the AAC(6'). The presence of a chromosomal gene specifying amikacin-resistance in enteric bacteria might be related to the isolation of the multiresistance transposon Tn 2424 that codes for resistance to amikacin due to a 6'-N-acetyltransferase (17). It is an open question whether resistance to amikacin in the reported *S. marcescens* strains is mediated by the Tn 2424.

Second, the limited spread of amikacin-resistance among gram-negative bacteria in the University Hospital. Recently, a direct relation was observed between amikacin use and ocurrence of enzyme-mediated amikacin resistance in gram-negative bacteria. (16, 23, 26). Our data, however, do not support this hypothesis. Despite the use of amikacin for years we have detected an amikacin-modifying enzyme, the AAC(6'), in only two

isolates of the family *Enterobacteriaceae*. It is possible that the spre this resistance may not respond as rapidly to the pressure of amikacin use since the enzyme is chromosomically encoded.

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# Purification of two exoglucanases secreted by *Saccharomyces cerevisiae* and partial characterization of their protein moieties.

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**Summary**

Growth of *Saccharomyces cerevisiae* cells in a synthetic buffered culture medium resulted in the secretion of high levels of two soluble exoglucanases which were purified by a procedure involving one (exglucanase II) and two (exoglucanase I) steps, respectively. Once treated with endoglucosaminidase H (Endo H) both enzymes behaved indistinguishably when analyzed by SDS-PAGE, high pressure liquid chromatography (HPLC) and ionic exchange chromatography. Exoglucanase I, the isoenzyme with higher carbohydrate content, exhibited a higher Km against laminarin and a higher thermal stability than exoglucanase II. However, once the enzymes were deglycosylated *in vitro* these parameters turned out to be identical. These results suggest that both exoglucanases share a very similar, if not identical protein portion and accordingly may be product of either the same gene or a family of related genes.

*Key words: Exoglucanases, protein moieties,* Saccharomyces cerevisiae.

**Resumen**

Cuando *Saccharomyces cerevisiae* se multiplicó en un medio de cultivo sintético y tamponado secretó altos niveles de actividad exoglucanasa soluble. Esta actividad fue debida a la acción de dos isoenzimas (exoglucanasas II y I), las cuales fueron parcialmente purificadas en uno y dos pasos respectivamente. Una vez tratadas con endoglucosaminidasa H (Endo H), ambas enzimas resultaron indistinguibles al ser analizadas en SDS-PAGE, cromatografía líquida de alta presión (HPLC) y cromatografía de intercambio iónico. La exoglucanasa I, la isoenzima con mayor contenido en carbohidrato, presentó una mayor Km frente a laminarina y una mayor estabilidad térmica que la exoglucanasa

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II. Sin embargo, una vez que las enzimas fueron desglicosiladas *in vitro* ambos parámetros resultaron idénticos. Estos resultados sugieren que ambas exoglucanasas poseen una porción proteica similar, si no idéntica, y por tanto son probablemente productos del mismo gen o de genes relacionados.

## Introduction

Exoglucanases are extracellular yeast enzymes. They have been suspected to play a role in yeast morphogenesis due to their potential action on β-1,3 glucan, which is thought to represent the main structural component of the yeast cell wall. This rigid polymer should be modified in some way in order to allow changes in morphology during the events that take place during the yeast cell cycle. In this line we have shown that a direct relationship exists between yeast (*Saccharomyces cerevisiae*) growth and synthesis and secretion of active exoglucanase (9). However, another line of evidence suggests that this activity may not be essential since growth, as well as other events of the yeast cell cycle, took place normally in a mutant (*exb-1*) deficient in secreted activity (12).

Several workers have detected two exoglucanases in the culture medium of *S. cerevisiae* (1, 3, 10). This fact complicates even more the problem concerning the functional role of this activity. Accordingly, we have initiated a study aimed to elucidate the chemical nature of both enzymes. In the present work, we report preliminary evidence indicating that both isoenzymes have a similar, if not identical, protein portion and differ in the amount of their N-glycosydically-linked carbohydrate moieties.

## Materials and methods

### *Microorganism and culture conditions*

*S. cerevisiae* 2180 1A, haploid, was used throughout this work. It was grown in a synthetic medium containing 5 % glucose and buffered as indicated. The synthetic culture medium consisted of (g/l): $(NH_4)_2SO_4$, 5; $KH_2PO_4$, 5; $MgSO_4.7H_2O$, 0.5); $CaCl_2.2H_2O$, 0,13; $H_3BO_3$, 0,001; KI, 0.0002; $Na_2MoO_4$ 0.0004; $CuSO_4.5H_2O$, 0.0008; $FeCl_3.6H_2O$, 0.0004; $MnSO_4.4H_2O$, 0.008. This medium was suplemented with a solution of vitamins (0,5 ml to 1000 ml of medium) containing (g/l): calcium pantotenate, 0,8; inositol, 0.4; niacin, 1.6; pyridoxin, 1.6; thiamine, 4.6. All the solutions were prepared in distilled and deionized water.

### *Purification of exoglucanases*

The growth medium from exponentially grown cultures was separated from the cells by centrifugation at 3000xg for 10 min. Then, it was concentrated and dialyzed against 25mM sodium acetate buffer, pH 5.2 by ultrafiltration in Amicon PM-10 membranes (exclusion limit 10 Kd) and subjected to the following purification procedure:

– Step 1. The concentrate was applied to a DEAE-Bio-Gel A column (60 × 2.5 cm) equilibrated at 4 °C with the same acetate buffer. The column was washed with the equi-

librium buffer and, afterwards it was eluted with 500 ml of a linear NaCl gradient (0-0.5 M in acetate buffer) at a flow rate of 25 ml/h. As described elsewhere (6), this procedure separates two exoglucanases which eluted at 0.13 M (exoglucanase I) and 0.18 M (exoglucanase II) NaCl, respectively. After this step, exoglucanase II was pure, whereas exoglucanase I was still contaminated with some proteinaceous material.

– Step 2. Fractions containing exoglucanase I were pooled and concentrated in Amicon cells as described before. The concentrate was applied to a column (100 × 2.5 cm) of Sephacryl S-200 equilibrated at 4 °C with acetate buffer containing 0.1 M NaCl, and eluted with the same solution at a flow rate of 25 ml/h. The exoglucanase activity eluted as a single peak at a $V_e/V_0$ of 1.33 which coincided with a peak of protein. The contaminant mentioned above was retarded in the column. During purification, exoglucanases were assayed using p-nitrophenyl-β-D-glucoside (p-NPG) as substrate as described before (9). One unit of activity releases 1 μmol p-nitrophenol per h at 30 °C.

*Criteria of purity*

SDS-PAGE was performed on slabs containing 4 % acrylamide stacking gel and 8 % acrylamide running gel according to Laemmli (5). Determination of $M_r$ was performed as described by Weber *et al.* (14). After electrophoresis, protein was stained by the silver method (8).

High pressure liquid chromatography (HPLC) was carried out in a Pack-TSK-G3000-SW (60 cm) column. Samples of 50 μl were applied and eluted with 0.1 M acetate buffer at a flow rate of 0.5 ml/min and at a pressure of 18 bars. $M_r$ were calculated by a standard procedure (12), once the column was calibrated with the standards suggested by the manufacturer (LKB).

*Other methods*

Protein was determined according to Lowry *et al* (7). Endoglucosaminidase H (Endo H) digestions were performed as described by Trimble and Maley (13) in the absence of SDS.

## Results

*Production of exoglucanase activity in a minimal buffered culture medium*

Fig. 1 shows the rate of secretion of exglucanase activity into the culture medium by *S. cerevisiae* cells when incubated in a synthetic medium supplemented with 50 mM citrate-100 mM phosphate buffer, pH 5.2 (Fig. 1A) or 300 mM citrate-600 mM phosphate buffer, pH 6.35 (Fig. 1B). In the first case, it is observed that, following an initial rise, the exoglucanase activity starts to decrease when the pH of the culture dropped below 4.8. By contrast, when the pH was regulated by use of citrate-phosphate buffer of higher molarity and pH (Fig. 1B) the exoglucanase activity steadily increased during the exponential phase and its levels remained constant for at least the first 15 h of the stationary phase. Under these conditions the pH of the culture medium never dropped below 5.

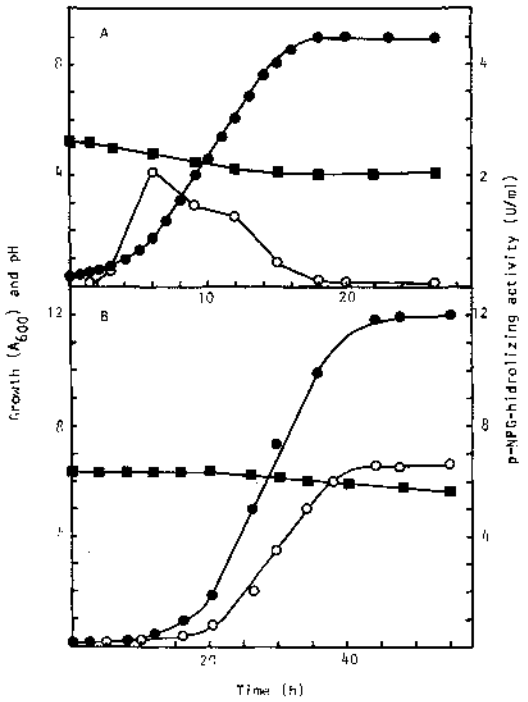


Fig. 1. Influence of pH regulation in the production of exoglucanase activity in a synthetic culture medium. Cells were inoculated in the minimal medium buffered with 50 mM citrate-100 mM phosphate, pH 5.2 (A) or 300 mM citrate-600 mM phosphate, pH 6.35 (B). Aliquots were taken at the indicated times to determine absorbance at 600 nm (●). They were then centrifuged to separate cells. Culture fluids were used to measure exoglucanase activity against p-NPG (○), and pH (■).

*Purification of exoglucanases I and II*

10 liters of culture medium collected from an exponentially growing culture under the conditions described in Fig. 1B, were subjected to the purification procedure described

TABLE I
PURIFICATION OF EXOGLUCANASES I AND II FROM CULTURE MEDIUM OF THE EXPONENTIAL PHASE OF GROWTH

| Purification step | Total Activity (U) | | Protein (mg) | | Specific activity (U/mg protein) | | Purification factor | |
|---|---|---|---|---|---|---|---|---|
| | Exo I | Exo II | Exo I | Exo II | Exo I | Exo II | Exo I | Exo II |
| Supernatant | 980 | | 18 | | 54 | | 1 | |
| DEAE-Bio-Gel | 72 | 586 | 1.2 | 1.2 | 60 | 326 | 1.1 | 6 |
| Sephacryl S-200 | 64 | 485 | 0.7 | 1.45 | 91 | 334 | 1.7 | 6 |

Purification factor is expressed as the ratio of the specific activities of each exoglucanase and the specific activity of the original culture medium.

under Materials and Methods. Table 1 summarizes the results obtained referring to one liter of the initial material.

*Criteria of purity*

Exoglucanase II, when analyzed by gel filtration in HPLC (Fig. 2) eluted as a single peak with a $M_r$ of 54,000. Exoglucanase I eluted in two fractions (Fig. 2). The first one peaked at an elution volume corresponding to a $M_r$ of 67,000. The other, which was a minor peak, eluted at the same position as exoglucanase II.

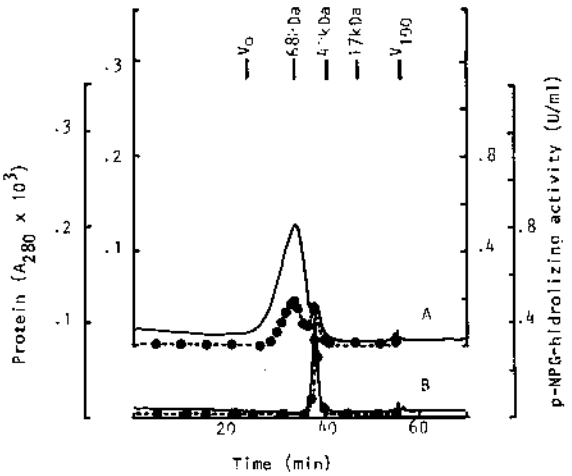


Fig. 2 Filtration in HPLC of purified exoglucanases. Elution profile of purified exoglucanases I (A) and II (B) in the TSK-G3000 SW column. (-) absorbance at 280 nm; (- -) exoglucanase activity. The position of authentic standards is indicated with bars: gammaglobulin ($M_r$ 160,000), bovine serum algumin ($M_r$ 68,000), ovoalbumin ($M_r$ 45,000) and myoglobin ($M_r$ 17,000).

When analyzed by SDS-PAGE (Fig. 3), exoglucanase II migrated as a single protein band centered at a $M_r$ of 53.000. Exoglucanase I was heterogeneous in nature, its size ranging from 56 to 93 KDa. As expected it contained a small contamination of exoglucanase II.

*In vitro deglycosylation of exoglucanases I and II*

Treatment of both exoglucanases with Endo H converted them into products that were indistinguishable when analyzed by SDS-PAGE (Fig. 3) and HPLC (not shown). The deglycosylated products exhibited a $M_r$ between 48,000 (SDS-PAGE) and 49,000 (HPLC). They bound to DEAE-Bio-Gel with higher affinity than any of the native enzymes and both eluted at the same NaCl molarity (0.35 M).

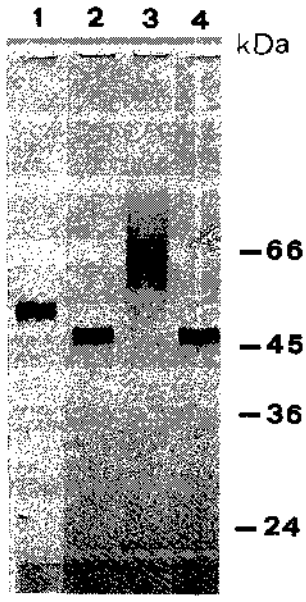


Fig. 3. Comparison of native and *in vitro* deglycosylated exoglucanases by SDS-PAGE. Lanes 1 and 3: purified exoglucanases II and I, respectively. Lanes 2 and 4: Endo H-treated exoglucanases II and I respectively.

*Kinetic parameters and resistance to temperature of exoglucanases*

The above results strongly suggested that both exoglucanases have a very similar protein moiety. In order to gain insight into this problem, we compared the Kms and resistance to temperature of both exoglucanases and their in vitro deglycosylated products.

As shown in Table 2 exoglucanase I exhibited a higher Km (20 mg/ml) against laminarin than exoglucanase II (10 mg/ml). However, once deglycosylated both exoglucanases exhibited the same Km (10 mg/ml). When tested against p-NPG the four enzymes showed identical Km (4 mg/ml).

TABLE 2
COMPARISON OF Kms OF PURIFIED EXOGLUCANASES AND THEIR *IN VITRO* DEGLYCOSYLATED PRODUCTS

| Enzyme | Endo H treatment | Km | |
|---|---|---|---|
| | | Laminarin (mg/ml) | p-NPG (mM) |
| Exoglucanase I | – | 20 | 4 |
| Exoglucanase I | + | 10 | 4 |
| Exoglucanase II | – | 10 | 4 |
| Exoglucanase II | + | 10 | 4 |

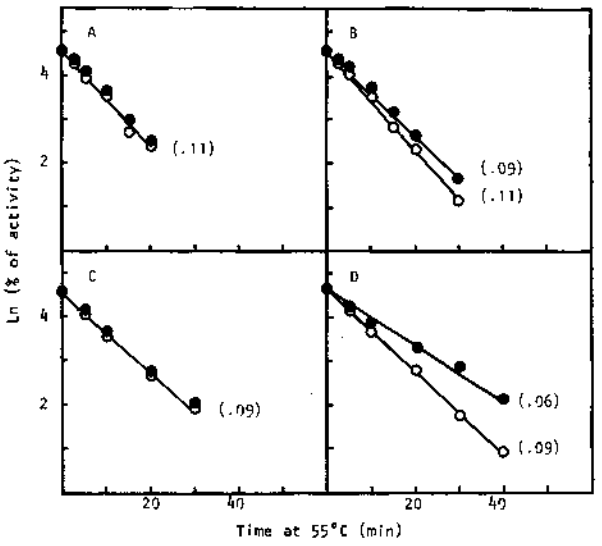


Fig. 4. Thermal stability of purified exoglucanases I and II (•) and their *in vitro* deglycosylated products (○. Exoglucanases I (B, D) and II (A, C) were incubated at 55 °C in the absence (A, B) or in the presence (C, D) of the substrate (p-NPG). In the first case (A, B), a sasmple was incubated at 55 °C. At the indicated times aliquots were withdrawn, taken at 30 °C and the activity determined by addition of the substrate. Incubations in the presence (C, D) of the substrate (1 ml of enzymatic solution plus 0.2 ml of 40 mM p-NPG) were stopped by addition of 0.5 ml $Na_2CO_3$. Under these conditions the activity was lineal for at least one hour when assayed at 30 °C. In all cases residual activty is expressed as the natural logarithm of the percentage of the initial activity. Numbers in parenthesis indicate the slopes of the corresponding lines.

Fig. 4 shows the inactivation kinetics at 55 °C of native and deglycosylated exoglucanases, in the absence (panels A, B) or in the presence (panels C, D) of the substrate (p-NPG). In the first case, the stability of exoglucanase I was higher (slope 0.09) than that of exoglucanase II (slope 0.11) probably due to the higher carbohydrate content of the former. By contrast, Endo H-treated exoglucanases showed the same thermal stability (slope 0.11). Similar results were obtained when thermal inactivation was conducted in the presence of p-NPG. Exoglucanase I was again more stable (slope 0.06) than exoglucanase II (slope 0.09), but both were inactivated at the same rate after Endo H treatment (slope 0.09).

## Discussion

The presence of two exoglucanases in the culture medium of *S. cerevisiae* (1, 3, 10), as well as the purification of the major one by a procedure involving five steps (11) has been described before. In this regard the novelty of the present work lies in a) the use of a synthetic buffered culture medium which improved significantly (x400) the yield of the exoglucanase activity as compared with the value reported before (11) and b) the subsequent reduction of the purification procedure to a single step. An additional step (Sephacryl S-200 chromatography) allowed the purification of exoglucanase I.

The finding that Endo H-treated exoglucanases were indistinguishable when analyzed by SDS-PAGE, HPLC and DEAE-Bio-Gel should be taken as a preliminary evidence that they are similar polypetides. This result contrasts with those reported by Sánchez *et al* (10) who detected two forms of deglycosylated exoglucanase in culture supernatants of cells grown in the presence of tunicamycin. These discrepancies deserve further analysis but we can advance that initial experiments in our laboratory have indicated that tunicamycin induces the synthesis and secretion of a sole deglycosylated enzyme. In addition, this enzyme appears to be identical to the *in vitro* deglycosylated products from both exoglucanases when analyzed by the techniques mentioned above (manuscript in preparation).

Our suggestion about the identity of the protein moieties of both exoglucanases was reinforced by measurement of kinetic parameters of native and deglycosylated enzymes. Thus, deglycosylated products showed the same Km against laminarin, in spite of the fact that exoglucanase I showed half of the affinity of exoglucanase II. This behaviour might be ascribed to the longer sugar chains present in the former which in turn would cause steric hindrance in its binding to substrates of high (laminarin) but not low (p-NPG) molecular weight. By contrast, the short sugar chains of exoglucanase II would not have effect on its affinity towards laminarin, since the Km was not modified by treatment of the enzyme with Endo H.

Similar observations were made with regard to thermal stability. Deglycosylated forms exhibited a similar stability at 55 °C although native exoglucanase I was more resistant than native exoglucanase II. Again, the higher carbohydrate content of exoglucanase I might account for this behaviour. Another collateral conclusion from these experiments is that the low carbohydrate content of exoglucanase II does not modify its thermal stability as compared with the *in vitro* deglycosylated form.

An exoglucanase gene from *S. cerevisiae* has recently been cloned (4). This approach may help to elucidate whether it codes for the protein moiety of both exoglucanases or there exists a family of related genes coding for similar proteins.

**Acknowledgements**

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MICROBIOLOGIA SEM 3 (1987), 205 - 207 NOTA

# Detección de cepas de *Penicillium variabile* resistentes a Imazalil y Prochloraz procedentes de un almacén de comercialización de cítricos

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**Summary**

"In vitro" sensitivity of two strains of *Penicillium variabile* to different concentrations of Imazalil and Prochloraz has been studied. Both strains were isolated from a citrus packing-house in which Imazalil was used. These strains were resistant to both compounds showing a $ED_{50}$ higher than 100 ppm.

*Key words:* Penicillium, *citrus, fungicide*

**Resumen**

Se realizaron ensayos «in vitro» con concentraciones de 1, 10, 100 y 1000 ppm de Imazalil y Prochloraz frente a dos cepas de *Penicillium variabile* aislados de un almacén de comercialización de cítricos que utilizaba Imazalil como fungicida.

Las cepas fueron resistentes a ambos compuestos con una $ED_{50}$ superior en ambos casos a 100 ppm.

Uno de los grupos de sutancias activas frente a formas de *Penicillium* resistentes a fungicidas comerciales es el de los imidazoles, particularmente el 1-[2(2,4 diclorofenil)-2-(2 propeniloxi) etil]-1H-imidazol (Imazalil). El uso de este fungicida va en aumento en los almacenes de comercialización de citricos españoles debido a que se ha mostrado eficaz tanto contra cepas de *Penicillium* spp resistentes a los bencimidazoles, como sobre otros patógenos de cítricos, entre los que cabe citar *Phytophthora citrophthora, Alternaria* spp y *Phomopsis* spp.

* A quien debe dirigirse la correspondencia.

En este mismo grupo de imidazoles se incluye también el N-propil-N[2-(2,4,6 triclorofenoxi) etil] carbamoil imidazol (Prochloraz); es un fungicida de amplio espectro, activo contra Ascomicetes y Fungi Imperfecti.

En el presente trabajo se estudia la resistencia de dos cepas de *P. variabile* frente a estos fungicidas, aisladas de un almacén de comercialización de cítricos, localizado en Vila-Real (Castellón), en el que se utilizaba Imazalil como fungicida.

Para su aislamiento se utilizó el medio Agar Suero de Naranja adicionado con 1 o 10 ppm de Imazalil. Las placas empleadas se expusieron al ambiente del almacén durante 30 segundos, incubándose a continuación a 25 °C y humedad relativa ($H^aR$) elevada durante

Las dos cepas se caracterizaron como *P. variabile* de acuerdo con el criterio de Pitt (8).

Los ensayos de resistencia se efectuaron en Agar Suero de Naranja al que se añadió Imazalil y Prochloraz a las concentraciones de 0, 1, 10, 100 y 1000 ppm. Las placas se inocularon con discos fúngicos de 5 mm ∅ procedentes de los bordes de la colonia objeto de prueba y cultivada durante 7-10 días en condiciones óptimas de humedad y temperatura (80-90 % $H^aR$ y 25 °C). Los inóculos se realizaron por cuadruplicado. A los 7 días de incubación se procedió a medir los halos de crecimiento, utilizando para ello una plantilla de círculos comprendidos entre 1 y 36 mm. Se calcularon las medias y, por comparación con el testigo, los porcentajes de crecimiento en cada caso para la posterior obten-

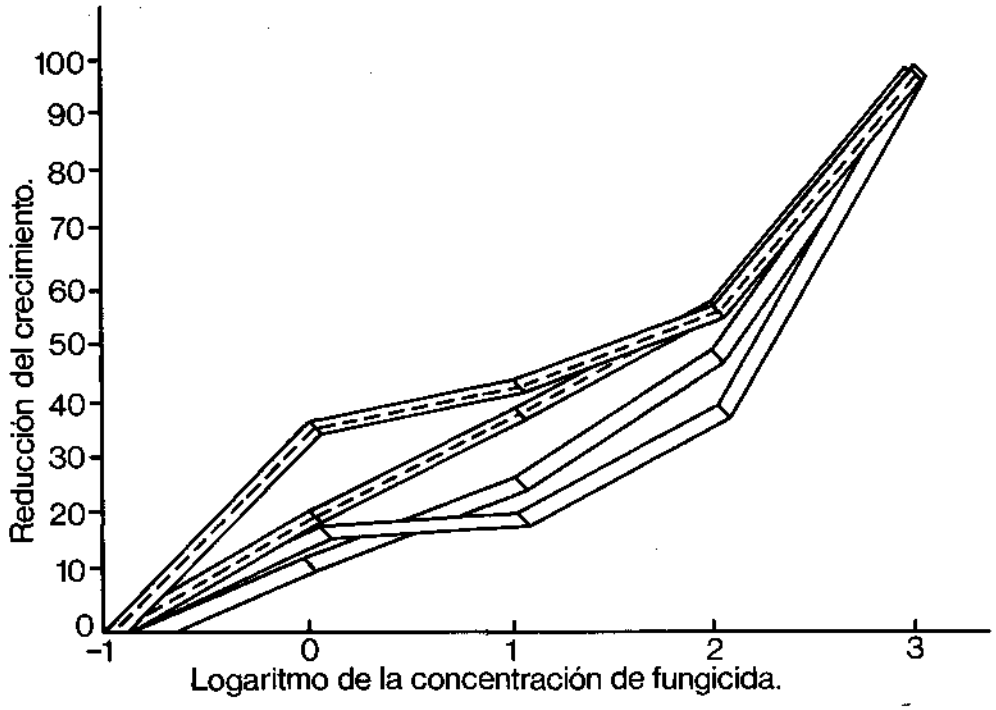


Figura 1. Reducción del crecimiento para las cepas 4R y 5R de *Penicillium variabile* frente a Imazalil y Prochloraz. ▭ Respuesta frente a Imazalil. ▭ Respuesta frente a Prochloraz.

ción de la $ED_{50}$ (concentración del fungicida necesaria para reducir en un 50 % el crecimiento del hongo sobre el agar).

Igualmente se realizaron ensayos con los fungicidas Ortofenilfenato sódico, 2-(4-thiazolil) bencimidazol (Thiabendazol) y metil-1-(butilcarbamoil)-2-bencimidazol (Benomilo), a las concentraciones de 0, 50, 100, 200 y 400 ppm. La secuencia de concentraciones señalada, es la considerada (3) como indicadora de una resistencia media de *Penicillium italicum* frente a los fungicidas bencimidazólicos; de la misma forma, la dosis de 50 ppm. se considera como el umbral entre formas sensibles y resistentes a Ortofenilfenato sódico.

Tal como se sospechaba, al haber sido aislados de placas conteniendo Imazalil, se confirmó la resistencia de estos mohos frente al mismo, así como frente a Prochloraz.

Los resultados se recogen en la Figura 1 donde se representan los porcentajes de reducción de crecimiento de las dos cepas en relación con las concentraciones de estos imidazoles ensayadas.

Como puede observarse, la $ED_{50}$, se estableció en todos los casos alrededor de 100 ppm. Se trata de una concentración notablemente elevada para estos imidazoles considerados hasta el momento de gran eficacia antifúngica. De la misma forma se aprecia un fenómeno de resistencia cruzada entre estos dos compuestos. Este es un fenómeno ya conocido entre los fungicidas sistémicos, como los bencimidazoles (2, 4, 5, 6, 7), pero no citado anteriormente para los imidazoles.

Por otra parte, las dos cepas de *P. variabile* se mostraron sensibles a las concentraciones ensayadas de Ortofenilfenato sódico, Thiabendazol y Benomilo.

La existencia de cepas de *P. variabile* resistentes a Imazalil en los locales de comercialización de cítricos, puede considerarse como una observación de gran trascendencia, avance de posibles fenómenos de resistencia que pueden producirse en las especies de *Penicillium*, causantes habituales de la podredumbre de los cítricos (*P. digitatum* y *P. italicum*), sin descartar la posibilidad, en determinadas circunstancias, de causar lesiones *per se* (1) en los frutos.

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MICROBIOLOGIA SEM 3 (1987), 209 - 212 NOTA

# Aislamiento extraintestinal de *Salmonella* en gallinas: estudio epidemiológico de dos brotes de salmonelosis por consumo de huevo crudo

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*(Recibido Abril 8/Julio 27, 1987)*

**Summary**

A taxonomic analysis of the malolactic microflora present in nine grape samples from different zones of Galicia, was carried out. Nineteen strains were isolated and identified as *Lactobacillus plantarum* (42 %), *L. brevis* (10.6 %), *L. casei* (5.25 %), *L. hilgardii* (5.25 %), *Streptococcus cremoris* (5.25 %), *Leuconostoc lactis* (5.25 %), *L. oenos* (10.6 %), and *Pediococcus acidilactici* (15.8 %).

*Key words: enology, malolactic bacteria, grape, taxonomy.*

**Resumen**

En dos brotes familiares de Salmonelosis, producidos por *S. enterica I* serotipos *typhimurium* y *enteritidis*, se aísla el agente causal en cloaca, hígado, oviducto y ovario de las gallinas implicadas, así como de la yema de un huevo.

Estas observaciones sugieren que ambos serotipos de *Salmonella* pueden encontrarse internamente en los huevos de gallina.

Es un hecho demostrado que los alimentos de origen animal constituyen un importante vehículo en la transmisión de *Salmonella*. En particular la presencia de estos gérmenes en huevos es un factor de riesgo que, en condiciones favorables a la multiplicación de los mismos (temperatura, tiempo, etc.), puede convertir un alimento a base de huevo crudo en un vehículo transmisor de una toxinfección alimentaria, en ocasiones mortal (4,5)

* A quien se enviará la correspondencia

Se admite generalmente que es la contaminación externa de la cáscara de los huevos causada por su paso a través de la cloaca, la responsable de los problemas sanitarios asociados al empleo de derivados no pasterizados de los huevos. En la presente nota recogemos información microbiológica y epidemiológica obtenida en el seguimiento de dos brotes de salmonelosis causados por el consumo de alimentos a base de huevos crudos. Esta información sugiere la contaminación interna del huevo antes de que la cáscara sea formada.

El primero de los brotes estudiados se produjo en Navarra el 11 de junio de 1986, con una familia de cuatro personas adultas afectadas, registrándose el fallecimiento del miembro de mayor edad (86 años). La causa aparente fue el consumo de natillas adornadas con clara batida cruda preparada con huevos procedentes de la pequeña explotación familiar. Aunque no quedaron restos del alimento sospechoso, lo limitado del caso permitió un análisis microbiológico más riguroso de lo habitual. Se analizaron heces de los enfermos, alimentos, huevos y órganos de gallinas (cloaca, hígado, ovario y oviducto) después de proceder a la autopsia de éstas.

La presencia de *Salmonella* en los huevos se investigó tanto en la cáscara como en el interior. Para ello se aplicó el procedimiento de Baker y Goff (1) con ligeras modificaciones. Los huevos se introducían en bolsas de polietileno estériles, cubriéndolos totalmente con caldo tripticasa de soja (TSB) y agitando suavemente con movimientos de rotación durante 1 minuto aproximadamente.

Posteriormente el huevo se retiraba de la bolsa esterilizando su superficie por inmersión en alcohol de 95% y flameando la cáscara. Después de apertura aséptica, se vertía su contenido en una nueva bolsa de polietileno, adicionando 100 ml de TSB. Esta bolsa, junto con la que contenía la solución de lavado de la cáscara, cerradas ambas mediante aplicación de calor, se incubaban a 37 °C durante 18 h. A partir de aquí se pasaban alícuotas de 1 ml a los diferentes medios de enriquecimiento de *Salmonella* (caldo tetrationato de Muller-Kauffmann y Rappaport modificado) que se incubaban a 43 °C. (3, 6). Se aisló *S. enterica I* serotipo *typhimurium* en las heces de los afectados, así como en cloaca, oviducto, ovario e hígado de algunas gallinas de la granja familiar. Igualmente fue aislada de la solución de lavado de la cáscara y del interior de uno de los 16 huevos restantes del lote empleado en la preparación del alimento. Todas las cepas aisladas pertenecían al mismo fagotipo 96 (Tabla 1).

TABLA 1
DISTRIBUCION DE LOS AISLAMIENTOS DE *SALMONELLA* EN ORGANOS DE AVES

| Brote | Gallina | Cloaca | Oviducto (1) | Oviducto (2) | Ovario | Hígado | Serotipo (Fagotipo) |
|---|---|---|---|---|---|---|---|
| 1 | 1 | + | – | – | – | – | typhimurium (96) |
| | 2 | + | + | + | + | + | typhimurium (96) |
| | 3 | + | – | – | – | + | typhimurium (96) |
| | 4 | + | + | – | – | – | typhimurium (96) |
| 2 | 5 | + | – | + | + | – | enteritidis |

(1) Mitad inferior.
(2) Mitad superior.

En el segundo brote, ocurrido el 18 de agosto en otra localidad, resultaron afectados seis miembros de una familia de siete, con síntomas más leves que en el caso anterior y sin hospitalizaciones. El alimento implicado fue un helado casero cuyos restos, conservados en el congelador, pudieron ser analizados. De igual manera se analizaron huevos del lote empleado en su confección y las gallinas de la granja familiar. Aunque en este brote no se aislaron salmonelas de los huevos, identificada *S. enterica I* serotipo *enteritidis* en las heces de los afectados, restos del helado ($3,2 \times 10^5$ ufc/g) y en la cloaca, mitad superior del oviducto y ovario de una de las gallinas analizadas (Tabla 1). El fagotipo del germen causal de este brote está en vías de determinación.

La presentación de ambos brotes, por su carácter familiar y el rápido acceso a casi todos los puntos de la cadena epidemiológica, ha permitido un análisis preciso de los mismos. Por esta razón los datos obtenidos pueden ser significativos ya que, al tratarse de dos brotes distintos causados por dos gérmenes diferentes, cabe pensar que la localización extraintestinal de Salmonelas relacionadas con toxinfecciones alimentarias no es tan infrecuente en las aves de corral. En este sentido, hay que señalar que la localización en el ovario de *S. pullorum* y *S. gallinarum* ha sido ya ampliamente demostrada, así como en hígado y bazo (1), si bien estos serotipos están escasamente relacionados con infecciones en humanos. De igual manera, existe información (Carranza, J., y col. 1985. Res. XXIII Symp. de Avicultura, p. 349) (Saco, M. y San Gabriel, A. 1986. Res. XXIV Symp. de Avicultura, p. 95) que confirma nuestros resultados en lo relativo a la presencia de estos serotipos en órganos internos de aves de corral. Es también llamativa la presencia de *S. enterica I* serotipo *typhimurium* en el interior de uno de los huevos analizados en el primer brote. Aunque no se aisló *S. enterica I* serotipo enteritidis en esta ocasión, tenemos igualmente evidencia de su aislamiento en el interior del huevo (Goñi, P. y col 1986. Res. V Reunión Nac. Microbiología de Alimentos, p. 75) (Perales, I. y Audicana, A. 1986. Res. V Reunión Nac. de Microbiología de Alimentos. p. 119). Estos hechos, junto a los relativos a la presencia de estos serotipos en órganos relacionados con la ovogénesis, sugiere la posibilidad de la contaminacióndel huevo antes del desarrollo de la cáscara.

Si se considera que en el período 1976-1984 el 47,63 % de los brotes de toxinfección alimentaria tuvieron como vehículo la mayonesa casera y que en el 91,72 % de estos brotes se aislaron salmonelas (Boletín Epidemiológico Semanal del Ministerio de Sanidad y Consumo, n.º 1755/86), es evidente el riesgo que representa en sí mismo el consumo del huevo crudo independientemente de las manipulaciones incorrectas desde el punto de vista higiénico a que pueda ser sometido.

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# Estudio taxonómico de bacterias lácticas epifitas en uvas de Galicia

Dolores Agrelo, Elisa Longo, Pilar Combarro, Manuel J. Garrido, Tomás G. Villa*

(Recibido 15 Diciembre 1986/1 Septiembre 1987)

**Summary**

A taxonomic analysis of the malolactic microflora present in nine grape samples from different zones of Galicia, was carried out. Nineteen strains were isolated and identified as *Lactobacillus plantarum* (42 %), *L. brevis* (10.6 %), *L. casei* (5.25 %), *L. hilgardii* (5.25 %), *Streptococcus cremoris* (5.25 %), *Leuconostoc lactis* (5.25 %), *L. oenos* (10.6 %), and *Pediococcus acidilactici* (15.8 %).

*Key words: enology, malolactic bacteria, grape, taxonomy.*

**Resumen**

Se analizó taxonómicamente la flora maloláctica epífita de 9 muestras de uvas recogidas en distintas comarcas vitivinícolas gallegas. De 19 cepas aisladas se identificaron, las especies: *Lactobacillus plantarum:* 42 %; *Lactobacillus brevis:* 10,6 %; *Lactobacillus casei:* 5,25 %; *Lactobacillus hilgardii:* 5,25 %; *Streptococcus cremoris:* 5,25 %; *Leuconostoc lactis:* 5,25 %; *Leuconostoc oenos:* 10,6 %; *Pediococcus acidilactici:* 15,8 %.

Al considerar las comarcas vinícolas de Galicia, se observa que el problema de la fermentación maloláctica presenta aspectos de diferente importancia y significación según la región de que se trate. Así, en las comarcas del ecosistema de transición (Ribeiro) no suelen alcanzarse niveles elevados de ácido málico (1), por lo que la aplicación de la fermentación maloláctica es discutible; sin embargo, en las comarcas del ecosistema atlántico (Condado y Rosal), los vinos contienen altas concentraciones de ácido málico (8 g/l) (1), y en ellos la desacidificación es un factor de notable utilidad en la mejora de la calidad vínica. En el presente trabajo se llevó a cabo el aislamiento e identificación de la

(*) A quien se dirigirá la correspondencia.

flora maloláctica epífita en uvas de diferentes variedades, cultivadas en Galicia, en los dos ecosistemas anteriormente citados. Hasta donde sabemos, los resultados presentados en este trabajo, constituyen el primer estudio taxonómico de la flora maloláctica epifita de uvas de Galicia.

En la vendimia de 1985 se recogió una muestra de cada una de las siguientes variedades de uva: Godello, Palomino y Treixadura de la comarca del Ribeiro, Loureiro, Albariño y Treixadura de la comarca del Rosal y Negrón, Torrontés y Espadeiro, de la comarca del Condado. De la variedad Treixadura se tomaron dos muestras, correspondientes a las zonas Rosal y Ribeiro. Las muestras se tomaron en varias bodegas inmediatamente después de recogida la uva; cada una de ellas se compuso de cuatro racimos semejantes elegidos al azar que fueron tomados asepticamente y transportados al laboratorio en recipientes estériles.

El aislamiento se realizó, para cada muestra, enriqueciendo en medio líquido el mosto obtenido al prensar los cuatro racimos; éste se repartió en volúmenes de 20 ml, en matraces con idéntico volumen de medio, compuesto por mosto y zumo de tomate (5). Tras incubación a 30 °C durante 5 días en atmósfera de $CO_2$, se sembró una alicuota de cada matraz en placas de agar nutritivo W. L. (Oxoid) con 20 mg/l de cicloheximida. Tras otros 5 días de incubación en las mismas condiciones, se aislaron las colonias de morfología diferenciada. Los cultivos puros fueron sembrados en estría en tubos de medio MRS (Oxoid) y mantenidos a 4 °C.

Las cepas se identificaron según los criterios establecidos en el Manual de Bergey (7, 8), empleando las siguientes pruebas taxonómicas: tinción de Gram, reacción de la catalasa (3). Fermentación de compuestos carbonados: se usó el método de Hugh y Leifson (4) con tropeolina como indicador; a una concentración del 1 % se ensayaron los azúcares: arabinosa, celobiosa, fructosa, galactosa, glucosa, inulina, lactosa, maltosa, manitol, manosa, melibiosa, rafinosa, sacarosa, salicina, sorbitol, trehalosa y xilosa; los resultados se leyeron después de 9 días de incubación a 30 °C en cultivos de 5 ml cubiertos con aceite de parafina. Formación de gas de glucosa: se observó en medio YP (Bacto-Yeast Extract, 5 $g.l^{-1}$; Bacto-Peptone, 1,5 $g.l^{-1}$ ; pH 5,0) adicionado de glucosa al 5 % y repartido en tubos con campana Durham, manteniendo el cultivo a 30 °C durante 4 días. Producción de etanol y ácidos D- y L-láctico: se cuantificaron mediante tests enzimáticos (Boehringer Mannheim) siguiendo las instrucciones proporcionadas por el fabricante. Crecimiento a diferentes valores de pH: para completar la identificación del género *Pediococcus*, se observó el crecimiento en medio MRS (Oxoid) a pH 7,0, pH 9,0 y pH 9,0 con 5 % de ClNa, a las temperaturas de 30 °C y 50 °C. Determinación del punto térmico letal: para la identificación de las especies del género *Pediococcus* se determinó el crecimiento en medio MRS (Oxoid), incubando distintas suspensiones celulares a 60 °C y a 70 °C durante 10 minutos y a 65 °C durante 8 minutos. Crecimiento a diferentes temperaturas y valores de pH: para confirmar la pertenencia de algunos aislados al género *Streptococcus*, se determinó su crecimiento en medio MRS (Oxoid) (adicionado con NaCl al 6.5 %) ajustado el pH a 5,0 e incubando a 10 °C y a 30 °C y a pH 9,6 incubando a 45 °C y a 30°C.

De las 9 muestras estudiadas se obtuvieron 19 cepas, aislando una media de 2 cepas por variedad de uva. La Tabla 1 indica el número de aislamientos de cada especie en cada variedad de uva, junto con su valor porcentual referido al total de aislados. Se observa que la variedad Treixadura del Rosal, permitió el mayor número de aislamientos, con 5 de un total de 19 (26,3 %). Las siguientes variedades son Palomino y Loureiro con un

15,7 % de los aislamientos en ambas. Para el resto, la frecuencia de aparición es de 5.3 %, que equivale a un aislamiento de cada variedad. La baja proporción de aislamientos en cada variedad de uva, confirma la dificultad ligada al aislamiento de éstas bacterias, ya mencionada en otros estudios sobre diversas regiones vinícolas del mundo (2, 6). Los problemas de aislamiento de bacterias malolácticas tienen su origen en la relativa minoría numérica de estas poblaciones respecto a otros grupos microbianos y en sus exigencias nutricionales. Esta situación ha creado notables dificultades de aislamiento, principalmente cuando éste se realiza en uvas, en las que la densidad de población es mucho menor que en la fermentación maloláctica y en vinos jóvenes. Señalamos, además, las condiciones particulares de las comarcas bajo estudio: el clima, frecuentemente adverso en Galicia para el viñedo, obliga a varios tratamientos antifúngicos durante el año y la acusada pluviosidad provoca un efecto de «lavado» de la uva. Además, las temperaturas moderadas y el bajo número de horas solares, impiden la total madurez de la uva en el momento de la vendimia. Todos estos factores reducen las poblaciones microbianas, lo que contribuye a explicar los bajos porcentajes de aislamiento encontrados.

El análisis taxonómico de los 19 aislados, revela la presencia de 8 especies, distribuidas en los siguientes géneros: *Lactobacillus, Leuconostoc, Pediococcus* y *Streptococcus*. Las más frecuentes son: *Lactobacillus plantarum* y *Pediococcus acidilactici* que representan un 42 % y un 16 % respectivamente del total de aislados, apareciendo en 3 variedades viníferas de las 9 estudiadas (Tabla 1). Se observa también que la variedad Treixadura del Rosal presenta la mayor diversidad poblacional siendo la única que permitió el aislamiento de tres especies bacterianas diferentes, aunque todas del género *Lactobacillus*. Esto contrasta con el resto de las variedades, excepto Loureiro, para las que se detectó una sola especie.

TABLA 1
ESPECIES BACTERIANAS MALOLACTICAS AISLADAS A PARTIR DE 9 VARIEDADES DE UVA

| VARIEDADES DE UVA | BACTERIAS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Lactobacillus | | | | Leuconostoc | | Pediococcus | Streptococcus |
| | *L. plantarum* | *L. brevis* | *L. casei* | *L. hilgardii* | *L. lactis* | *L. oenos* | *P. acidilactici* | *S. cremoris* |
| Albariño | 2.[a] (10,5)[b] | – | – | – | – | – | – | – |
| Espadeiro | – | – | – | – | – | – | 1 (5,3) | – |
| Godello | – | – | – | – | – | 2 (10,5) | – | – |
| Loureiro | – | 2 (10,5) | – | – | 1 (5,3) | – | – | – |
| Negrón | – | – | – | – | – | – | – | 1 (5,3) |
| Palomino | 3 (15,8) | – | – | – | – | – | – | – |
| Torrontés | – | – | – | – | – | – | 1 (5,3) | – |
| Treixadura del Rosal | 3 (15,8) | – | 1 (5,3) | 1 (5,3) | – | – | – | – |
| Treixadura del Ribeiro | – | – | – | – | – | – | 1 (5,3) | – |

[a]: Número de cepas
[b]: % del total de cepas aisladas

Aunque el número de aislados no permite obtener conclusiones definitivas, resaltamos las diferencias en cuanto a las especies aisladas en variedades de uvas del ecosistema atlántico y de transición, particularmente los obtenidos con las dos muestras de Treixadura procedentes de la comarca del Ribeiro y la del Rosal. Los aislamientos en uvas del ecosistema de transición son siempre monoespecíficos.

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# AUTHOR INDEX

## VOLUMEN 3

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Tomasz, A. (1984). Building and breaking in the cell wall of bacteria - The role for autolysins. *In:* C. Nombela (ed.) Microbial Cell Wall Synthesis and Autolysis. pp. 3-12. Elsevier Science Pub. B. V. Amsterdam.

References to thesis, manuscripts not accepted for publication or Meetings should be indicated in the text as follows: (Garcia, P. *et al.* 1985. in preparation), (Smith, T. 1985.

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NOTAS. Las Notas, que no deberán exceder de seis páginas mecanografiadas incluyendo figuras y tablas, tienen por objeto la presentación de observaciones experimentales, descripción de técnicas o modificaciones metodológicas de interés. Su redacción se efectuará ateniéndose a las Normas previamente descritas para los trabajos, pero suprimiendo las divisiones con encabezamiento y con resúmenes no superiores a 50 palabras. Sólo incluirán, como máximo, dos figuras y una tabla o viceversa.

ARTICULOS DE REVISION. Los artículos de revisión versarán sobre temas de microbiología de gran interés, y su redacción se solicitará a especialistas. Podrán incluir además del Resumen un índice de contenido.

PRUEBAS. Los autores recibirán pruebas que deberán devolver en plazo no superior a una semana. Transcurrido dicho plazo sin devolución de las pruebas, éstas serán corregidas por la revista y publicado el trabajo. Las correcciones se limitarán a errores tipográficos, gramaticales o de datos incorrectos. Modificaciones más importantes que impliquen recomposición del texto, deberán ser abonadas por el autor. Se enviarán 25 separatas gratuitas por artículo; si se desearan más, deberá indicarse por escrito cuando se devuelvan las pruebas corregidas. Las separatas adicionales serán facturadas a precio de coste.

Dos copias de cada manuscrito se enviarán a: «Microbiología» (Publicación de la SEM). c/ Vitruvio, 8. 28006 Madrid o al Editor de la Revista que este más relacionado con el contenido del trabajo.

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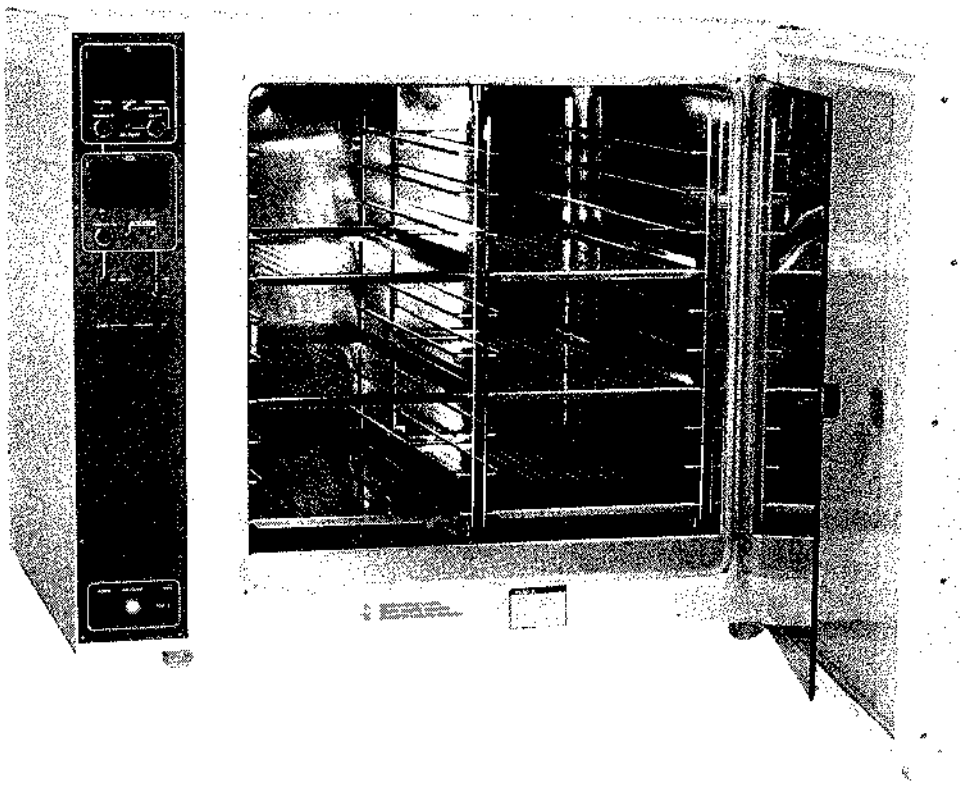

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**AHORRA ESPACIO, AHORRA TIEMPO.** Al diseñar la TL-100 (y su gama de rotores) específicamente para muestras tan pequeñas, hemos podido reducir también el tiempo de centrifugación. En trabajos como; aislamiento de plásmidos de DNA, separación de gradientes, estudios de ligando, determinaciones de pesos moleculares, ensayo de receptores o separación de proteínas, se tarda mucho menos tiempo.

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**MUY ECONOMICA DE MANTENER.** A pesar de que el tamaño se ha reducido, la calidad no lo ha sido. Se ha asegurado la fiabilidad de la TL-100 en el diseño, incluyendo el sistema de giro por inducción, que elimina el delicado sellado de vacío y las escobillas. Esta sencillez de diseño evidente en la TL-100 crea una nueva categoría de máquina.

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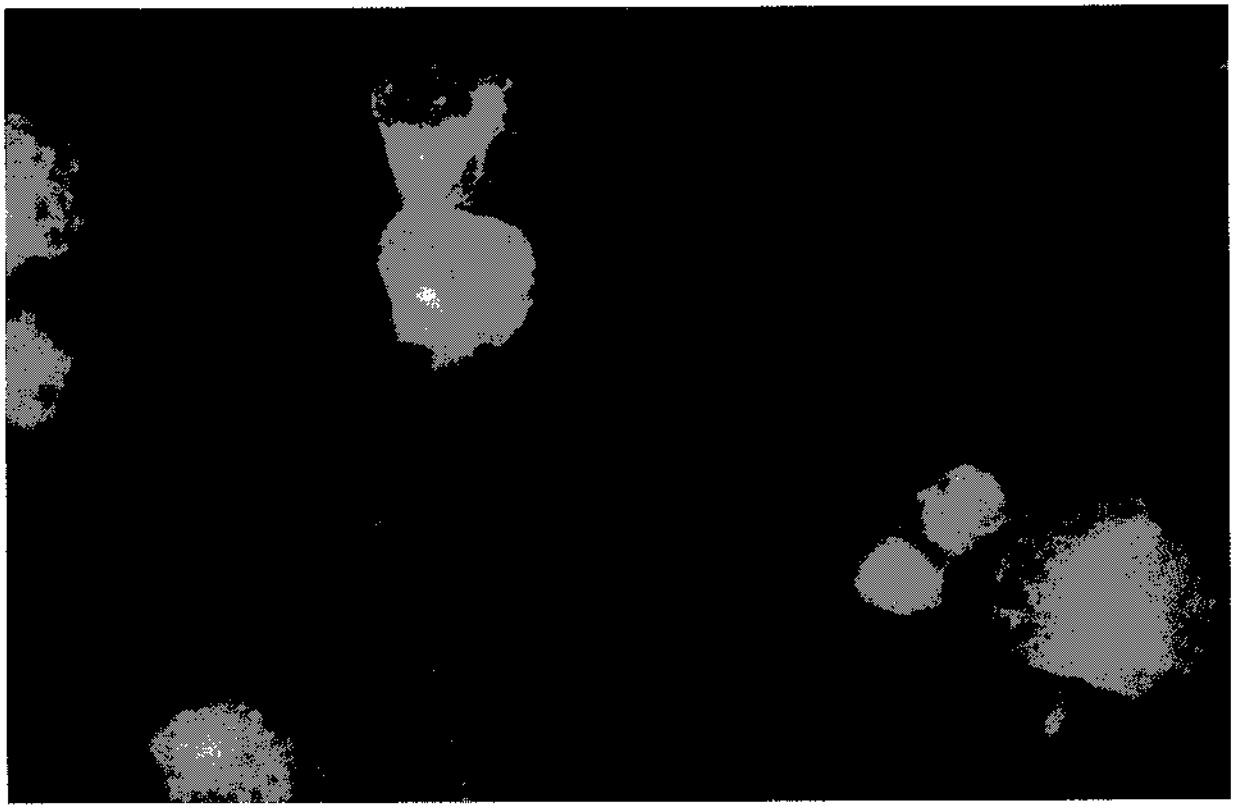
## Determinación de INMUNOFLUORESCENCIA INDIRECTA para la determinación de "HTLV III"



### DETERMINACIONES mediante INMUNOFLUORESCENCIA INDIRECTA

* Anticuerpos anti - mitocondriales
* Anticuerpos anti - nucleares
* Anticuerpos anti - nDNA
* Anticuerpos frente a Chlamydia Trachomatis.
* Anticuerpos frente al Citomegalovirus.
* Anticuerpos frente al V. de Epstein - Barr
* Anticuerpos frente al Treponema (FTA - ABS)
* Anticuerpos frente al V. Respiratorio Sincitial.
* Anticuerpos frente al V. Herpes Simplex 1
* Anticuerpos frente al V. Herpes Simplex 2
* Anticuerpos frente al V. del Sarampión.
* Anticuerpos frente al V. de las Paperas.
* Anticuerpos frente al V. de la Rubeola.
* Anticuerpos frente al Toxoplasma.
* Anticuerpos frente al V. Varicela - Zoster.

* DETERMINACION IFA para "HTLV - III"

### DETERMINACIONES mediante INMUNOFLUORESCENCIA DIRECTA

Test de identificación y tipificación del Virus Herpes Simplex, tipos 1 & 2.

### DETERMINACIONES mediante SISTEMA "ELISA"

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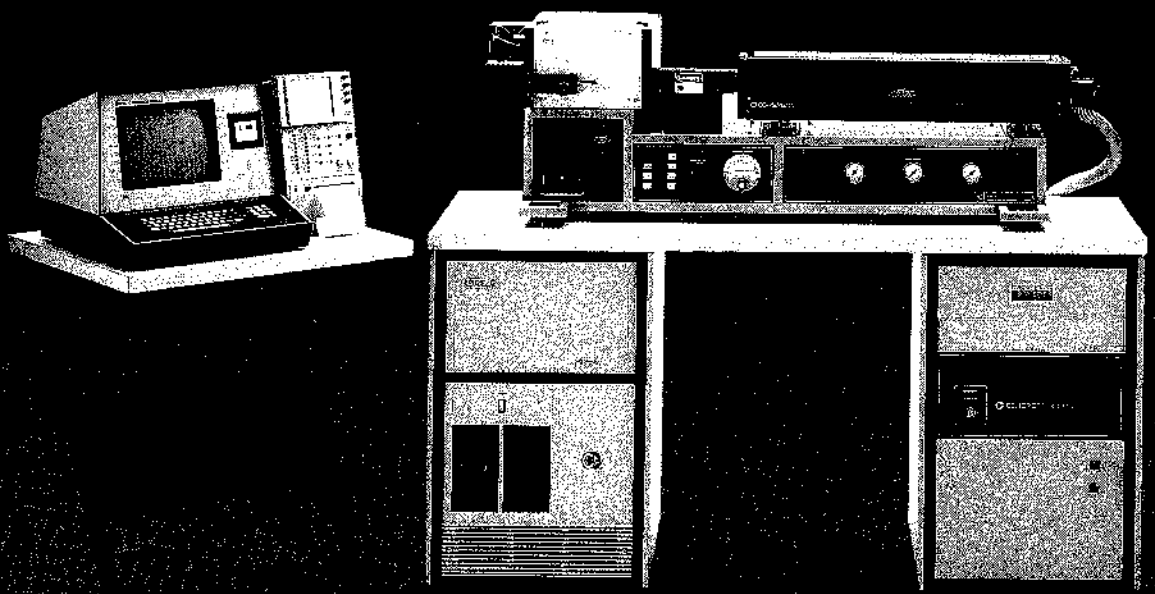
# LA CITOMETRIA DE FLUJO



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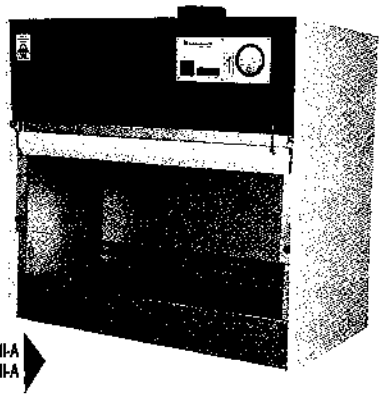
# Cabinas de seguridad biológica «Biohazard»



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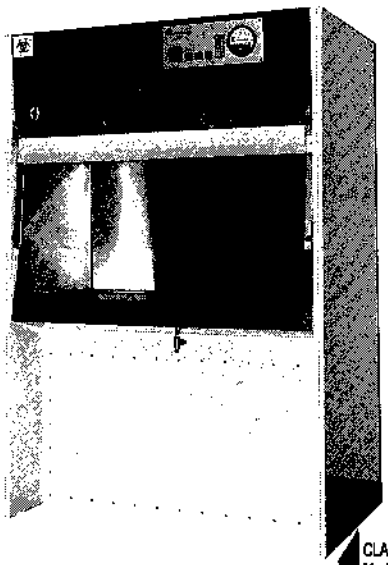
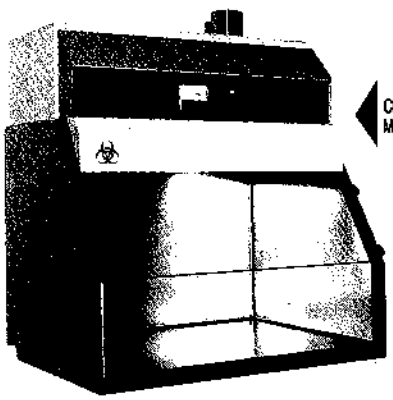


CLASE II-B
Mod. BIO-II-B



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