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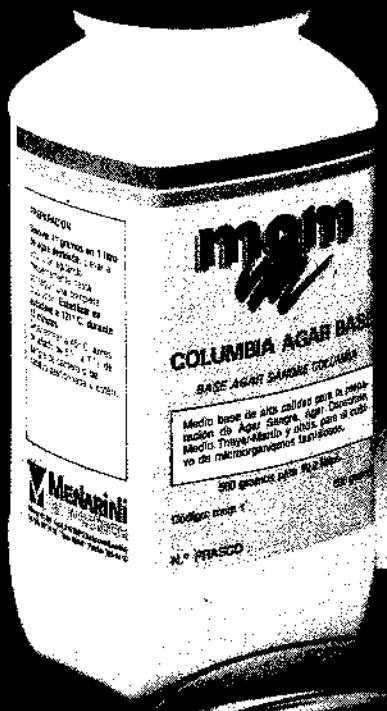

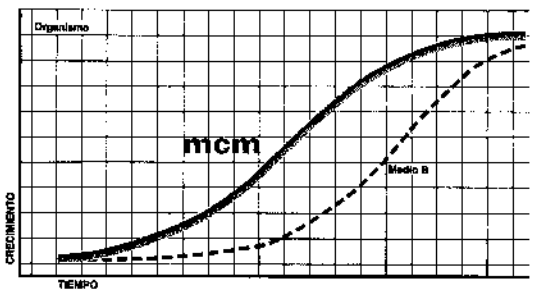

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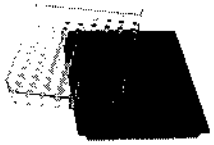

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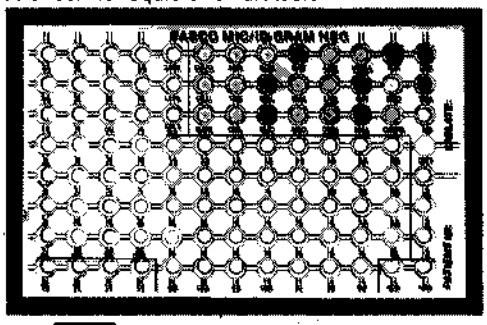

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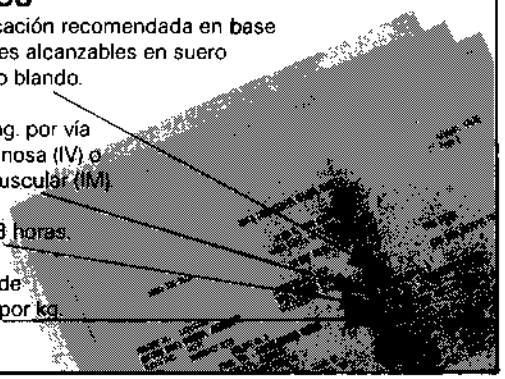

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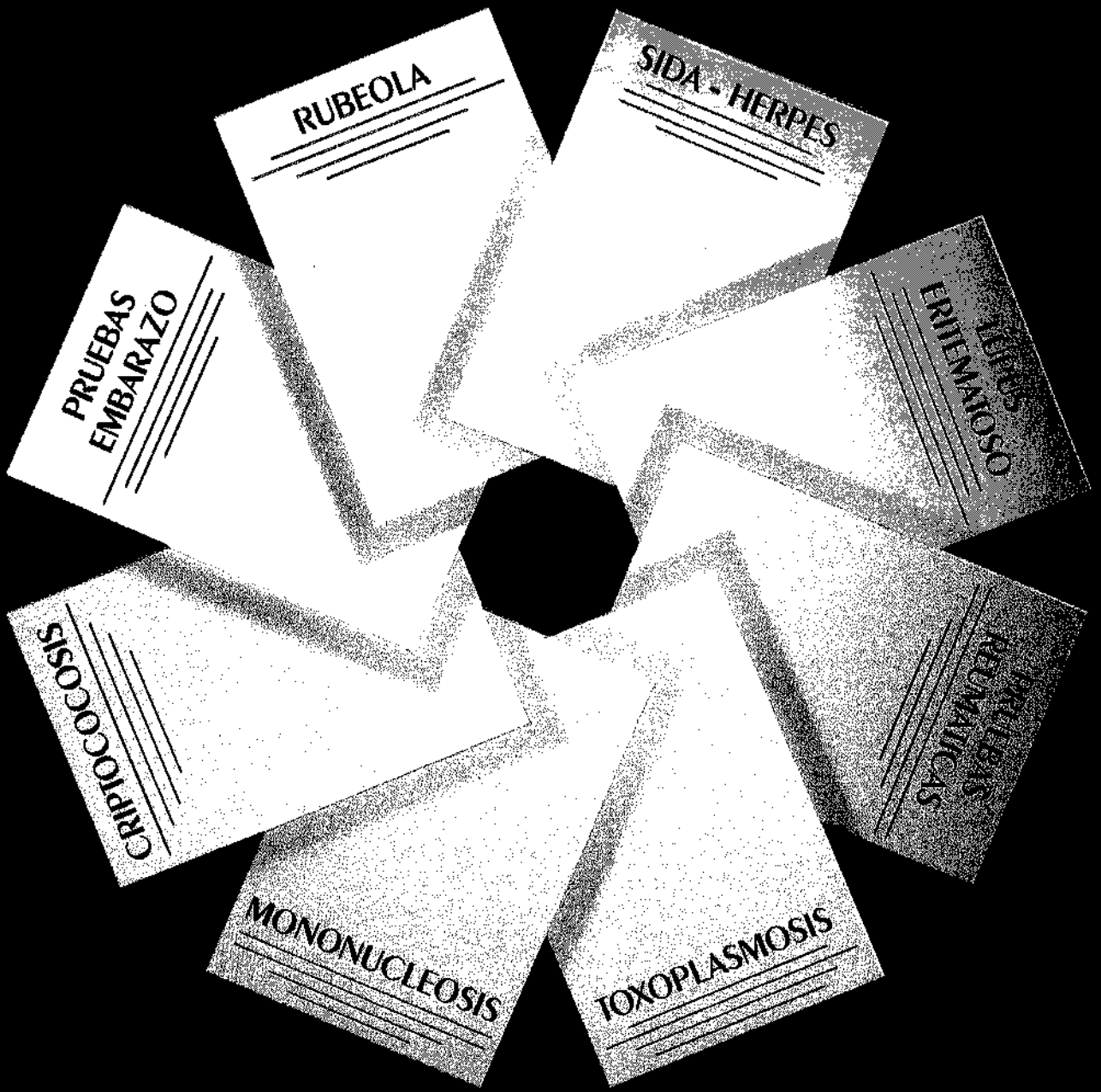

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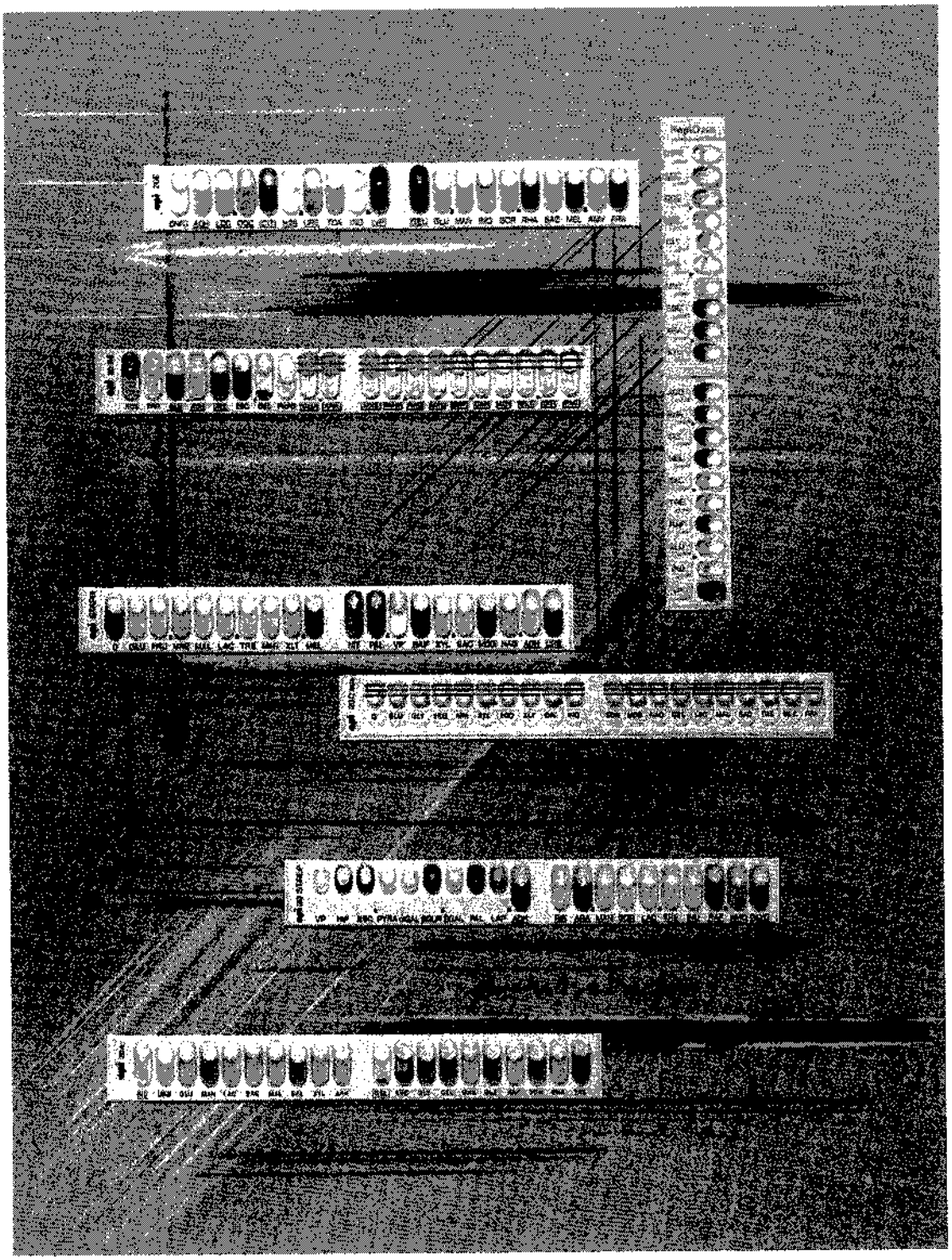

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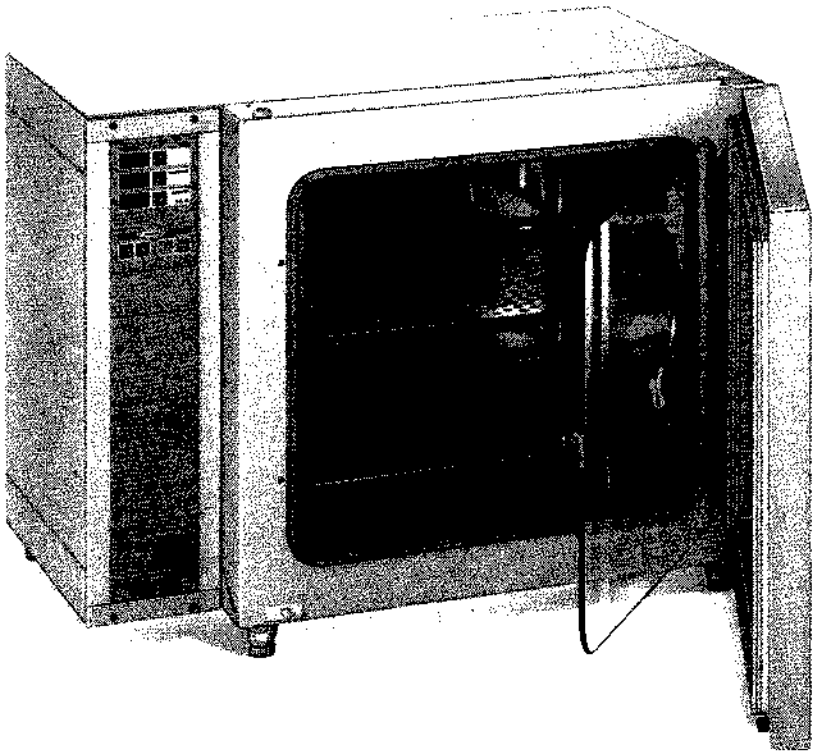

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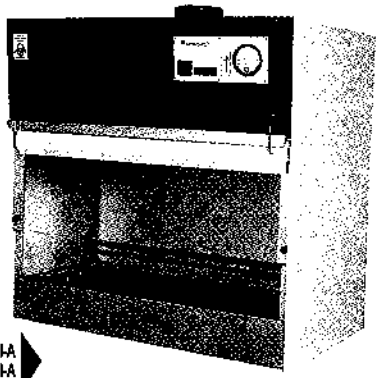

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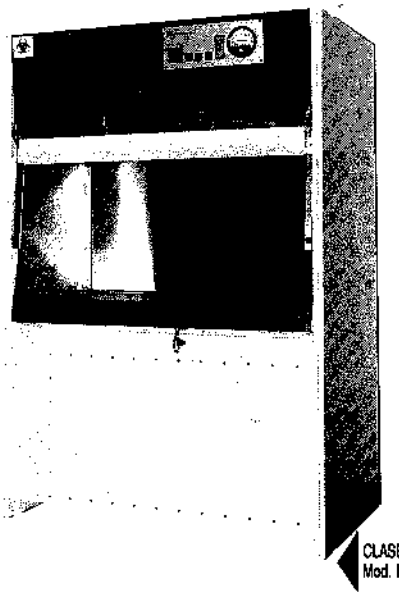

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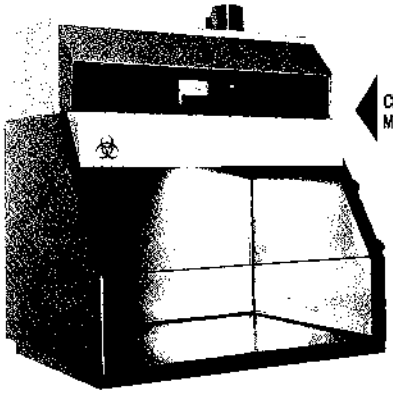

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MICROBIOLOGIA SEM 6 (1990), 1-10 **MINIREVIEW**

Genomic imprinting in microorganisms

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(Received December 14, 1989)

Summary

Genomic imprinting is an epigenetic mark introduced on a DNA molecule without alteration of the base sequence. Upon replication, the primary mark is propagated to the daughter DNA molecules. Epigenetic DNA modification often serves as a regulatory signal and may play a crucial role in many developmental processes. Although this mode of gene regulation was first discovered in multicellular eukaryotes, cases of imprinting have been recently found in lower eukaryotes, bacteria and phage. Thus it may be reasonable to list DNA modification among the major mechanisms that regulate gene expression.

Key words: DNA modification - gene regulation.

Resumen

La impronta genómica es una marca epigenética que se introduce en una molécula de ADN sin alterar la secuencia de bases. Cuando el ADN se replica, la marca primaria se propaga a las moléculas hijas. La modificación epigenética del ADN puede servir de señal reguladora y probablemente desempeña una función crucial en muchos procesos de desarrollo. Aunque este tipo de regulación se descubrió en eucariotas multicelulares, también existe en eucariotas inferiores, bacterias y fagos. Por tanto, tal vez sea hora de incluir a la modificación de ADN entre los mecanismos generales de regulación genética.

Introduction

Today's paradigm of genetic regulation emphasizes the role of trans-acting molecules (usually proteins) that switch on and off the intracellular flow of genetic information at a certain level (often acting upon transcription). Under this paradigm, the ADN sequences that embody the genetic information play a passive role: their activation or repression is decided by elements external to the sequence. This review explores a completely different mechanism of gene regulation, in which DNA sequences are turned on and off by signals present on the DNA itself. The classical example is the inactivation of one chromosome X in female mammals; other cases are known in a variety of organisms, from phage to higher eukaryotes. The mechanisms underlying genetic inactivation are still poorly understood; whatever they are, all must be based on «genomic imprinting», that is, the

(*) Corresponding author

inheritable modification of DNA without alteration of the nucleotide sequence. The increasing number of reports on differential, sex-dependent gene expression suggests that genomic imprinting may be more common than previously expected. Moreover, the recent discovery of cases of genetic inactivation in bacteria *(Bacillus, Azotobacter)* and phage (P22) indicates that this mode of gene regulation is not confined to eukaryotes.

A brief history of genomic imprinting

In sexually-reproducing organisms, deviations from Mendelian inheritance are often due to the influence of cytoplasmic factors. However, examples exist where non-Mendelian inheritance of certain traits is known to be caused by differences between the male and the female nuclear genomes. The first of such cases, the R-mottled phenotype of maize, was described by R. Emerson in 1918, although its proper interpretation has taken more than half a century (32). Unlike animals, flowering plants reproduce sexually by double fertilization: the embryo develops from two sperm cells, one of which fertilizes the egg, while he other fuses with the two haploid nuclei of the central cell in the embryo sac, giving rise to the triploid endosperm. The two sperm of one pollen grain are isogenic; likewise, the two nuclei of the central cell derive from the same meiotic product. The R locus controls the synthesis of pigments in the aleurone layer of the kernel, derived from the triploid endosperm. In reciprocal crosses between colored and colorless varieties, Emerson found that the R-phenotype of the offspring was parentally sex-biased: kernels receiving R from the mother were colored, while those receiving R from the father were mottled. This result was first interpreted as a consequence of unequal gene dosage: since the central cell is binucleate, reciprocal crosses between pollen and ears from strains carrying or not the allele R yield different doses in the endosperm. However, detailed analysis carried out by J. L. Kermicle has provided a completely different explanation: the asymmetry is caused by uniparental imprinting of the R locus. In the female gametophyte, the color-producing allele R is modified. The modification causes absolute turn-off of the gene. Imprinting of the R locus is stable, but can be erased if the inactive allele is passed through the male gametophyte in the following generation (24).

Six decades after Emerson's experiments, many examples of differential genetic contribution by paternal and maternal gametes are known. In animals, the most extreme situation is found in some mealy bugs, in which the paternal set of chromosomes is completely inactivated during development: therefore, the father does not contribute to gene expression in somatic tissues (40). Mealy bugs are not a bizarre exception: imprinting seems to be extremely common in insects (see ref. 6 for a review). However, the classical and most thoroughly studied example of epigenetic modification of DNA is the inactivation of the X chromosome in female mammals. By the thousandcell stage of embryogenesis, one X chromosome undergoes condensation and becomes transcriptionally silent (26). This inactivation is stably inherited but fully reversible: during oogenesis the inactive X becomes reactivated (8). In marsupials, the inactive X chromosome is always paternal (22). In the embryos of eutherian mammals, X inactivation occurs randomly, so that the inactive chromosome may be from paternal or maternal origin. However, inactivation always affects the paternal X in the extraembryonic membranes of eutherian mammals (22).

Mammalian imprinting not only occurs at the chromosome X. Differential expression of paternal and maternal genomes appears to exist for many autosomal loci of both the mouse and the human genome (33, 39). For instance, in the mouse, the paternal set of autosomes seems to be more important for the development of extraembryonic tissues, while the maternal genome is more important for embryonic development (33). Examples of individual markers whose expression seems to be impaired by their paternal or maternal origin are also known (17, 33, 39). To be differentially expressed when contributed by the paternal or the maternal genome, sex-dependent imprinting of

genes must occur. Imprinting must be established before or during gametogenesis and persist stably throughout DNA replication and somatic cell division. Later, imprinting must be erased in the germ line to be differentially established once again in sperm and egg genomes. Cyclic imprinting and erasure may have been of evolutionary importance as a mechanism to force organisms to undergo sexual reproduction. It may also account for the developmental failure of parthenogenetic embryos in mammals (39). Sex-dependent imprinting may be also the clue to explain the odd patterns of inheritance found for a number of human diseases (17, 38, 39).

Imprinting in eukaryotic microbes: Mating type interconversion in fission yeast

The yeast *Schizosaccharomyces pombe* usually exists in a haploid state and has two mating-cell types, called P («plus») and M («minus»). The mating type is controlled by alternate alleles of the *mat1* locus. These alleles can interchange («switch»), following a nonrandom pattern: of a pair of sister cells, only one member gives rise to switched progeny. Recently switched cells cannot produce switched progeny in the next generation: an unswitchable cell gives rise to one unswitchable and one switchable cell; the latter will switch in the following generation. The mechanism of switching is a transposition-substitution event involving loci *mat2-P* and *mat2-M*. These loci respectively contain «plus» and «minus» information, but both are transcriptionally silent; they serve only as donors of the «plus» and «minus» information used to transform the *mat1* locus. The switch is a gene conversion of the transcriptionally active *mat1* locus with a copy of either *mat2-P* or *mat2-M*.

Why does only one member of a pair of sister cells produce switched progeny? Fascinating studies carried out by Amar Klar (25) indicate that the competence for switching is acquired by epigenetic marking at the *mat1* locus. According to Klar's hypothesis, one of the two strands of the DNA receives a special strand-and sequence-specific modification that allows the DNA modecule to be cut «in vivo». Site-specific cutting is a requisite for the gene conversion process. In the absence of strand modification, *mat1* DNA is not cleaved and switching does not occur. The chemical nature of the signal is not yet known; Klar speculates with several possibilities: (I) methylation or some other base modification; (II) an unrepaired RNA primer of Okazaki fragments; (III) a protein complex that segregates with a specific strand; and (IV) a site-specific single-stranded nick that becomes a double-stranded break in the next round of replication. Whatever the signal is, it works in an opposite mode to the restriction-modification system of prokaryotes, where imprinting of specific sequences prevents DNA cutting by restriction endonucleases (see below).

Genomic imprinting in prokaryotes

Host-controlled restriction and modification

The best known (and probably the simplest) case of genomic imprinting described in bacteria is the modification of DNA sequences recognized by restriction enzymes. The terms «restriction» and «modification» describe the different behavior of bacteriophage on different strains of host bacteria (1, 3). The basic observation, first made by Giuseppe Bertani in the fifties, was that the plaquing efficiency of a given preparation of bacteriophage largely depended on the last host where the bacteriophage had replicated. For instance, a phage λ suspension grown on *E. coli* K will successfully multiply on strain K, but will grow poorly on strain C. If the few phage particles obtained on strain C are used to reinfect the same strain, they will now produce a spectacular burst; however, these particles will produce a poor infection on strain K. It appears that *Escherichia coli* K, known as the restricting host, possesses the ability to reduce the biological activity of phage DNA; howe-

ver, the restricting host *Escherichia coli* K also appears to be able to effectively modify some of the infecting λ DNA molecules in such a way that they will now grow efficiently on this host in subsequent infections. The former process is known as «restriction»; the latter is called «modification». Elegant experiments carried out at Werner Arber's laboratory during the sixties showed that restriction is caused by cleavage of infecting DNA by restriction endonucleases, while modification results from DNA methylation (2). Methylation of specific bases in the target sequences recognized by restriction endonucleases protects an organism's own DNA from being digested by its own nucleases. Unmethylated «foreign» DNA is recognized as soon as it enters the bacterial cell; it is immediately digested and rendered biologically inactive. This explains why infecting phage DNA performs so poorly in a restricting host and why the few DNA molecules that have escaped restriction will grow efficiently in further infections (2). The small fraction of surviving DNA is replicated in the presence of modifying methylases and becomes methylated. In subsequenct infections of the same host this DNA will, therefore, be protected from attack by restriction endonucleases. Of course, the process affects not only phage DNA but other genetic transfer processes such as conjugation, transduction and transformation.

Restriction-modification systems are common throughout the prokaryotic world, giving rise to the variety of restriction enzymes used in recombinant DNA techonology. All restriction systems consist of an endonuclease and a methylase, encoded either by the bacterial chromosome or by phage or plasmid DNA's. Three types of restriction endonucleases, designated types I, II and III, are currently known (4). Moreover, restriction-modification systems that do not fit the current classification appear to exist, thus leaving open the possibility of defining additional types in the near future (4).

Modification of bacteriophage Mu DNA: Imprinting against restriction

The temperate bacteriophage Mu specifies a DNA modification function encoded by a gene designated *mom* (41). Expression of the *mom* gene renders Mu insensitive to many restriction systems «in vivo»; it also protects Mu DNA from digestion by many restriction endonucleases «in vitro» (20). The ability to overcome restriction-modification systems probably has an enormous selective advantage since Mu can infect many gram-negative bacteria. The nature of the epigenetic mark introduced into Mu DNA is not yet known; whatever it is, it is certainly different from methylation (20, 21). However, methylation is involved in the modification process, albeit at another level: transcription from the *mom* promoter requires methylation of an upstream GATC sequence by the host's Dam methylase (21).

The «non-complementing diploids» of Bacillus subtilis

A well documented case of imprinting has been found in the gram-positive bacterium *Bacillus subtilis*: when protoplasts from multiply-marked strains are fused using the polyethylene glycol procedure, certain colonies of the diploid offspring only express markers from one of the parents (18). However these isolates are able to segregate clones that express markers from the other parent; thus they were true diploids and contained chromosomes from both parents, but one of two parental chromosomes was phenotypically silent (18). Unstable non-complementing diploids (henceforth, «unstable NCDs») are also able to segregate recombinants between the two parental genomes, indicating that recombination can occur between the active and the inactive chromosome (12). The possibility of recombination indicates that chromosome inactivation is not caused by compartmentation: to be able to recombine, the active and the inactive nucleoid must coexist in the same

cellular milieu. Thus inactivation must be achieved through an epigenetic mark that labels one of the chromosomes, turning off its expression. There is evidence that expression is turned off at the transcriptional level: a prophage resident on the silent chromosome does not confer immunity; direct measurements of RNA hybridizable to phage DNA led to the conclusion that the prophage on the silent chromosome is transcriptionally silent (14).

Little is known on the nature of the mechanism that turns transcription off. In transformation experiments using DNA from unstable NCDs, transformants are obtained for markers located on both the active and the silent chromosome (18). This result suggests that the epigenetic mark is not a covalent modification, but a looser label that can be easily erased when the DNA is purified and introduced in the recipient cell. If crude nucleoid preparations are used, the frequency of transformation is higher for markers located on the active chromosome. However, transformation by markers located on the silent chromosome can be enhanced by treating the transforming nucleoid DNA with proteinase K. This suggests that chromosomal inactivation may be caused by proteins associated with the nucleoid (5).

However, the phenomenon is probably more complex than described so far. A subclass of «stable» NCDs has been found (15). These revert at frequencies below 10^{-7} and their DNA has low transforming activity for markers located on the silent chromosome. This activity is not enhanced by treatment with proteinase K; even highly-purified DNA from stable NCDs has low transforming ability. These results can be taken as an evidence that the silent chromosome of stable NCDs contains an additional epigenetic mark not present on the DNA of unstable NCDs. Studies on the expression of genes cloned onto bifunctional plasmids that can be transferred forth and back between *E. coli* and *Bacillus* seem to indicate that the silent DNA of stable NCDs is modified: passage through *E. coli* efficiently causes reactivation of cloned genes transferred from stable NCDs. This result suggests that the modification is erased when the DNA is replicated in an unrelated host such as *E. coli* (28). The physical nature of the hypothetical DNA modification is unknown. The existence of unstable and stable NCDs and their different requirements for epigenetic erasure of the inactivating signal suggest that the imprinting system of *Bacillus subtilis* comprises two or more steps, perhaps operating serially. Unstable NCDs may represent the looser stage of inactivation, where the epigenetic mark responsible for inactivation can still be erased by proteinase K. The tighter inactivation observed in stable NCDs may be a DNA modification that can only be erased if the silent DNA is replicated in an unrelated host.

The biological significance of NCD formation in *Bacillus* is unclear. However, current evidence suggests that the phenomenon might be related to the formation of condensed chromosomes during the process of sporulation. This hypothesis is supported by the fact that the *spoOA* gene, previously discovered as a sporulation gene (29), is required for the establishment and maintenance of chromosome inactivation (40). If a NCD containing a cloramphenicol actetyl transferase cassette on the silent chromosome is transformed with DNA from a *spoOA* mutant, reactivation (detected as the appearance of Cami isolates) is easily observed (11). Cami isolates are not obtained when transforming DNA from a *spoOA*$^+$ strain is used (11). In addition to *spoOA*, other genes may be involved in the regulation of gene expression on the silent chromosome: for instance, the *recE* gene, functionally analogous to the *recA* locus of enteric bacteria (30), seems to be able to induce a prophage located on the silent chromosome after treatment with DNA damaging agents (16).

Genetic inactivation in Azotobacter vinelandii

Azotobacter vinelandii is a gram-negative bacterium, widely known for its ability to fix atmospheric nitrogen. The organization of the *Azotobacter* genome is quite unusual in the bacterial

world: each vegetative cell contains 40-80 copies of a single chromosome (34, 35). This extreme polyploidy has been envisaged as a possible strategy to cope with the enormous respiratory activity displayed by the bacterium (23). However, the introduction of transposon technology and the subsequent development of neoclassical genetic analysis in *Azotobacter* has raised doubts about the existence of functional polyploidy (9). Segregation patterns, UV sensitivity studies and analysis of the expression of selectable recessive mutations indicate that *A. vinelandii* is a moderately polyploid bacterium in functional terms (7, 31). The number of active copies can be estimated between 4 and 16, depending on the marker considered and the estimation method used (31). Thus a large fraction of the redundant genome seems to be inactive.

The hypothesis of genetic inactivation is supported by genetic and physical data. False homozygotes, formally analogous to the NCDs of *Bacillus subtilis,* can be isolated after gene transfer and rapid segregation (7, 9 31). These false homozygotes carry silent alleles that reactivate spontaneously at very low frequencies. Moreover, reactivation can be triggered by genetic transfer (transformation or conjugation). The existence of induced reactivation is a fortunate finding that makes the system more amenable to genetic and physical analysis (31).

Inactive alleles carried by false homozygotes can be detected by Southern hybridization. Many silent alleles show altered hybridization patterns with loss of restriction sites, suggesting the existence of a DNA modification that renders the silent DNA insensitive to restriction enzymes (31). However, cases have been found where an inactive allele still shows a wild-type hybridization pattern, indicating that imprinting can exist without DNA modification. Thus it is tempting to hypothesize that genetic inactivation in *Azotobacter* comprises several steps, in an analogous fashion to the formation of stable NCDs in *Bacillus.* Interestingly, altered hybridization patterns are never found in a mutant strain that shows higher reactivation frequencies; this strain might be deficient in one of the imprinting steps, thus becoming more prone to undergo reactivation.

The imprinting system of *A. vinelandii* still has another interesting feature: the likely existence of biased segregation. Alleles recently introduced in a strain seem to have a high, statistically unaccountable chance of appearing in homozygotic strains after a few generations (R. Maldonado and J. Casadesús, unpublished data). In practice, biased segregation complicates the study of genetic inactivation, since both processes seem to occur simultaneously in batch cultures (Figure 1). However, biased segregation may also uncover a subtle mechanism to avoid segregation of inactive genomes: only DNA molecules labeled as «active», such as those recently recombined and expressed, will be recognized by the partition mechanism of the cell. The model, still speculative, may explain how a polyploid bacterium containing inactive genomes can ensure proper partition of the active ones.

The biological significance of imprinting in *Azotobacter* is unknown. Taking the *Bacillus* system as a model (40), it is conceivable that DNA inactivation might be related to cyst formation. However, it might also be a flexible mechanism controlling gene expression. The latter hypothesis is supported by the observation that gene dosage may slightly vary depending on the marker considered (7, 31).

The «archived» P22 prophage of Salmonella typhimurium

An unusual mode of lysogeny was recently discovered by Diana Downs and John Roth (10). Under certain conditions, all of which seem to perturb purine metabolism, a variety of cryptic phages are released from the standard strain LT2 of *Salmonella typhimurium.* These phages were first classified into several phenotypic groups according to plaque morphology; however, physical analysis proved that all were undistinguishable from P22 (10). Downs and Roth suggest that the variety

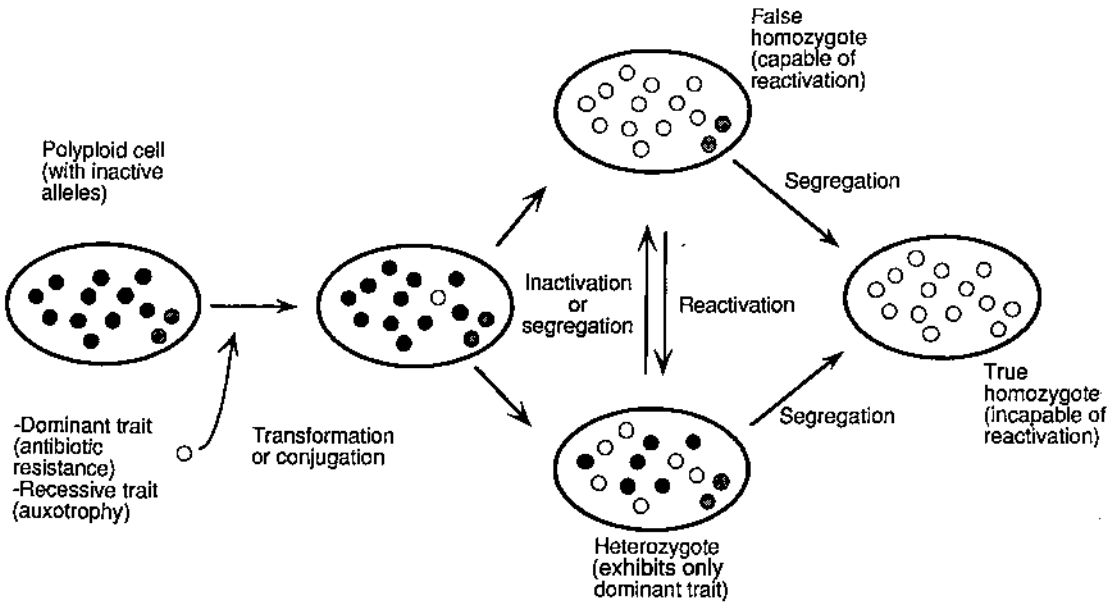

Fig. 1.—A model for the alternative processes of biased segregation and genetic inactivation and their respective roles in the generation of true and false homozygotes in the polyploid bacterium *Azotobacter vinelandii*. When a transposon-induced mutation is introduced into the wild-type strain by transformation or conjugation, transient heterozygotes are obtained. These can segregate either false homozygotes that contain inactive alleles or true homozygotes produced by biased segregation (see refs. 9 and 31).

of types may arise from mutagenesis of the cryptic prophage during the induction proccess (which, interestingly, is *recA*-independent, unlike the induction of normal P22 lysogens). The cryptic P22 prophage does not confer immunity, indicating that its lysogenic repression functions are not expressed. Moreover, physical analysis of genomic DNA from the strains from which the phage was released provides puzzling results. Southern hybridization against P22 probes shows bands with unusually high mobility, whose origin is not yet understood. Anyway, abnormal hybridization patterns clearly suggest the existence of DNA modification. Thus the «archived» prophage seems to be held in an inactive state by an imprinting mechanism of unknown nature. The imprinting system must be regulated by a global regulatory network related to purine metabolism. For this reason, environmental conditions that perturb purine synthesis are able to lift the «archived» state (10).

Physical mechanisms of imprinting

With the exception of DNA methylation associated to host-controlled modification, none of the imprinting systems described above has been fully characterized at the molecular level. Methylation has been ruled out as the mark that modifies Mu DNA (20), but it likely plays a major role in eukaryotic imprinting. For instance, the methylation of deoxycitosines in CG doublets of mammalian DNA seems to be a key developmental process, often invoked in cell differentiation models (17, 37). However, it is not clear whether methylation is the primary signal for imprinting or just a maintenance signal for patterns established by other mechanisms (27). Tagging of the mammalian genome with transgenes can now be used to examine sex-dependent imprinting, since in appropriate crosses the transgene is inherited either from the mother or the father (36). In general, transgenes are less methylated when they are inherited from the father; as the transgene is passed from one

generation to another, the methylation is reversed again and again, depending on the parental origin (33, 36, 39). In some cases, a correlation between methylation and gene expression is found: for instance, in a transgenic mouse line containing a translocated *c-myc* gene, the transgene is hypomethylated and expressed in the myocardium when inherited from the father, but hypomethylated and silent when inherited from the mother. However, methylation seems to be a necessary but not sufficient factor in regulating expression, since the paternally-inherited transgene is equally undermethylated in other tissues but is only expressed in the heart (39). Some authors suggest that the role of DNA methylation in mammalian imprinting might be only to «lock in» the process triggered by other factors (22). At this point, it is interesting to recall that the imprinting systems described in bacteria, albeit poorly understood at the physical level, all seem to comprise several steps, one of which probably consists in DNA modification (7, 15, 31, 40). Thus many, if not all, imprinting systems used for modulating gene activity may require two or more serially operating mechanisms. Hypothetical models for such mechanisms are extensively discussed in refs. 13, 18 and 40; some of them are summarized in Figure 2.

Future prospects

The recent discovery of genomic imprinting in fission yeast, bacteria and phage suggests that epigenetic DNA modification may be a widespread mode of gene regulation. Moreover the increa-

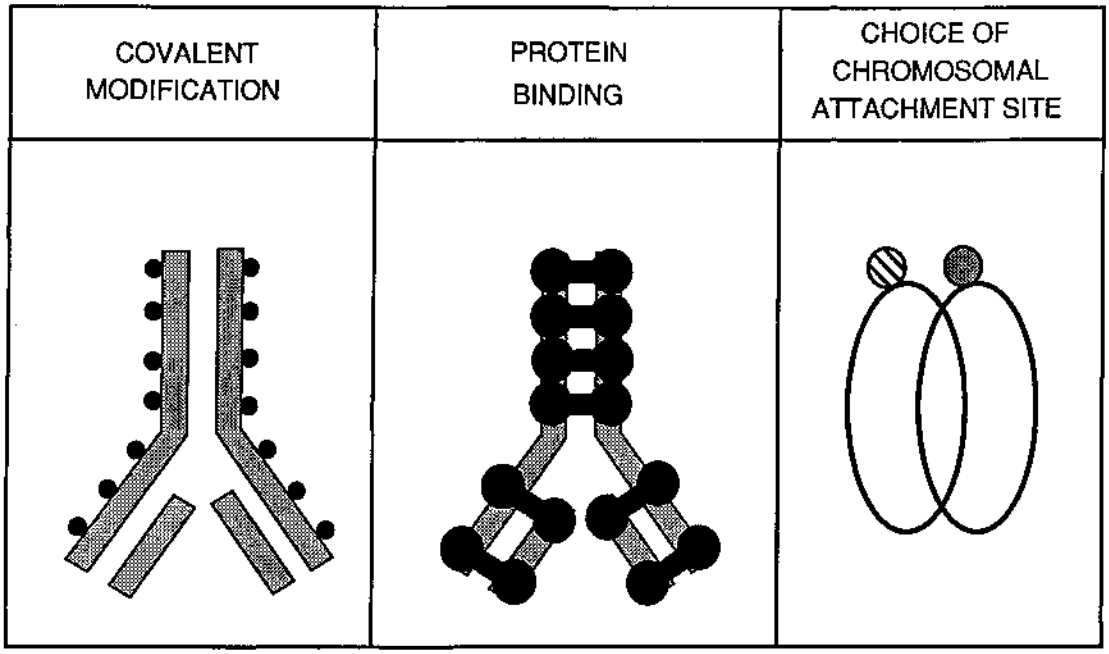

Fig. 2.—Hypothetical mechanisms of imprinting, according to Thaler et al. (ref. 40). (I) Covalent modification of each chain of a double helix leaves hemimodified daughter helices after semiconservative replication. The old, modified chain of each daughter helix is then recognized by a maintenance enzyme that modifies the recently synthesized chain. (II) Monomers of a highly cooperative DNA-binding protein associate with the double helix. During replication, each daughter DNA molecule receives about half of the protein units. Cooperativity will atract new protein units towards the half-coated helices. (II) Each chromosome binds to a macromolecular complex (such as the membrane-bound segregation apparatus proposed by F. Jacob et al. for bacterial chromosomes: ref. 19). The macromolecular complex has alternate states that confer activity or inactivity to the attached chromosome. Replicated chromosomes are provided with a new macromolecular complex, alike in conformational state to the old one.

sing number of reports on sex-dependent expression suggests that imprinting is extremely common in sexually-reproducing organisms. These findings broaden the traditional image of imprinting: DNA modification should not be longer viewed as uncommon or restricted to certain biological systems, but as a general regulatory mechanism. Thus we should not be surprised if new cases of imprinting are discovered in the coming years. In the case of bacteria, where few genome organizations have been studied in detail, imprinting might regulate genes needed in a crucially low dosage, such as the loci that regulate cell division or any other genes encoding potentially harmful products. In polyploid bacteria it might also adjust chromosomal dosage to physiological needs. On the other hand, the study of imprinting in microbial systems can be expected to be simpler and more amenable than in complex eukaryotic systems. Work on both higher organisms and microbes will undoubtely be of mutual benefit to unravel the regulatory networks based on epigenetic modification of DNA. The solution to this problem may prove to be of general biological significance.

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References

1. Arber, W. and Dussoix, D. (1962). Host specificity of DNA produced by *Escherichia coli*. I. Host controlled modification of bacteriophage λ J. Mol. Biol. **5,** 18-36.
2. Arber, W. and Linn, S. (1969). DNA modification and restriction. Annu. Rev. Biochem. **38,** 467-500.
3. Bertani, G. and Weigle, J. J. (1953). Host-controlled variation in bacterial viruses. J. Bacteriol. **65,** 113-121.
4. Bickle, T. (1987). DNA restriction and modification systems. *In:* F. C. Neidhardt (ed.) *Escherichia coli* and *Salmonella typhimurium*. Cellular and Molecular Biology, pp. 692-696. American Society for Microbiology. Washington D. C.
5. Bohin, J. P., Ben Khalifa, K., Guillén, N., Schaeffer, P. and Hirschbein, L. (1982). Phenotypic expression «in vivo» and transforming activity «in vitro»: two related functions of folded bacterial chromosomes. Mol. Gen. Genet. **185,** 65-75.
6. Brown, S. P. and Sarat-Chandra, H. (1977). Chromosome imprinting and the differential regulation of homologous chromosomes. *In:* L. Goldstein and D. M. Prescott (eds.). Cell biology: A comprehensive treatise. Vol. I. Genetic mechanisms of cells, pp. 110-189. Academic Press, New York.
7. Casadesús, J., Maldonado, R., Contreras, A. and Garzón, A. (1989). Organización del genomio de *Azotobacter vinelandii*. *In:* M. Megías (ed.) Avances en al biología de la fijación de nitrógeno. EUS. Sevilla (in press).
8. Chapman, V. M. (1986). X-chromosome regulation in oogenesis and early mammalian development. *In:* J. Rossant and R. A. Petersen (eds.) Experimental approaches to mammalian embryonic development, pp. 365-398. Cambridge University Press, New York.
9. Contreras, A. and Casadesús, J. (1987). Tn*10* mutagenesis in *Azotobacter vinelandii*. Mol. Gen. Genet. **209,** 276-282.
10. Downs, D. M. and Roth, J. R. (1987). A novel P 22 prophage in *Salmonella typhimurium*. Genetics. **117,** 367-380.
11. Ferrari, F. A., Trach, T., LeCoq, D., Spence, J., Ferrari, E. and Hoch, J. A. (1985). Characterization of the *spoOA* locus and its deduced product. Proc. Natl. Acad. Sci. USA **82,** 2647-2651.
12. Gabor, H. M. and Hotchkiss, R. D. (1983). Reciprocal and non-reciprocal recombination in diploid clones from *Bacillus subtilis* protoplast fusion: Association with the replication origin and terminus. Proc. Natl. Acad. Sci. USA **80,** 1246-1250.
13. Grant, S. G. and Chapman, V. M. (1988). Mechanisms of X-chromosome regulation. Annu. Rev. Genet. **22,** 199-233.
14. Guillén, N., Sánchez-Rivas, C. and Hirschbein, L. (1983). Absence of functional RNA encoded by a silent chromosome from non-complementing diploids obtained from protoplast fusion in *Bacillus subtilis*. Mol. Gen. Genet. **191,** 81-85.
15. Guillén, N., Amar, R. and Hirschbein, L. (1985). Stabilized non-complementing diploids (NCD) from fused protoplast products of *Bacillus subtilis*. EMBO J. **4,** 1333-1338.
16. Guillén, N., Zahraoui, A., D'Ari, R. and Hirschbein, L. (1986). RecE-dependent lysogenic induction in the absence of repressor in *Bacillus subtilis* non-complementing diploids. J. Gen. Microbiol. **132,** 1703-1707.
17. Holliday, R. (1987). The inheritance of epigenetic defects. Science **23,** 163-170.

18. Hotchkiss, R. D. and Gabor, M. H. (1980). Biparental products of bacterial protoplast fusion showing unequeal parental chromosome expression. Proc. Natl. Acad. Sci. USA **77,** 3553-3557.
19. Jacob, F., Brenner, S. and Cuzin, F. (1963). On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. **28,** 339-348.
20. Kahmann, R. (1984). The *mom* gene of bacteriophage Mu. Curr. Top. Microbiol. Immunol. **108,** 29-47.
21. Kahmann, R. and Hattman, S. (1987). Regulation and expression of the *mom* gene. *In:* N. Symonds, A. Toussaint, P. van de Putte and M. M. Howe (eds.) Phage Mu, pp. 93-109. Cold Spring Harbor Laboratory, Cold Spring Harbor.
22. Kaslow, D. C. and Migeon, B. R. (1987). DNA methylation stabilizes chromosome inactivation in eutherians but not in marsupials: Evidence for multistep maintenance of mammalian X dosage compensation. Proc. Natl. Acad. Sci. USA **84,** 6210-6214.
23. Kennedy, C. and Toukdarian, A. (1987). Genetics of Azotobacters: Applications to nitrogen fixation and related aspects of nitrogen metabolism. Annu. Rev. Microbiol. **41,** 227-258.
24. Kermicle, J. L. (1978). Imprinting of gene action in maize endosperm. *In:* D. B. Walden (ed.) Maize breeding and genetics, pp. 357-371. John Wiley & Sons, New York.
25. Klar, A. J. S. (1987). Differentiated parental DNA strands confer developmental asymmetry on daughter cells in fission yeast. Nature **326,** 466-470.
26. Lyon, M. F. (1961). Gene action in the X-chromosome of the mouse (*Mus musculus* L.). Nature **190,** 372-373.
27. Lock, L. F., Takagi, N. and Martín, G. R. (1987). Methylation of the *Hprt* gene on the inactive X occurs after chromosome inactivation. Cell **48,** 39-46.
28. López, F., Guillén, N. and Hirschbein, L. (1986). Further characterization of the inactive chromosome from *Bacillus subtilis* stabilized non-complementing diploids. *In:* A. T. Ganesan and J. A. Hoch (eds.) Bacillus molecular genetics and biotechnology applications, pp. 73-86. Academic Press, New York.
29. Losick, R., Youngman, P. and Piggot, P. J. (1986). Genetics of endospore formation in *Bacillus subtilis*. Annu. Rev. Genet. **20,** 625-669.
30. Love, P. E. and Yasbin, R. E. (1986). Induction of the *Bacillus subtilis* SOS-like response by the *Escherichia coli* RecA protein. Proc. Natl. Acad. Sci. USA **83,** 5204-5208.
31. Maldonado, R., Contreras, A., Garzón, A. and Casadesús, J. Genetic inactivation in *Azotobacter vinelandii*. Manuscript in preparation.
32. Messing, J. (1989). Broadening our understanding of genetic information: Beyond base pairing. ASM News **5,** 255-258.
33. Monk, M. (1987). Memories of mother and father. Nature **328,** 203-204.
34. Nagpal, P., Jafri, S., Reddy, M. A. and Das, H. K. (1989). Multiple chromosomes of *Azotobacter vinelandii*. J. Bacteriol. **139,** 3133-3138.
35. Sadoff, H. L., Shimel, B. and Ellis, S. (1979). Characterization of *Azotobacter vinelandii* deoxyribonucleic acid and folded chromosomes. J. Bacteriol. **138,** 871-877.
36. Sapienza, C., Peterson, A. C., Rossant, J. and Balling, R. (1987). Degree of methylation of transgenes is dependent on gamete of origin. Nature **328,** 251-254.
37. Silva, A. J. and White, R. (1988). Inheritance of allelic blueprints for methylation patterns. Cell **54,** 145-152.
38. Solter, D. (1987). Inertia of the embryonic genome in mammals. Trends Genet. **3,** 23-37.
39. Solter, D. (1988). Differential imprinting and expression of maternal and paternal genomes. Annu. Rev. Genet. **22,** 127-146.
40. Thaler, D., Roth, J. R. and Hirschbein, L. (1989). Imprinting in prokaryotes. *In:* K. Drlica and M. Riley (eds.). The bacterial chromosome. John Wiley & Sons, New York (in press).
41. Toussaint, A. (1976). The DNA modification of phage Mu-1. Virology **70,** 17-27.

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Susceptibilidad de células inmunocompetentes a virus animales

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Abreviaturas

DMEM: Medio de Eagle modificado por Dulbecco.
ECP: Efecto citopático.
EMC: Virus de la encefalomiocarditis.
HSV-1: Hermes simplex tipo 1.
m.d.i.: Multiplicidad de infección.
PBS: Tampón salino fosfato.
SFB: Suero de ternera fetal.
SFV: Virus del bosque de Semliki.
u.f.p.: Unidades formadoras de placas.
VSV: Virus de la estomatitis vesicular.

Summary

The infection of several cell lines of the immune system by animal viruses has been studied. In general, those cell lines derived either from the myeloid or from the lymphoid differentiation pathways were poorly affected by these viruses. Only Semliki Forest Virus (SFV) and poliovirus were able to replicate in most of the cell lines assayed, inhibiting the cellular protein synthesis. However, this inhibition was not accompanied by a significant expression of viral proteins. These effects were not observed with UV irradiated virus suggesting that intact viral particles were required to interfere with the host macromolecular synthesis.

Key words: Virus, immune system, susceptibility.

Resumen

Se ha estudiado la infección de diferentes líneas celulares, derivadas del sistema inmune, por virus animales. En general, las líneas celulares, procedentes tanto de la serie de diferenciación mieloide como de la linfoide, fueron poco susceptibles a los diferentes virus ensayados. Sólo el virus del bosque de Semliki (SFV) y poliovirus fueron capaces de replicarse de forma significativa, inhibiendo la síntesis de proteínas totales y produciendo la muerte celular a tiempos tardíos en la mayoría de las líneas celulares analizadas. Sin embargo, esta inhibición no fue acompañada de una síntesis acusada de proteínas virales. Estos efectos no fueron observados con virus irradiados con luz UV, lo que indica que se necesitan partículas virales intactas para que se produzca una interferencia con el metabolismo celular.

Introducción

Puesto que tanto los linfocitos B y T como las células fagocíticas mononucleares representan factores claves en la limitación de la expansión de las infecciones por virus, resulta de interés estu-

diar las interacciones (directas o indirectas) que los virus mantienen con tales células inmunocompetentes. Tales estudios han revelado que, según los casos, la infección viral «in vivo» puede conducir a una inmunopotenciación o inmunosupresión (22).

El estudio de la infección de células inmunocompetentes presenta al menos un doble interés: por un lado, se puede realizar un estudio de la infección de estas células *per se,* observando, por ejemplo, cómo en unos casos se puede producir la transformación celular, como es el caso del virus Epstein-Barr (19), mientras que otras veces la infección viral puede ser productiva, conduciendo a la producción de partículas virales y la destrucción celular o de tipo abortivo (22). Por otra parte, es obvio que cualquier infección que altere la funcionalidad de las células del sistema inmune lleva aparejada una alteración en la reactividad y defensa frente a otros agentes patógenos no relacionados con el virus que ha provocado tal estado. Un ejemplo de palpitante actualidad es la infección por el retrovirus HIV-1 de células que expresan en su membrana el antígeno CD4, que produce una incapacidad para responder a antígenos, dando lugar al cuadro clínico denominado síndrome de inmunodeficiencia adquirida o SIDA (27).

Nosotros hemos realizado un estudio sobre la infección de diferentes líneas derivadas del sistema inmune por diversos virus animales, observando que la susceptibilidad en este tipo de células está, en general, muy modificada con respecto a otras líneas celulares no inmunocompetentes (4, 5, 13, 15, 16).

Materiales y métodos

Células y virus

Las células se crecieron en medio de Eagle modificado por Dulbecco (DMEM) suplementado con 10 % de suero de ternera fetal (SFB). Los experimentos se llevaron a cabo en medio DME conteniendo 1 % de suero de ternera fetal. Los virus de la polio tipo I (cepa Mahoney) y de la vacuna fueron crecidos en cultivos de células HeLa en medio DME con 1 % de suero de ternera recién nacida; el virus de la encefalomiocarditis (EMC) se creció en células L929 de ratón; el virus de la estomatitis vesicular (VSV) se creció en céluls BHK-21 (células de riñón de hamster de un día de vida); herpes simplex tipo 1 (HSV-1) se creció en células vero de mono verde africano.

Estimación de la destrucción celular

El estudio de la destrucción celular se realizó observando mediante microscopía óptica el efecto citopático (ECP) producido en las células infectadas, observando la morfología y el aumento de restos celulares en el medio con respecto a un control de células sin infectar. El contaje se efectuó mezclando un volumen determinado del medio con las células a analizar con otro volumen igual de una solución de azul-tripán (colorante aniónico que penetra sólo en las células muertas) en PBS. La estimación del número de células vivas se determinó mediante un hematocitómetro, utilizándose una cámara de contaje Neubauer. La evaluación de este ECP se indicó según los siguientes símbolos:

\+ 20-25 % de destrucción con respecto al control.
++ 25-60 % de destrucción.
+++ > 60 %.

Valoración de las partículas virales infectivas producidas mediante el método de la placa de lisis

La determinación del número de unidades formadoras de placa (u.f.p.) por este método se llevó a cabo según lo descrito por Dulbecco y Vogt en 1954 (6). Brevemente se hicieron diluciones se-

riadas de las muestras a titular en PBS total, infectando a continuación monocapas confluentes de células susceptibles al virus en cuestión en medio conteniendo agar. Se incubó durante 48 h, contándose seguidamente las placas de lisis producidas y multiplicando por los factores de dilución adecuados para obtener el número de u.f.p./ml de la preparación viral.

Marcaje de proteínas y análisis por electroforesis en geles de poliacrilamida

El marcaje «in vivo» de las proteínas sintetizadas se efectuó dando pulsos de 1 hora con 10 µCi de (^{35}S)-metionina (1.450 Ci/mmol; Amersham) en cultivos celulares con medio DME sin metionina. Transcurrido el tiempo de marcaje se retiró el medio, se lavaron dos veces las células con PBS y se suspendieron en 100 µl de 0,02 M NaOH/1 % dodecil sulfato sódico (SDS) y 200 µl de tampón de muestra (62,5 Tris pH 6,8/2 % SDS/0,1 M ditiotreitol/17 % glicerol/0,024 % azul de bromofenol). Se aplicaron volúmenes de 10 µl en geles de poliacrilamida 15 % y se llevó a cabo la electroforesis a 100 v durante toda la noche.

Inactivación de partículas virales por radiación ultravioleta

Se preparó una solución viral de 1 ml en una placa Petri de 35 ó 60 mm y se colocó a una distancia de 3 cm por debajo de una lámpara UV de General Eléctrica G8T5, que emite a 260 nm. Durante el tiempo de exposición, que fue de 3 minutos, se colocó la placa sobre un recipiente con hielo para evitar el aumento de temperatura debido a la radiación.

Valoración de la síntesis de DNA

La síntesis de DNA se analizó midiendo la incorporación de (^{3}H)-timidina. Para ello las células se incubaron con 5 µCi/ml de (^{3}H)-timidina en medio DME con SFB al 2 %. La incorporación se realizó en placas multipocillos. Transcurrido el tiempo de incorporación, las células fueron recolectadas con multifiltrador «Cell Harvester», de Titertek, que succionó los medios de cultivo de cada pocillo con flujos de agua destilada, llevándolos hasta un filtro de fibra de vidrio. Los filtros fueron secados y sumergidos en líquido de centelleo, realizándose el contaje de la radiactividad incorporada.

Resultados

Viabilidad celular tras la infección

En el presente estudio se utilizaron las siguientes líneas celulares humanas: K-562 (eritroleucémica), U-937 (monocítica), HL-60 (promielocítica), Daudi (linfoblastoide), Molt-4 (linfocitos T) y RPMI-1788 (linfocitos B). Estas líneas celulares fueron infectadas por virus que poseían como material genético RNA (polio, EMC, VSV y SFV) o DNA (HSV-1 y virus de la vacuna). En la Tabla 1 se muestra la sensibilidad celular a la infección viral medida por la inducción del efecto citopático observado mediante microscopía óptica. Este ECP fue difícil de valorar al tratarse de células que crecen en suspensión, siendo normalmente uno de los datos considerados al estimar dicho ECP precisamente la liberación y pérdida de adherencia al plástico de la monocapa celular infectada. A pesar de ello, y considerando el aumento de restos celulares en el medio de cultivo, o la vacuolización de las células, se observó que, en general, las líneas celulares ensayadas fueron poco sensibles a los

TABLA 1
SUSCEPTIBILIDAD DE DIFERENTES LINEAS CELULARES INMUNOCOMPETENTES A VIRUS ANIMALES

Virus	m.d.i.	Efecto citopático (ECP)[a]					
		K-562	Daudi	U-937	HL-60	RPMI 1788	Molt-4
Polio	0,5	– –	+	+	– –	– –	– –
	10	– –	++	++	– –	+	– –
EMC	0,5	– –	– –	– –	– –	n.e.	n.e.
	10	– –	– –	– –	+	n.e.	n.e.
VSV	0,5	– –	+	– –	– –	n.e.	n.e.
	10	– –	+	– –	– –	n.e.	n.e.
SFV	0,5	– –	– –	+	– –	+	n.e.
	10	+	– –	+++	– –	++	n.e.
Vacuna	0,5	– –	+	– –	+	n.e.	n.e.
	10	– –	++	+	++	n.e.	n.e.
HSV-1	0,5	– –	– –	– –	– –	+	– –
	10	– –	– –	– –	– –	++	– –

[a] A las 48 horas p.i. se determinó el efecto citopático producido mediante microscopía óptica según se indica en materiales y métodos. Los valores indican la media de 4 experimentos.
n.e. No ensayado.

diferentes virus 48 horas después de la infección. Sólo SFV produjo ECP y muerte celular en la mayoría de las líneas celulares ensayadas a una multiplicidad de infección (m.d.i.), incluso de 0,5 u.f.p./célula. Poliovirus indujo ECP en varias de las líneas celulares empleadas. Por otra parte, la línea K-562 apenas mostró sensibilidad a la infección, siendo sólo sensible a altas m.d.i. de SFV. En cualquier caso, se observó cierta especificidad de infección, mostrando cada línea celular un perfil de susceptibilidad diferente a las demás.

Síntesis de proteínas

Siguiendo con el presente estudio, analizamos la síntesis proteica producida tras la infección, determinando la incorporación de (^{35}S)-metionina a proteínas precipitables con ácido tricloroacético. En la Tabla 2 se puede observar nuevamente cómo SFV produce el mayor efecto, inhibiendo a las 24 horas p.i. la síntesis de proteínas totales en la mayoría de las líneas celulares y presentando un efecto dosis dependiente. U-937 vio inhibida su síntesis de proteínas tras la infección con poliovirus o SFV ya a m.d.i. de 0,5 u.f.p/célula. El resto de las líneas celulares no modificaron significativamente la incorporación de (^{35}S)-metionina, incluso en la línea celular Molt-4 ésta aumenta de forma acusada tras la infección.

El análisis de las proteínas sintetizadas mediante electroforesis en geles de poliacrilamida en condiciones desnaturalizantes (SDS-PAGE) se observa en la Figura 1. Cuando se compara con la infección de la línea epitelial humana HeLa a una m.d.i. de 10 u.f.p./célula se puede observar en las líneas celulares del sistema inmune una inhibición menos drástica de la síntesis de proteínas totales a

TABLA 2
EFECTO DE LA INFECCION VIRAL EN LA SINTESIS DE PROTEINAS DE DIFERENTES LINEAS CELULARES INMUNOCOMPETENTES

Virus	m.d.i.	(^{35}S) metionina incorporada (% control)[a]				
		K-562	U-937	HL-60	RPMI 1788	Molt-4
Polio	0,5	100	89	100	100	187
	10	100	47	80	84	231
EMC	0,5	100	100	100	n.e.	n.e.
	10	82	100	83	n.e.	n.e.
VSV	0,5	100	93	100	n.e.	n.e.
	10	98	88	72	n.e.	n.e.
SFV	0,5	78	67	100	100	n.e.
	10	36	16	89	32	n.e.
Vacuna	0,5	100	100	100	n.e.	n.e.
	10	100	131	89	n.e.	n.e.
HSV-1	0,5	100	100	100	56	139
	10	100	95	83	20	249

[a] A las 24 horas p.i. se midió la incorporación de (^{35}S) metionina mediante pulso de una hora según se detalla en materiales y métodos. Los valores indican los porcentajes medios de 4 experimentos.
n.e. No ensayado.

las 24 horas p.i. Además, en células HeLa se pudo detectar síntesis de proteínas virales a partir de la hora 5 p.i. Las células K-562 son capaces de sintetizar, tras la infección con SFV, algunas de las proteínas virales mayoritarias, sin que apenas se inhiba la síntesis de las proteínas celulares. Asimismo, la línea RPMI-1788 resultó ser susceptible a HSV-1, sintetizándose algunas de las proteínas virales. No obstante, en ningún caso se observó, incluso a tiempos muy tardíos (hasta 72 horas p.i.), una aparición de proteínas virales comparable a la que tiene lugar en HeLa. La línea U-937 sufrió una parada de la síntesis de proteínas celulares al infectarse con SFV o poliovirus, mientras que Molt-4 incrementó la síntesis de proteínas celulares tras la infección sin que estos hechos llevasen consigo síntesis de proteínas virales. En el resto de los casos la infeción no produjo ningún efecto notable, poniéndose de manifiesto la resistencia a la infección por virus de las líneas celulares inmunocompetentes empleadas.

Efecto de virus inactivado en la viabilidad celular

Para comprobar si los efectos observados en algunas de las líneas celulares analizadas requerían virus con capacidad replicativa, se infectaron las células con partículas virales irradiadas con luz UV. Los resultados se muestran en la Tabla 3. En la mayoría de los casos, la infección con partículas virales infectivas no modificó bruscamente la incorporación de (^{3}H) timidina a DNA. En el caso de SFV, la infección produjo un aumento considerable en la síntesis de DNA total. En otros casos el virus inhibió dicha síntesis, pero aquí la inhibición no excedió del 50 % con respecto a células sin infectar.

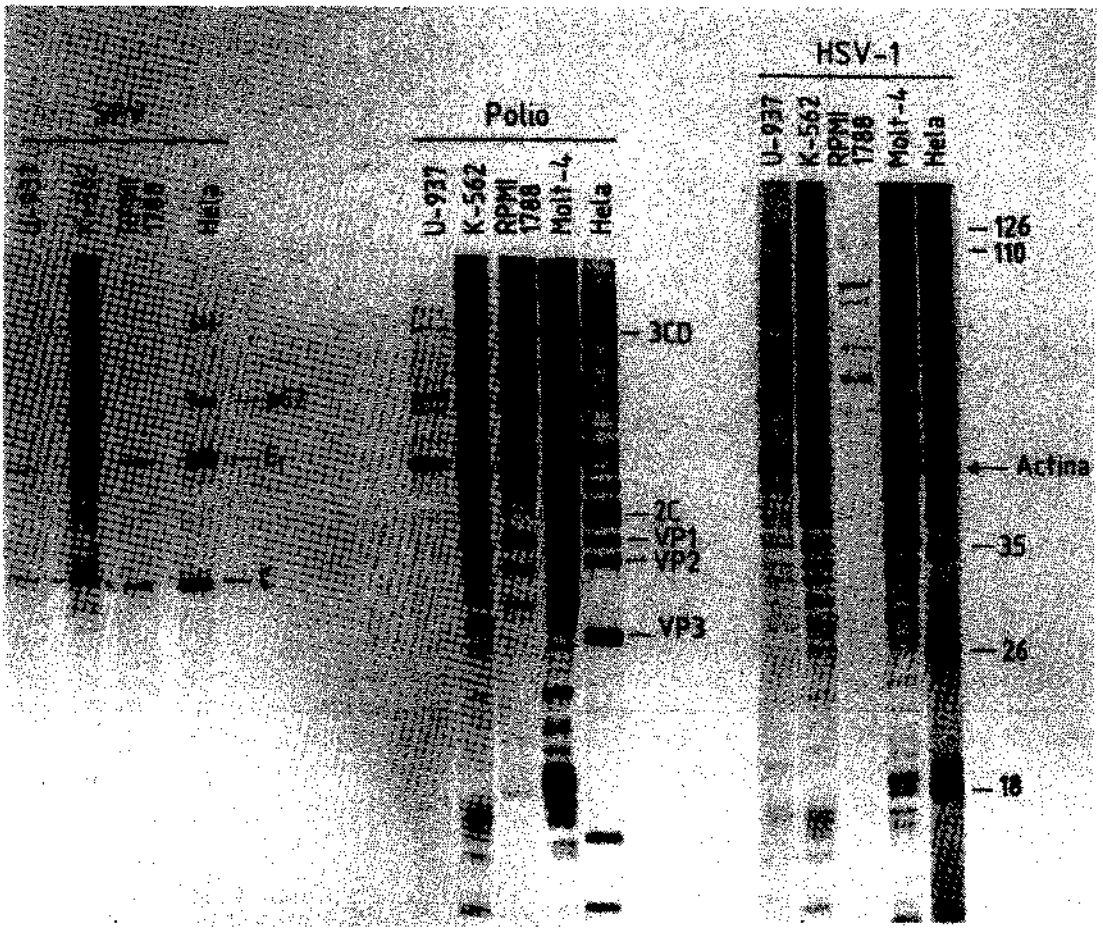

Fig. 1.—Efecto de la infección viral en la síntesis de proteínas de diferentes líneas celulares inmunocompetentes. Las líneas celulares se infectaron con los virus indicados a 10 m.d.i. A las 6 h p.i. (para células HeLa infectadas con poliovirus o SFV), 16 h p.i. (en el caso de células HeLa infectadas con HSV-1) o 24 h p.i. (para el resto de líneas celulares) se marcaron las proteínas con 20 μCi/ml de (^{35}S) metionina durante 1 h y se analizaron en SDS-PAGE. Se indican las posiciones y Pm de algunas proteínas virales y de actina.

En cualquier caso, todos estos efectos anteriormente señalados desaparecían cuando se utilizaban partículas virales irradiadas con luz UV. Este hecho puede sugerir que la modificación de la síntesis de macromoléculas celulares producidas por el virus podría ser un fenómeno activo con requerimiento de replicación viral, y no sólo un efecto producido por la entrada y descapsidación del virus *per se.*

Producción de partículas virales infectivas

Por último, una vez estudiado el efecto en la síntesis de macromoléculas celulares producido por los diferentes virus utilizados, analizamos si a pesar de los pocos efectos observados existía producción de partículas virales infectivas. Para ello se infectaron las células con una m.d.i. de 10 u.f.p./célula, ensayándose la producción viral a diferentes tiempos p.i.

Como muestra la Tabla 4, en todos los casos analizados la producción viral fue muy escasa

TABLA 3
SUSCEPTIBILIDAD DE DIFERENTES LINEAS CELULARES A VIRUS IRRADIADO

Virus	m.d.i.	(^{3}H) timidina incorporada (% control)[a]					
		Virus sin irradiar			Virus irradiado UV[b]		
		U-937	K-562	Daudi	U-937	K-562	Daudi
Polio	0,5	88[c]	100	98	93	100	100
	10	41	89	83	96	115	100
EMC	0,5	93	100	99	100	112	n.e.
	10	88	71	87	103	100	102
VSV	0,5	73	100	100	102	99	n.e.
	10	94	107	109	100	106	100
SFV	0,5	137	130	78	107	100	n.e.
	10	102	128	67	108	93	88
Vacuna	0,5	109	100	89	99	90	n.e.
	10	100	74	82	103	94	107
HSV-1	0,5	92	95	100	98	100	n.e.
	10	91	119	87	100	101	92

[a] A las 48 h p.i. se midió la incorporación de (^{3}H)-timidina durante 4 h. Los valores indicados representan la media de 4 experimentos.
[b] Los virus fueron irradiados durante 3 minutos con una lámpara UV a una longitud de onda de 260 nm.
n.e. No ensayado.

cuando se comparó con la línea epitelial HeLa. A pesar de ello, en el caso de la infección de células U-937 por poliovirus, HSV o SFV se observó una producción significativa de partículas infectivas. Asimismo, poliovirus y SFV fueron capaces de replicarse en la línea eritroleucémica K-562, a pesar de que el primero no inducía aparición de ECP ni modificaba la síntesis de proteínas totales (Tablas 1 y 2, respectivamente).

Discusión

El presente estudio muestra la escasa susceptibilidad que las células de origen hematopoyético presentan a la infección por diversos virus animales, sobre todo cuando la comparamos con los resultados obtenidos en líneas celulares no inmunocompetentes, independientemente del tipo de virus empleado. Este hecho parece lógico si se tiene en cuenta el importante papel que desempeñan las células del sistema inmune en el organismo. Los linfocitos T, B y las células del sistema mononuclear fagocítico representan papeles claves en el control de las infecciones virales. La infección de diferentes subpoblaciones celulares del sistema inmune por virus produce una alteración no sólo de la reactividad contra el mismo virus, sino que lleva aparejada una alteración de la respuesta inmune contra otros antígenos (10, 18, 22).

Aunque hoy día se dispone de mucha información sobre la respuesta inmune frente a las infecciones virales, es mucho menos lo que se conoce sobre los efectos que las infecciones virales tienen en las funciones del sistema inmune, debido principalmente a que los síntomas detectados «in vivo»

TABLA 4
PRODUCCION DE PARTICULAS INFECTIVAS TRAS LA INFECCION DE DIFERENTES LINEAS CELULARES CON VIRUS ANIMALES

Virus	Tiempo (h.p.i.)	Partículas infectivas producidas (UFP/ml)[b]		
		HeLa[a]	U-937	K-562
Polio	14	n.e.	$5{,}0 \times 10^6$	n.e.
	20	$2{,}0 \times 10^8$	$1{,}2 \times 10^7$	$3{,}0 \times 10^6$
	44	n.e.	$3{,}0 \times 10^7$	$8{,}9 \times 10^6$
HSV-1	20	$8{,}0 \times 10^7$	$5{,}6 \times 10^4$	$< 10^4$
	44	$7{,}0 \times 10^8$	$3{,}2 \times 10^5$	$< 10^4$
EMC	20	$5{,}5 \times 10^7$	$< 10^4$	$< 10^4$
Vacuna	20	$1{,}5 \times 10^7$	$< 10^4$	$< 10^4$
SFV	20	$6{,}5 \times 10^8$	$3{,}4 \times 10^7$	$1{,}0 \times 10^7$

[a] Las células se infectaron a una m.d.i. de 10 u.f.p./célula.
[b] A los tiempos indicados se analizó la producción de partículas virales mediante el ensayo de las placas de lisis, según se detalla en materiales y métodos.
n.e. No ensayado.

raramente han sido relacionados con una infección de células inmunocompetentes. Es sorprendente que este área haya recibido tan poca atención, sobre todo cuando se sabe desde hace tiempo que un gran número de virus son capaces de replicarse en células del sistema linforreticular (11, 21, 22, 24).

Por todo esto se ha hecho necesario un acercamiento al problema «in vitro», utilizando cultivos de células derivadas del sistema inmune. Nosotros hemos realizado un estudio utilizando como sistema líneas celulares establecidas, no poblaciones celulares extraídas directamente del animal, que presentan el problema de la falta de homogeneidad.

A pesar de la escasa susceptibilidad que en general muestran las líneas celulares derivadas del sistema inmune a la infección por virus, se ha demostrado que en algunos casos el virus puede replicarse y acabar destruyendo las células (2, 7, 8, 23). Algunos virus lo hacen tan rápidamente que la aparición de la inmunidad celular o humoral llega a ser inefectiva para eliminar el desarrollo del virus. El hecho de que los macrófagos sean capaces de eliminar partículas virales por fagocitosis y de que las células citotóxicas naturales (NK) destruyan células infectadas con virus produce un mecanismo accesorio para prevenir la expansión del virus a nuevas dianas (3, 12, 17).

Esta menor susceptibilidad, que en general muestran las líneas celulares derivadas del sistema inmune, puede no ser indicativa de la resistencia de un cierto tipo celular a la infección por un determinado virus. Obviamente, varias causas pueden contribuir a este fenómeno, entre ellas la no existencia de receptores virales o la falta de adaptación de estos virus a las líneas celulares estudiadas. Aunque no podemos descartar ninguna de ellas, no creemos que sean las responsables de lo observado en nuestro caso. Así, los virus estudiados producen ciertos efectos apreciables en las células, pudiendo incluso haber una pequeña producción de partículas infectivas virales (Tabla 4). Es tos efectos, en general, desaparecen cuando se utilizan virus irradiados con luz UV, indicando que se requiere un genoma viral intacto y posiblemente su replicación para que tengan lugar.

Por otra parte, la producción de partículas virales y su adaptación a cultivos celulares mediante sucesivos pases no incrementaron su infectividad (resultados no mostrados).

Los estudios de la susceptibilidad celular a la infección por virus se han realizado por varios métodos: ECP, incorporación de (^{3}H)-timidina y de (^{35}S)-metionina.

En algunos casos hemos observado una falta de correlación entre los valores obtenidos por estos métodos.

Varias pueden ser las causas de este hecho: en primer lugar, la asignación de un valor que indique estado general celular, tras una infección viral en células que crecen en suspensión, es dificultosa. Por otra parte, el cambio morfológico, observado al microscopio, que sufren las células después de la infección no tiene por qué deberse a un deterioro celular. Asimismo, la incorporación de (^{3}H)-timidina a DNA total tras la infección viral puede verse afectada de varias maneras. De hecho, se conocen ciertos tipos de virus que sólo se réplican en leucocitos activados. Además, ciertos tipos de virus, como es el caso de HSV-1, inducen la división celular para poder replicarse (9, 22). Por lo tanto, la diferencia entre efectos citopáticos parciales e incorporación de timidina podría explicarse por un incremento en la división celular durante la infección.

Puesto que el presente estudio se ha realizado con células transformadas, cabe preguntarse si los resultados obtenidos son extrapolables a sistemas celulares primarios. En este sentido, Sarmiento (25), estudiando la infección de macrófagos y fibroblastos de ratones por HSV, sugirió que los mecanismos de resistencia de líneas celulares establecidas pueden estar alterados y no ser representativos del tejido original o aislados celulares primarios. Nosotros hemos realizado estudios comparativos con la línea celular monocítica U-937 y cultivos celulares primarios infectados con poliovirus y HSV, obteniendo, en todos los casos, resultados similares, con inhibición parcial de la síntesis de proteínas celulares y falta de detección de síntesis de macromoléculas virales (15, 16).

Actualmente se tiende a relacionar el grado de diferenciación celular con la susceptibilidad a la infección por virus. En este sentido, Okada y col. (21) observaron, estudiando la infección de un gran número de líneas celulares inmunocompetentes por poliovirus, que las líneas más diferenciadas eran a su vez las más susceptibles a la infección. En este mismo sentido, Tenney y Mornhan (26), o más recientemente Albers y col. (1), comprobaron que cultivos de monocitos humanos veían aumentada su susceptibilidad a HSV-1 tras la diferenciación a macrófagos. Sin embargo, recientemente, y en nuevos trabajos de Morahan y col. (20) con macrófagos de ratón, no se pudo realizar la misma observación. Los estudios realizados en nuestro laboratorio sobre el efecto de la diferenciación de células monocíticas humanas a macrófagos en la infección por poliovirus o HSV-1 (13, 14, 15), no revelaron diferencias significativas en cuanto al grado de susceptibilidad al virus, tanto durante como tras la diferenciación celular. Por lo tanto, se hace necesario el planteamiento de nuevos enfoques que permitan clarificar el tipo de interacción que presentan las células inmunocompetentes con los virus animales, puesto que en ello se encuentra la clave para comprender los mecanismos de defensa del organismo frente a las infecciones de agentes patógenos.

Por último, se puede resumir que aunque ciertos virus interfieren de forma activa con la síntesis de macromoléculas de líneas celulares derivadas del sistema inmune, este efecto no es muy acusado, permitiendo a la célula sobrevivir durante largos períodos de tiempo o acabar constituyéndose en una infección de tipo persistente.

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Bibliografía

1. Albers, I., Kirchner, H. and Domke-Opitz, I. (1989). Resistance of human blood monocytes to infection with herpes simplex virus. Virology. **169,** 466-469.
2. Braun, R.W. and Reiser, H. C. (1986). Replication of human cytomegalovirus in human peripheral blood T cells. J. Virol. **60,** 29-36.
3. Buchmeier, N. A., Gee, S. R., Murphy, F. A. and Rawls, W. E. (1979). Abortive replication of vaccinia virus in activited rabbit macrophages. Infect. Immun. **26,** 328-338.
4. Carrasco, L. (1977). The inhibition of cell functions after viral infection: A proposed general mechanism. FEBS Letters. **76,** 11-15.
5. Carrasco, L. and Smith, A. E. (1976). Sodium ions and the shut-off of host cell protein synthesis by picornaviruses. Nature. **264,** 807-809.
6. Dulbecco, R. and Vogt, M. (1954). Ptaque formation and isolation of pure lines with poliomyelitis viruses. J. Exp. Med. **99,** 167-182.
7. Esparza, I., González, J. C. and Viñuela, E. (1988). Effect of interferon-α, interferon-γ and tumor necrosis factor on African Swine Fever virus replication in porcine monocytes and macrophages. J. Gen. Virol.. **69,** 2973-2980.
8. Gendelman, H. E., Narayan, O., Kennedy-Stoskopf, S. and Pezeshkpour, G. (1986). Tropism of sheep lentiviruses for monocytes: Susceptibility to infection and virus gene expression increase during maturation of monocytes to macrophages. J. Virol. **58,** 67-74.
9. Hirsch, M. S., Zisman, B. and Allison, A. C. (1970). Macrophages and age-dependent resistance to herpes simplex virus in mice. J. Immunol., **104,** 1160-1165.
10. Klatzmann, D., Barré-Sinoussi, F., Nugeyre, M. T. and Montagnier, L. (1984). Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. Science. **225,** 59-63.
11. Lagwinska, E., Stewart, C., Adles, C. and Schlesinger, S. (1975). Replication of lactic deshydrogenase virus and Sindbis virus in mouse peritoneal macrophages. Induction of interferon and phenotypic mixing. Virology. **65,** 204-214.
12. López-Guerrero, J. A., Alarcón, B. and Fresno, M. (1988). Mechanism of recognition of herpes simplex virus type-1-infected cells by Natural Killer cells. J. Gen. Virol. **69,** 2859-2868.
13. López-Guerrero, J. A., Cabañas, C., Bernabéu, C., Fresno, M. and Alonso, M. A. (1989). Poliovirus infection interferes with the phorbol ester-induced differentiation of the monocytic U-937 cell line. Virus Res. **14,** 65-72.
14. López-Guerrero, J. A., Cabañas, C., Bernabéu, C., Fresno, M. and Alonso, M. A. (1990). Effects of poliovirus replication on undifferentiated and differentiated monocytic U937 cells. Comparative studies with human macrophages. Intervirology (en prensa).
15. López-Guerrero, J. A., Fresno, M. and Alonso, M. A. (1989). Inhibición de la replicación del virus de la polio en células del sistema mononuclear fagocítico. Inmunología (en prensa).
16. López-Guerrero, J. A., Martínez-Abarca, F., Carrasco, L., Fresno, M. and Alonso, M. A. (1989). Restriction of poliovirus RNA translation in a human monocytic cell line. Eur. J. Biochem. **186,** 577-582.
17. López-Guerrero, J. A., Redondo, J. M., Alarcón, B., Sánchez-Madrid, F., Rodríguez, M., de Landázuri, M. O., Bernabéu, C. and Fresno, M. (1989). Different functional domains on the transferrin receptor molecule defined by monoclonal antibodies. Immunology. **66,** 252-257.
18. McDougal, S. J., Mawle, A., Cort, S. P., Nicholson, J. K. A. and Sligh, J. (1985). Cellular tropism of the human retrovirus HTLV-III/LAV. I. Role of T cell activation and expression of the T4 antigen. J. Immunol. **135,** 3151-3162.
19. Miller, G., Lisco, H., Kohn, H. and Stitt, D. (1971). Establishment of cell lines from normal adult human blood leukocytes by exposure to Epstein-Barr virus and neutralization by human sera with Epstein-Barr virus antibody. Proc. Soc. Exp. Biol. Med. **137,** 1459-1465.
20. Morahan, P. S., Mama, S., Anaraki, F. and Leary, K. (1989). Molecular localization of abortive infection of resident peritoneal macrophages by herpes simplex virus type 1. J. Virol. **63,** 2300-2307.
21. Okada, Y., Toda, G., Oka, H., Nomoto, A. and Yoshikura, H. (1987). Poliovirus infection of established human blood cell lines: Relationship between the differentiation stage and susceptibility or cell killing. Virology. **156,** 238-245.
22. Oldstone, M. B. A. (1979). Immune responses, immune tolerance and viruses. Comprehensive Virology. **15,** 1-29.
23. Rodgers, B. C. and Mims, C. A. (1982). Role of macrophage activation and interferon in the resistance of alveolar macrophages from infected mice to influenza virus. Infec. Immun. **36,** 1154-1159.
24. Sabin, A. B. (1956). Pathogenesis of poliomyelitis: Reappraisal in the light of new data. Science. **123,** 1151-1157.
25. Sarmiento, M. (1988). Intrinsic resistance to viral infection: Mouse macrophage restriction of herpes simplex virus replication. J. Immunol. **141,** 2740-2748.
26. Tenney, D. J. and Morahan, P. S. (1987). Effects of differentiation of human macrophage-like U937 cells on intrinsic resistance to herpes simplex virus type 1. J. Immunol. **139,** 3076-3083.
27. Wong-Staal, F. and Gallo, R. C. (1985). Human T-lymphotropic retroviruses. Nature (London). **317**, 395-403.

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Fish viral infections in Northwest of Spain

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Summary

During a three years survey, a total of 149 samples from 20 farms of rainbow trout, salmon and turbot were examined for the presence of virus with the purpose to study the viral infections affecting cultured fish and their incidence in the fishfarms of Northwestern Spain.

Infectious pancreatic necrosis virus (IPNV) was the only viral agent isolated from salmonid fish. Fry and fingerlings of trout showed the highest infection rate (24 %). This virus was not detected in broodstock or embrionated eggs, although it was isolated from ovaric and seminal fluids and from juvenile carriers. From 24 samples of salmon analyzed, IPNV was only detected in one sample of juveniles. Examination of turbot led the isolation of a new virus belonging to the reoviridae family, which affected to the ongrowing population. All of the IPNV tested belonged to serotype Sp regardless of the origin of the trout stocks.

During the monitorization of imported embrionated eggs, no virus was detected from any of the samples. However, in some case, IPNV was isolated when testing the fry obtained in our laboratory from those samples of imported eggs. Our findings indicate that: i) the analysis of fingerlings increase the probability to detect viral infections allowing us an optimal control of importations, and ii) most of the viral infections of fish take place in the own fish farms. The detection of mixed viral and bacterial infections emphasize the importance of carriyng out an integral microbiological analysis to determine the causal agent(s) of fish mortalities.

Key words: Fish viruses, IPNV, trout, salmon, turbot.

Resumen

Con el fin de determinar el tipo e incidencia de infecciones virales en Galicia durante un período de 3 años, se examinaron 149 muestras correspondientes a 20 piscifactorías de trucha, salmón y rodaballo.

En peces salmónidos hemos detectado exclusivamente el virus de la necrosis pancreática infecciosa (IPNV), siendo los alevines de trucha los que presentaron una mayor tasa de infección (24 %). Aunque este virus fue también aislado en juveniles y muestras de fluido seminal y ovárico, no se detectó en vísceras de reproductores ni en muestras de huevos embrionados. De un total de 24 muestras de salmón analizadas, el virus IPNV fue aislado solamente en una muestra de juveniles. El análisis virológico de rodaballo permitió aislar un nuevo Reovirus, que afectó a poblaciones de peces de más de 250 g. Independientemente del origen geográfico de los peces, todos los IPNV estudiados pertenecen al serotipo Sp. Durante el estudio de las importaciones de huevos embrionados no se detectó virus directamente en los huevos; sin embargo, se aisló IPNV de una muestra de alevi-

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nes nacidos en el laboratorio a partir de una submuestra de dichos huevos. Los resultados sugieren que: i) el análisis de los alevines incrementa la probabilidad de detectar virus, permitiendo un óptimo control de las importaciones, y ii) la mayoría de las infecciones virales de peces tienen lugar en las propias piscifactorías.

La detección de infecciones mixtas de virus y bacterias en los peces examinados apoya la necesidad de realizar un análisis microbiológico integral con el fin de determinar el (los) agente(s) causales de las mortalidades.

Introduction

The development of aquaculture in the last decades has increased the microbiological problems related to fish cultures. This fact has determined an improvement in the investigation in fish pathology and the publication of a great number of papers about this subjetc (2, 33).

Among the microbiological infections affecting farmed fish the viral diseases cause the most important problems due to the lack of effective treatments and the persistence of viruses in asymptomatic carriers. The studies in this field have currently been focused on: i) the knowledge of the viruses affecting to a specific geografic area and the fish species susceptible to these viral agents, and ii) the incidence of that viral infections have in cultured fish in each area.

To date, the viruses discovered in teleost fishes are members of 12 established families, being the most relevant birnaviridae, herpesviridae, rhabdoviridae and more recently, reoviridae.

Within the birnaviridae family, the IPNV (infectious pancreatic necrosis virus) and other IPN-like viruses represent a very important group due to their high pathogenicity as well as their wide host range and geografic distribution (2, 8, 32).

The channel catfish virus (CCV) (11), *Herpervitus salmonis* (HS) (29), *Herpesvirus scophthalmi* (6) and Pacific salmon herpesviruses such as OMV (16) and NevTa (26) are relevant members of the herpesviridae family. Whereas most of them have been extensively studied and characterized because their importance in cultures of catpish and salmonids. *Herpesvirus scophthalmi* has only been detected by electron microscopy in one occasion (6). Therefore the real implication of this virus in the turbot culture is not known.

Erythrocytic necrosis virus (ENV), is one of the most studied iridovirus (10, 23). This virus affects mainly to seawater fish species and at a lower extend to freshwater fishes. However, until present ENV has not been isolated in cell culture.

Among rhabdoviruses, the infections haematopoietic necrosis virus (IHNV) and the viral haemorrhagic septicaemia virus (VHSV) produce the most important economic losses in the culture of salmonids throughout the world (2, 32). While the IHNV affects mainly to young salmonids and it is considered endemic of the Pacific coast of the United States, the VHS has been only detected in Europe causing mortalities in older fishes (2, 32).

The reoviridae family has acquired importance in aquaculture because from 1979 ar least eleven reo-like viruses were detected among fresh and sea water fishes as well as in molluscs and crustacea (20, 24, 27). The implication of al these reo-like viruses in aquaculture as well as their taxonomic position within the family is still not clear.

The occurrence of vertical transmission in some viruses has produced their spreading caused mainly by the uncontrolled importations of embrionated eggs and asymptomatic carrier fishes. In fact, the IPN virus has been recently detected in China (35), probably due to an importation of rainbow trout from the United States.

The transmission of IPNV and IHNV from eggs and sex products has been clearly supported by several authors (21, 30). However, until now, the vertical transmission of other viruses like CCV and VHS has not been demonstrated, although there are some evidences of its existence. Therefo-

re, the diagnostic of viruses in embrionated eggs and sex products allows the detection of important viral diseases without destroying the broodstock.

The purpose of this work was to determine the viral infections affecting to cultured fish species and their incidence in the fish farms of our area. In order to investigate the origin of these diseases a study of the virological quality of imported embrionated eggs was also conducted.

Materials and methods

Cell culture and media

The CHSE-214 (chinook salmon embryo) and EPC (epithelioma papullosum cyprini) cell lines were used for the primary isolation of viruses. These fish cell lines were cultured as monolayers using minimal essential medium (MEM) with Earle's salts supplemented with 10 % of newborn calf serum (NBS) and containing 100 IU/ml of penicillin and 100 µg/ml of streptomycin.

CHSE-214 cultures were grown at 15° C and EPC at 25° C. Both cell lines were maintained at 15° C when monolayers were confluent.

Virus isolation

Fish, sex products and embrionated eggs of different fish farms from the Northwest of Spain were examined for the presence of viruses. Samples were taken regularly as well as during epizootics Kidney, liver and spleen were removed from juveline and adult fishes, and in the case of breeders, sperm and eggs were also tested. When analyzing fry or fingerlings, the whole fish was sampled. The samples were homogenized in Hanks balanced salt solution (HBSS). The number of the fish per sample was determined according to the Ossiander and Wedemeyer tables (22). Assuming a minimal infection rate of 5 % and a confidence limit of 95 %, each sample was composed by 60 fish. The samples were divided into six pools which were analyzed separately. The homogenate was diluted 1:10 in HBSS and centrifugated at 2000 x g for 15 min. The supernatant was collected and the microbial contamination was eliminated by treatment with antibiotics (penicillin, 800 UI/ml, streptomycin, 800 µgr/ml) for 1-3 hours.

The samples were inoculated onto monolayers of CHSE and EPC cells grown in 24 well plates. For each sample four wells were inoculated with 0.1 ml aliquots. One row on each plate served as control receiving only maintenance medium. The plates were incubated at 15° C. If no cythopatic effect (CPE) was observed within 7-14 days, fluids were removed from the wells and used to inoculate fresh monolayers (blind passage). When no CPE was detected after ten days after the blind passage, the samples were considered negative for virus. Samples were only considered positives for virus presence when CPE was observed in two consecutive passages.

Virus titration

The titrations of the viruses were carried out in 96 well microculture plates. After seeding the plates with approximately 5×10^4 cells per well, 0.1 ml amounts of serial 10-fold virus dilutions were added, eigth wells being used per dilution. Assays of the different isolates were incubated for 14-20 days at 15° C. Cultures were examined periodically for cythopatic effect and the results were expressed as the number of tissue culture infectious dose ($TCID_{50}$) per ml according to the Reed and Müench method (25).

Physicochemical characterization of the viral isolates

i) *Chloroform sensitivity assay.*

This test was performed following basically the procedure of Jørgensen (13). Briefly, virus suspensions treated with chloroform were titrated along with untreated control suspensions. Losses of infectivity greater than 1 $\log_{10}$ indicated susceptibility to the treatment. IPNV (non-enveloped birnavirus) and IHNV (enveloped rhabdovirus) served as negative and positive controls respectively.

ii) *Acridine orange stain*

To elucidate the nature of the genome of our viral isolates, inoculated CHSE-214 cells were stained with the acridine orange stain performed according to Kuchler procedures (15). IPNV and NeVTA (RNA and DNA viruses respectively) as well as non-inoculated CHSE-214 cells were used as controls.

iii) *Serological characterization*

Neutralization test with our viral isolates were performed using specific rabbit antisera against IPNV serotypes Sp, Ab and Vr-299. Stocl preparations of the isolates were diluted to contain about 10^2-10^3 $TCID_{50}$/ml and mixed with sera dilutions (1/20,000 for Vr-299 and 1/500,000 for Sp and Ab), according with their previously determined titer. Mixtures were incubated at room temperature during one hour and then inoculated onto confluent CHSE-214 monolayers. A group of 6 wells was used per mixtures and an inoculum of 0.1 ml per well. Control cultures were inoculated with IPNV serotypes Sp, Ab and Vr-299 and IHNV treated with the three antisera and normal rabbit serum. Untreated viruses were also inoculated as control of viral infectivity.

iv) *Electron microscopy*

Morphological studies of the viral isolates were carried out by electron microscopy using negative staining (8). Viral samples were concentrated by centrifugation at 100,000 x g for 2 hours at 4° C and the pellet resuspended in a minimun volume of PBS. A small drop of the concentrate was placed onto a carbon coated grid and after 3-5 minutes, the excess was rinsed off and the grid and covered with 2 % phosphotungstic acid for 40 sec. Negatively stained virions were the viewed with a JEOL CX electron microscope.

Virological studies of eggs importations

With the aim to determine if importations of eggs introduce viral infections in our fishfarms, samples of embrionated eggs imported from different geografic areas, were collected at their arrival at the custom. These samples were divided in two pools. One of them was processed immediately for virological analysis and the other one was maintained to hatch in a laboratory aquarium with virus free spring water maintained at 15 ± 1° C. During this period, samples were periodically observed to eliminate dead eggs. Fry born from these eggs were examined for virus after 30 days post hatching. Simultaneously, fry from the same stocks of eggs were sampled from the fish farms and analyzed with comparative purposes. During the incubation, the temperature of the water was measured daily. The age of the fish was expressed as X/day (sum of the temperature of each day).

Results

During the period 1986-1988, a total of 149 samples from 20 fishfarms of rainbow trout, salmon and turbot were examined for the presence of virus using two different cell lines, CHSE-214 and EPC, which allow the detection of a wide range of fish viruses. In the present work infectious pancreatic necrosis virus (IPNV) was the only viral agent isolated from salmonid fishes. Virological examination in farms of turbot led the isolation of a new virus, which was identified as a member of the reoviridae family (Table 1).

With respect to the samples of trout (Table 2), the highest percentage of viral isolations was obtained from fry and fingerlings which showed an infection rate of 24 %, while only 4 of 23 samples of yearlings were infected. From sex products, only three samples yielded IPNV. Virus was not detected in broodstock or in the embrionated eggs examined, but a short number of these samples were available for investigation.

Among the samples of salmon analyzed, IPNV was only isolated once from a sample of juveniles but no virus was isolated from the remaining sample types.

Table 3 shows the annual distribution of positive IPN isolates among the farms of trout analyzed. In the majority of them, IPNV was isolated occasionally in the samples analyzed but, interestingly, we have isolated this virus from two farms (n.° V and VI) in the two consecutive years with a high infection rate (44 % and 32 %, respectively).

All the virus isolated from salmonids were identified as IPNV according to their resistance to chloroform treatment and seroneutralization test with a polivalent antiserum of IPNV. Furthermore, some of these viral strains were serotyped by neutralization test using specific antisera against the three classic serotypes of IPNV (Vr-299, Sp and Ab). The results revealed that all the isolates serotyped belong to serotype Sp regardless of the origin of the fish stocks.

From a total of 38 samples of turbot studied (Table 2), a reovirus was detected twice from the ongrowing population (average weight 250 grams) of different fishfarms. These viral isolates produced a slow CPE in CHSE cell line, typified by formation of plaque inrolving syncitia containing 5 to 10 nuclei and surrounded by a clear area. When the CPE became extensive, large syncitia of 25 or more detected in different samples of turbot three putative viral agents which are actually being investigated.

The results obtained from the virological study of 7 egg importations (Table 4) revealed that although no virus were detected in embrionated eggs, IPNV was isolated in one sample of 30 days old fry which were born in the laboratory from eggs imported from USA. In addition, the fry reared from these importations in the fishfarms were also tested after 30 days, and three of them were infected with IPN virus.

The simultaneous analysis for viruses and bacteria in 24 samples, revealed the presence of a 25 % of mixed infections, being *Yersinia, Aeromonas* and *Renibacterium* the bacterial genera more frequently detected.

TABLE 1
ISOLATION OF VIRUSES DURING A THREE YEAR PERIOD (1986-1988)

Specie	No of farms examined	No of samples analyzed	No of positive samples	Virus isolated
Trout	12	87	18 (20.6 %)[a]	IPNV
Salmon	2	24	1 (4.1 %)	IPNV
Turbot	6	38	2 (5.2 %)	Reovirus

[a] Percentage of positive samples

TABLE 2
DETECTION OF VIRUS IN EGGS, SEX PRODUCTS AND DIFFERENT STAGES OF FISH GROWTH

Species	Fingerlings	Juveniles	Broodstock	Sex products		Eggs
				Seminal fluid	Ovarian fluid	
Trout	11/45[a](24.4)[b]	4/23 (19.0)	0/6	1/6 (16.6)	2/6 (33.3)	0/4
Salmon	0/3	1/14 (7.1)	0/4	ND	0/1	0/2
Turbot	0/20	2/10	0/2	0/3	0/2	0/1

[a] Number of positive samples/ Total samples analyzed.
[b] Percentage.
ND: No determined.

Discussion

The farming industry of salmonids and turbot in the Northwestern Spain has a considerable potential for the production of those fish species. Although we have previously detected infectious pancreatic necrosis virus (IPNV) among trout and salmon reared in fishfarms (17), disease outbreaks attributed to viruses have not been reported to date in cultured turbot in Spain.

Due to the impossibility to obtain balanced samples from the fishfarms both within and between species, no statistical analysis was performed. In spite of this, the results of our study have revealed that only IPNV is detected among salmonid fishes. On the other hand, although the existence of VHS virus in Spain has been reported by other authors (12), we have not detected this virus during the present study. In addition, although a high number of importations of trout eggs comes from enzootic areas in USA for IHNV, this virus has not been isolated among the samples analyzed. These results are more unexpected because the viral analysis was carried out in cell lines with high

TABLE 3
INCIDENCE OF IPNV IN THE TWELVE TROUT FARMS ANALYZED DURING THIS SURVEY

Farm	No of positive samples/ Total samples analyzed	1986	1987	1988
I	1/3 (33.3)[a]	1/1	ND	0/2
II	1/13 (7.6)	1/2	0/1	0/10
III	0/5 (0)	0/2	ND	0/3
IV	0/8 (0)	0/1	0/5	0/2
V	4/9 (44.4)	0/1	2/3	2/5
VI	8/25 (32)	ND	5/18	3/7
VII	0/3 (0)	ND	0/3	ND
VIII	1/3 (33.3)	ND	1/3	ND
IX	2/8 (25)	0/1	2/6	0/1
X	0/6 (0)	ND	0/4	0/2
XI	0/3 (0)	ND	0/3	ND
XII	1/1 (100)	ND	ND	1/1
TOTAL	18/87 (19.3)	2/8	10/46	6/33

[a] Percentage of positive samples.
ND: No determined.

TABLE 4
MONITORIZATION OF IMPORTATIONS BY VIROLOGICAL ANALYSIS OF EMBRIONATED EGGS AND FRY HATCHED IN THE LABORATORY AND IN THE FARMS

Origin of embrinated eggs		Virological analysis				
Species	Country	Embrionated[a] Eggs	Laboratory[b]		Farm[c]	
			Fry	Age (X/day)	Fry	Age(X/day)
Trout	USA	ND	–	460	–	368
Salmon	Norway	–	–	447	–	492
Trout	Denmark	–	–	407	+	751
Trout	Denmark	–	–	406	+	599
Trout	Spain	–	–	480	–	561
Trout	Spain	–	–	ND	–	ND
Trout	USA	–	+	ND	+	ND

[a] Eggs sampled at the arrival at the custom.
[b] Fry from eggs hatched in the laboratory.
[c] Fry from eggs hatched in the fish farms.
ND: No determined.
+: Sample of fry in which IPNV was isolated.

susceptibility to these viruses (14). The isolation of a virus of the Reoviridae family from diseased farmed turbot is a very interesting finding since there are few reports of viral infections in this species (6, 7). Until recently, few reo-like viruses were detected from aquatic organisms, being the most relevant: GSV (golden shiner virus) (24), chum salmon virus (CSV) (28), channel catfihs reovirus (CRV) (1), and striped bass reovirus (SRV) (3). However, the role of this viral group in aquaculture as well as their pathogenicity to cultured species has not yet clearly established. Recently results of experimental infections with the Turbot Reovirus (TRV) (18) performed in our laboratory demonstrated that this new virus is pathogenic for turbot, being reisolated from each inoculated dead fish (Lupiani, B., Ledo, A., Dopazo, C. P., Baya, A., Toranzo, A. E. and Barja, J. 1989 Prooc. 4rd Intern. Conf. Eur. Ass. Fish Pathol. p. 103).

IPN is considered as a disease of young salmonid fish (2, 8, 32). In fact, we have detected a higher percentage of isolations among fry and fingerlings than in yearlings of rainbow trout. However, the studies with respect to the incidence of carrier fish in populations vary depending on the species of fish involved. Although Billi and Wolf (4) and Yamamoto et al. (34) found that almost all brook trout (*Salvelinus fontinalis*) surviving from a IPNV epizootic were carriers, rainbow trout have a lower and rapidly decreasing carriers rate (31, 34). It is noteworthy that our IPNV isolates from adult rainbow trout were detected in fish showing high mortalities that would be unusual if were due to the IPNV infection. Interestingly, the presence of bacterial pathogens in those samples indicates that secondary infections were the main responsable cause of these mortalities.

Also, we have detected IPN virus in one sample of seminal fluid and two samples of ovarian fluid belonging to the same stock of breeders. The principal non-destructive method for the detection of virus carriers is the analysis of the sex products. Because asymptomatic carriers usually contain concentrations of virus lower than those found in fish dying from acute disease, it would be useful to improve the techniques to detect virus in sexual products. In fact, we have reported [Dopazo, C. P., Baya, A., Ledo, A., Lupiani, B. and Toranzo, A. E.. 1988 Prooc. 3rd Intern. Coll. Pathol. Marine Aquacult. (PAMAQ), p. 3-4] that the use of high speed and low time in the centrifugation

step of the analysis (conditions used in this study) facilitate the rapid detection of IPN virus in the ovarian fluid samples without loss of sensitivity.

All the samples of imported eggs analyzed were negative for virus presence. However, because in one case we isolated virus from fry obtained in our laboratory, we can suggest the analysis of fingerlings increase the probability to detect viral infections allowing us not only an optimal virological control of importations but also their detection at early stages before the appearance of outbreaks among fish population. In addition, the isolation of virus among trout reared in fish farms which were born from imported virus free eggs seems to indicate that these fish were infected in the hatchery, that reveal the necessity of good disinfection treatments in the fishfarms. These findings are supported by the fact that IPNV was isolated repeatedly in these fishfarms.

The detection of the same serotype of IPNV strains from fishes with different geographic origin could indicate the necessity of a revision of the epidemiological distribution of the three classic serotypes of this virus. In this sense, MacAllister et al. (19) have reported the isolation of IPNV serotype Ab in USA and Bovo et al. (5) the isolation of IPNV strains in Italy belonging to serotype Vr-299. Moreover, due to the great serological heterogenicity among the strains of this virus it has recently proposed a new serological classification in nine serotypes, according to the host specificity and serological relationships among strains.

Finally, the detection of mixed infections demonstrated the importance to carry out simultaneous analysis for virus and bacteria in diseased fish with the aim to determine the causal agent(s) of mortalities.

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References

1. Amend, D. F., McDowell, T. and Hedrick, R. P. (1984). Characteristics of a previously unidentified virus from channel cat fish. *(Ictalurus punctatus)*. Can. J. Fish Aquat. Sci **41,** 807-811.
2. Ahne, W. (1985). Viral infection in fishes: Aetiology, Diagnosis and Control. Zhl. Vet. Med. **32,** 237-264.
3. Baya, A., Toranzo, A. E., Núñez, S., Barja, J. L. and Hetrick, F. M. (1989). Association of a Moraxella sp. and a reo-like virus with mortalities of striped bass *(Morone saxatilis)*. *In:* C. F. O. Cheng & Perkins (eds.). Pathology in Marine Science, Academic Press, N. Y. pp 91-99.
4. Billi, J. L. and Wolf, K. (1969). Quantitative comparison of peritoneal wastes and feces for detecting infectious pancreatic necrosis (IPN) virus in a carrier brook trout. J. Res. Board. Can. **26,** 1459-1465.
5. Bovo, G., Giorgetti, G. and Ceschia, G. (1985). Comparative sensitivity of five fish cell lines to wild infectious pancreatic necrosis virus isolated in Northeastern Italy. *In:* E. Ellis (Ed.). Fish and Shellfish Pathology, Academic Press, London.
6. Buchanan. J. S., Richards, R. H., Sommerville, C. and Madeley, C. (1978). A herpes-type virus from turbot *(Scophthalmus maximus)*. Vet. Rec. **102,** 527-528.
7. Castric, J., Baudin-Laurencin, F., Coustans, M. F. and Auffret, M. (1978). Isolation of infectious pancreatic necrosis virus, Ab serotype from an epizootic in farmed turbot *(Scophtalmus maximus)*. Aquaculture **67,** 117-126.
8. Dobos, P. R., Hallet, R., Kells, D. T. C., Sorensen, O. and Rowe, D. (1977). Biophysical studies of infectious pancreatic necrosis virus. J. Virol. **22,** 150-159.
9. Dorson, M. (1982). Infectious pancreatic necrosis of salmonids: An overwiew of current problems. *In:* D. P. Anderson, M. Dorson and P. H. Dubourget (Eds.). Antigens of fish pathogens, pp. 7-32. Collection Fundation Marcell Merieux, Lyon, France.
10. Evelyn, T. P. T. and Traxler, G. S. (1978). Viral erythrocytic necrosis: natural occurrence in Pacific salmon and experimental transmission. J. Fish Res. Board Can. **35,** 903-97.

11. Fijan, N. (1968). Progress report on acute mortality of channel catfish fingerlings caused by a virus. Bull. Off. Inter. Epizoot. **69,** 1167-1168.
12. Jiménez de la Fuente, J., Marcotegui, M. A., San Juan, M. L. and Basurco, B. (1988). Diagnosis of viral diseases of salmonid farms in Spain. Bull. Eur. Ass. Fish Pathol. **8,** 1-2.
13. Jorgensen, P. E. (1972). Egtved virus: Antigenic variation in 76 virus isolates examined in neutralization test and by means of the fluorescent antibody technique. Symp. Zool. Soc. Lon. **30,** 333-340.
14. Kelly, R. K., Souter, B. W. and Miller, H. R. (1978). Fish cell lines. Comparisons of CHSE-214, FHM and RTG-2 assaying IHN and IPN viruses. J. Fish Res. Board Can. **35,** 1009-1011.
15. Kuchler, R. J. (1977). Biochemical methods in cell culture and virology. Dowden, Hutchinson & Ross, Inc. Pennsylvania, USA.
16. Kimura, T., Yoshimizu, T., Tanaka, M. and Sannoche, H. (1981). Studies in a new virus (OMV) from *Oncorhynchus masou* I. Characteristics and pathogenicity. Fish. Pathol. **15,** 143-147.
17. Ledo, A., Dopazo, C. P., Lupiani, B., Barja, J. L. and Toranzo, A. E. (1987). Incidencia del virus de la necrosis pancreática infecciosa (IPNV) en Galicia. Cuad. Marisq. Publ. Tec. **12,** 645-650.
18. Lupiani, B., Dopazo, C. P., Ledo, A., Fouz, B., Barja, J. L. Hetrick, F. M. and Toranzo, A. E. (1989). A new syndrome of suspected bacterial and viral aetiology in cultured turbot *(Scophthalmus maximyus, L.).* J. Aquac. An. Health, pp 197-204.
19. Mac Allister, P. E., Newman, M. W., Sauber, J. H. and Owens, W. J. (1984). Isolation of infectious pancreatic necrosis virus (Serotype Ab) from diverse species of stuarine fish. Helgoländer Meeresunters. **37,** 317-328.
20. Meyers, T. R. (1979). A reo-like virus isolated from juvenile American oyster *(Crassostrea virginica).* J. Gen. Virol. **43,** 203-212.
21. Mulcahy, D. and Pascho, R. J. (1985). Vertical transmission of infectious haematopoyetic necrosis virus in sockeye salmon. *Oncorhynchus nerka* (Walbaum): isolation of a virus from dead eggs and fry. J. Fish. Dis. **8,** 393-396.
22. Ossiander, F. J. and Wedemeyer, G. (1973). Computer program for sample sizes required to detemine disease incidence in fish populations. J. Fis. Res. Bd. Can. **30,** 1383-1384.
23. Pinto, R. M., Alvarez-Pellitero, P., Bosch, A. and Jofre, J. (1989). Occurrence of a viral erythrocytic infection in the Mediterranean seabass *(Dicentrarchus labrax, L),* J. Fish. Dis. **12,** 185-191.
24. Plumb, J. A., Bowser, P. R. Grizzle, J. M. and Mitchell, A. J. (1979). Fish viruses: A double stranded RNA icosahedral virus from north American cyprinid. J. Fish. Res. Board Can. **36,** 1390-1394.
25. Reed, L. J. and Muench, H. (1938). A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27,** 493-497.
26. Sano, T. (1976). Viral diseases of culured fishes in Japan. Fish. Pathol. **10,** 221-226.
27. Winton, J. R., Lannan, C. N., Fryer, J. L., Hedrick, R. P., Meyers, T. R., Plumb, J. A. and Yamamoto, Y. (1987). Morphological and biochemical properties of four members of a novel group of reovirus isolated from aquatic animals. J. Gen. Virol. **68,** 353-364.
28. Winton, J. R., Lannan, C. N., Fryer, J. L. and Kimura, T. (1981). Isolation of a new virus from chum salmon in Japan. Fish. Pathol. **15,** 155-162.
29. Wolf, K., Darlington, W. G., Taylor, W. G., Kimby, M. C. and Nagabayashi, T. (1978). Herpesvirus salmonis: Characterization of a new pathogen of rainbow trout. J. Virol. **27,** 659-666.
30. Wolf, K., Quimby, M. C. and Bradford, A. D. (1963). Egg-associated transmission of IPN virus of trouts. Virol. **21,** 317-321.
31. Wolf, K., Quimby, M. C., Carlson, C. P. and Bullock, G. L. (1968). Infectious pancreatic necrosis virus: Detection of virus free stock from a population of carrier trout. J. Fish. Res. Board Can. **25,** 383-391.
32. Wolf, K. (1984). Diseases of pisces. Diseases caused by microorganisms. In: O. Kinne (Ed.) Diseases of marine annimals, pp. 17-47. Biologische Anstalt Helgoland, Hamburg, F. R. G.
33. Wolf, K. (1988). Fish viruses and fish viral diseases. Cornell University Press, London.
34. Yamamoto, T. and Kilistof, J. (1979). Infectious pancreatic necrosis virus: Quantification of carriers in lake populations during a 6 year period. J. Fish. Res. Board Can. **36,** 562-567.
35. Yuling, J. and Zhengqiu, L. (1987). Isolation of IPN virus from imported rainbow trout *(Salmo gaidneri)* in P. R. China. J. Appl. Ichtiol. **3,** 191-192.

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Biovars of coagulase-positive staphylococci isolated from bovine mastitic milk

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Summary

Fifty strains of coagulase-positive staphylococci associated with bovine mastitis were biotyped. Of them, 20 were identified as biovar C (cattle & sheep), 17 as biovar B (poultry & swine), 2 as biovar D (hares) and 1 as biovar F (pigeon & fox). Of the remaining strains, 2 were closely related to human biovar A, 2 failed one property to be classified as biovar B, 3 shared properties of biovars B and D and 3 could not be identified. Bovine strains belonging to biovar C formed an heterogeneous group showing differences in crystal violet growth type and production of alpha haemolysin. Only strains associated with biovar A were Tween 80 positive. The highest incidence of lytic reactions amongst all biovars was with phages 42 E (III) and 102 (IV). Antibiotic resistance was most frequently found in biovar C (65%). The 2 strains in biovar D, one classed as biovar C and one as intermediate, were enterotoxigenic (C or D toxins).

Key words: Staphylococci, biotypes, bovine mastitic, milk.

Resumen

Un total de 50 cepas de estafilococos coagulasa positivos aisladas de mamitis bovinas fueron adscritas a biotipos (biovares o ecovares). De ellas, 20 pertenecían al biovar C (vacuno y ovino), 17 al biovar B (aves y cerdos), 2 al biovar D (liebres) y 1 al biovar F (palomas y zorros). Las propiedades de otras 2 cepas eran muy similares a las del biovar humano (A), 2 fallaban una propiedad del biovar B, 3 compartían características de los biovares B y D y 3 no pudieron ser identificadas. Las cepas del biovar C formaban un grupo heterogéneo con variaciones en el tipo de crecimiento en agar cristal violeta y en la producción de hemolisina α. Unicamente las cepas próximas al biovar humano hidrolizaban el Tween 80. Los bacteriófagos que lisaban mayor número de cepas en todos los biovares fueron el 42 E (III) y el 102 (IV). La mayor frecuencia de resistencia a los antibióticos se encontró en el biovar C (65%). Las 2 cepas del biovar D, una adscrita al C y otra de propiedades intermedias eran enterotoxigénicas (enterotoxinas C o D).

Introduction

Considerable host-specifity has been demonstrated by certain species of staphylococci (16, 18). Furthermore, some phenotypic characteristics of *S. aureus* seem to depend on the origin of the

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strains (humans or different animal species). On the basis of the latter observations, Meyer (21) proposed to subdivide *S. aureus* into three varieties according to the natural host (*S. aureus* var. *hominis, S. aureus* var. *canis* and *S. aureus* var. *bovis*) and Hajek & Marsalek (13, 14) distinguished six biovars or ecovars (biotypes) of *S. aureus,* three of which corresponded to the varieties of Meyer. Strains isolated from humans were placed in biotype A. Poultry and pig strains were biotype B, cow and sheep isolates usually belonged to biotype C and the predominant strains isolated from hares were placed in biotype D. Strains of biotype E (dogs, minks & horses) and F (pigeon & foxes) were later reclassified as *S. intermedius* (10).

Bovine mastitis is one of the most important animal diseases, in which coagulase-positive staphylococci may be the specific aetiological agent. The aim of this work was to identify the biovars or ecovars of coagulase-positive staphylococci associated with bovine mastitis in order to obtain ecological and epidemiological information.

Materials and methods

Cultures

A total of 50 strains of coagulase-positive staphylococci isolated either in pure culture or in numbers sufficient to be considered the cause of mastitis were studied. Each culture was obtained from one sample of milk taken from an individual cow suffering from acute, gangrenous or chronic mastitis. Most of the animals yielded pure cultures. Each strain was incubated in brain heart infusion broth (BHI) and streaked on BHI agar. One colony was picked, and the procedure repeated three times. Identification at the genus level and coagulase production were carried out according to Menes *et al.* (20).

Taxonomic scheme

Subdivision into ecovars was done according to the scheme proposed by Hajek and Marsalek (13, 14).

Tests

Fibrinolysin production was investigated by the plate method of Christie and Wilson (5).

Production of pigment was observed on P agar as proposed by Kloos (17).

Coagulase activity was determined by the tube assay described by Baird-Parker (2) using fresh human and bovine plasmas containing 0.1 % EDTA. Reactions were examined after 4 and 24 hours and scored from 0 to 4 + (29).

The production of alpha, beta and delta haemolysins was studied by the method of Nakagawa (23) using tryptose blood agar base (Difco) and three times washed rabbit, sheep, horse, and human erythrocytes (5 %, v/v). Filter paper strips soaked in anti-alpha-haemolysin serum (Burroughs Wellcome Co., England) were employed as indicated by Elek and Levy (8). *S. aureus* strains ATCC 8096 and ATCC 25178 were included as controls.

The crystal violet reaction was investigated according to Meyer (21).

Clumping factor was determined with rabbit plasma plus EDTA (Difco) according to the manufacturer's instructions and taking into account the recommendations of Devriese and Hajek (7).

Tellurite reduction and egg yolk production were observed after growth for 24 and 48 hours on Baird-Parker medium (Oxoid).

Hydrolysis of Tween 80 was examined as described by Gutiérrez *et al.* (9).

Phage typing was carried out using the international basic set of human *S. aureus* phages (28)

and the basic set for typing bovine *S. aureus* (6). The technique used was that of Blair and Williams (4) as modified by Parker (24a) and de Saxe *et al.* (26). Strains negative at the routine test dilution (RTD) were retested at 100xRTD.

Antibiotic susceptibility was determined by the disc diffusion method (3). The following antibiotics were used: penicillin G, cephalotin, kanamycin, tetracycline, streptomycin, methicillin, erythromycin and chloramphenicol (Difco). Special precautions were taken for detecting methicillin resistance (the medium was stabilized osmotically with 5 % NaCl and the temperature hold at 30° C). *S. aureus* strain ATCC 25923 and *E. coli* ATCC 25922 were included as controls.

Enterotoxin A-E production were assayed by the optimal sensivity plate method of Robbins *et al.* (25). The cellophane over agar method was used for enterotoxin production (25).

Results

The biovars found, their physiological and biochemical characteristics and the number of isolates belonging to each biovar are shown in Table 1.

Twenty strains were classified as biovar C, 17 as biovar B, 2 as biovar D, and 1 as biovar F. Of the remaining strains, 3 were considered intermediate (shared properties of biotypes B and D), 2 were similar to biovar A (failed one property), 2 were similar to biovar B and 3 could not be identified.

Only 3 strains showed lipolytic activity on Tween 80 and 9 produced egg yolk factor.

The number of antibiotic resistant strains was 22. Of them, 4 belonged to biovar B (penicillin, 3; streptomycin, 1), 13 to biovar C (chloramphenicol, 2; penicillin, 8, and penicillin + streptomycin, 3), and 1 to biovar D (penicillin). Table 1 shows that penicillin resistance was also observed in strains of patterns 9, 10 (Next A) and 16 (unidentified). The strain in pattern 11 (Next B) was resistant to streptomycin.

Sixteen strains belonging to biovar B were typable. The phage groups found were III-IV (9 strains), IV (4 strains), II-III-IV (2 strains) and I-III-IV (1 strain). The highest incidence of lysis corresponded to phages 102 (14 strains) and 42 E (12 strains). The number of typable strains in biovar C was 19. Of them, 10 were lysed by phages from groups III-IV, 7 by phages from group IV, 1 by phages from groups II-III-IV and 1 by phages from groups III-IV-M. All typable strains were lysed by phage 102, 15 by phage 42 E and 14 by phage 42D. The phage groups of both strains in biovar D were I-M. Strains classed as Next A were lysed exclusively by human phages 29/52/52A/81 (I-M). Patterns 11 and 12 were lysed by phages in groups IV and III-IV, respectively. Phage groups of isolates in pattern 13 were I-M, I-II-III, and I-M. Also, the unclassified strain of pattern 16 was typable by phages of III and IV groups.

Four strains were enterotoxigenic. Of them, 2 corresponded to biovar D (toxins C and D), one to biovar C (C toxin) an the remaining, also C producer, was identified as intermediate.

Discussion

Several studies have shown that certain infections of sheep (mastitis, abscesses, etc.) are mainly produced by ovine-adapted *S. aureus* strains which form an homogeneous and characteristic group within the biovar C (cattle & sheep). They could be recognized by being crystal violet type C (negative), usually enterotoxin C producers and typable by bovine phage 78 (11, 15, 20). In a previous work on *S. aureus* strains isolated from ovine mastitic milk (9), we obtained data which confirmed the above observations. In contrast, many of the coagulase-positive staphylococci included in this

TABLE 1
BIOCHEMICAL AND PHYSIOLOGICAL PATTERNS AND ECOVARS (BIOTYPES) OF 50 COAGULASE-POSITIVE STAPHYLOCOCCAL STRAINS AGENTS OF BOVINE MASTITIS

	Patterns															
Tests	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Fibrinolisyn	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	–
Pigment	+	+	+	+	+	+	–	–	+	+	–	+	+	+	+	+
Coagulase:																
Human plasma	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+
Bovine plasma	–	–	+	+	+	+	–	+	–	+	–	–	–	–	+	–
Haemolysins:																
Alpha	+	–	–	+	+	–	–	–	+	+	+	+	+	–	–	–
Beta	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–
Crystal violet type[a]	A	A	A	C	A	C	C	A	A	C	A	A	C	A	A	B
Clumping factor	+	+	+	+	+	+	+	–	+	+	+	–	+	–	+	–
Tellurite reduction[b]	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+
Phage typing[c]	V	V	V	V	V	V	V	–	H	H	B	H/B	V	–	–	H/B
Biotype[d]	B	B	C	C	C	C	D	F	NxtA	NxtA	NxtB	NxtB	IntB-D	U	U	U
N.° of strains	14	3	7	7	4	2	2	1	1	1	1	1	3	1	1	1
Tween 80	–	–	–	–	–	–	–	–	+	+	–	–	–	+	–	–
Egg yolk factor	6+	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
Enterotoxins	–	–	–	–	–	1C	1C+1D	–	–	–	–	–	1C	–	–	–

[a] A/B type, +; C type, –.
[b] ±, grey
[c] V, variable (human and/or bovine phages or none); H, lysed by human phages; B, lysed by bovine phages.
[d] Next, failed one property; Int, shared properties of ecovars B and D; U, unidentified.

study, isolated from mastitic cows in the same geographic area, belonged to biovars associated with other hosts.

Characteristic of our C biovar strains (Table 1, patterns 3 to 6) was their ability to coagulate human and bovine plasmas and to produce beta haemolysin; however, there were differences in crystal violet growth type (11 strains were type A and 9 strains were type C) and alpha haemolysin production (11 strains were positive). According to Hajek and Marsalek (12), the character of growth on crystal violet agar is useful for the differentiation of virulent bovine strains (type A) from carrier bovine strains (type C). Also, production of alpha haemolysin is often associated with the more virulent bovine strains (1). The most remarkable aspect of this group was its similar behaviour in additional tests: lack of lipolytic activity (egg yolk factor and Tween 80) and very low enterotoxigenicity. In addition, 65 % of the strains showed antibiotic resistance.

The differences in enterotoxin production and phage sensitivity of the bovine and ovine strains is quite marked. As many as 86.4 % of 59 *S. aureus* strains isolated by us from ovine mastitis (9) belonged to the ovine biotype C. Of them 80 % produced enterotoxin C and 57.6 % were lysed by phage 78. This compared to the bovine strains of this study: 40 % were identified as biovar C, and only one produced enterotoxin and was lysed by phage 78. The high sensitivity of our biovar C isolates to phages 102 and 42 E was shared by the strains of the remaining biovars.

Strains belonging to patterns 1 and 2 showed good correlation with the description of biovar B (swine and poultry). They were fibrinolysin negative, unable to coagulate bovine plasma, and crys-

tal violet type A. In addition, all of them produced beta haemolysin and only 3 isolates failed to produce alpha-haemolysin.

The two strains in pattern 7 were considered as biovar D (hares). The failed to produce pigment, were enterotoxigenic and lysed by human phages in group I and by the bovine phage 119 (Miscellaneous group). It must be noted that both strains were obtained from animals of different herds in the same village.

The strain identified as biovar F (*S. intermedius*) was isolated from a cow suffering from a very severe form of mastitis.

The two strains in patterns 9 and 10 were similar. In addition to being fibrinolysin positive, beta-haemolysin negative and typable by human, but not bovine, phages, they were egg yolk factor and Tween 80 positive. These properties are related to biovar A (human), but unlike strains in this biovar, one was crystal violet type A (positive), and the other coagulated bovine plasma.

Strains belonging to patterns 11 and 12 correlated in almost all characteristics with the description of biovar B but pattern 11 failed pigment production and pattern 12 was clumping factor negative.

Strains included in pattern 13 resembled biovar 13 but failed the crystal violet type of growth. Also, there was a significant difference between these strains and those belonging to biovar D (alpha haemolysin production). Although these cultures were isolated from animals in different places within the province of León (more than 100 kms away), all of three gave the phage pattern 29/52/52A/119.

Remarkable differences were observed between the strain of pattern 14 (fibrinolysin positive) and biovar A. The remaining cultures could not be identified with any of the currently recognized biovars.

A good correlation was found between fibrinolysin production and lipolytic activity on Tween 80. On the other hand, none of the strains classified as or close to biovars C or D produced egg yolk factor. According to Hajek and Marsalek (12), infectious and carrier bovine strains could also be differentiate by this latter property. The low incidence of egg yolk factor producers among coagulase-positive staphylococci isolated from bovine mastitis has also been reported by O'Toole (24).

The high frequency of bovine strains lysed by phages of the international basic sets for typing human and bovine *S. aureus* strains and the predominance of groups III (phage 42 E) and IV (phage 102) are in agreement with observations of other workers (22, 27). However, Mackie *et al.* (19) found that the majority of 617 *S. aureus* cultures isolated from subclinical mastitis cases in Northern Ireland, were typable with phages from groups I and III. Furthermore, bovine phage 102 did not lyse any of the strains studied by the latter authors. The above discussion suggest that sensitivity to phages varies from location to location.

Antibiotic resistance was noted in 65 % of strains in biovar C. This compared to 23.5 % in biovar B. The high incidence of resistance among strains belonging to biotype C could possibly be related to the fact that most of them were obtained from treated cows suffering from chronic forms of mastitis.

Although the low percentage of enterotoxigenic strains makes it impossible to relate this property to any of the other characteristics, it must be noted that they were the only ones lysed by phage 119 and one of them (C producer) was also sensitive to phage 78.

Finally, the prevalence of B, D, etc. biotype strains in bovine mastitis indicates transfer between animals and reflects the impact of poor herd management practice such as keeping together different animal species.

References

1. Anderson, J. C. (1976). Mechanisms of staphylococcal virulence in relation to bovine mastitis. Br. Vet. J. **132,** 229-235.
2. Baird-Parker, A. C. (1979). Methods for identifying staphylococci and micrococci. *In:* F. A. Skinner and D. W. Lovelock (eds.). Identification methods for microbiologists, 2nd edn, pp. 201-210. Academic Press, London.
3. Barry, A. L. (1976). The antimicrobic susceptibility test: Principles and practices. pp. 236. Lea and Febiger, Philadelphia.
4. Blair, J. E. and Williams, R. E. O. (1961). Phage typing of staphylococci. Bull. WHO. **24,** 771-784.
5. Christie, R. and Wilson, H. (1941). A test of staphylococcal fibrinolysis. Aust. J. Exp. Biol. Med. Sci. **19,** 329-332.
6. Davidson, I. (1971). Report of the working group on phage-typing of bovine staphylococci to the Subcommittee on phage-typing of staphylococci. Int. J. Syst. Bacteriol. **21,** 171.
7. Devriese, L. A. and Hajek, V. (1980). Identification of pathogenic staphylococci isolated from animals and food derived from animals. J. Appl. Bacteriol. **49,** 1-11.
8. Elek, S. O. and Levy, E. (1950). Distribution of haemolysins in pathogenic and non-pathogenic staphylococci. J. Pathol. Bacteriol. **62,** 541-554.
9. Gutiérrez, L. M., Menes, I., García, M. L., Moreno, B. and Bergdoll, M. S. (1982). Characterization and enterotoxigenicity of staphylococci isolated from ovine milk in Spain. J. Food Protect. **45,** 1282-1286.
10. Hajek, V. (1976). *Staphylococcus intermedius* a new species isolated from animals. Int. J. Syst. Bacteriol. **26,** 401-408.
11. Hajek, V. (1978). Identification of enterotoxigenic staphylococci from sheep and sheep cheese. Appl. Environ. Microbiol. **35,** 264-268.
12. Hajek, V. and Marsalek, E. (1969). A study of staphylococci of bovine origin. *S. aureus* var. *bovis.* Zbl. Bakt., I. Abt. Orig. **209,** 154-160.
13. Hajek, V. and Marsalek, E. (1971). The differentiation of pathogenic staphylococci and a suggestion for their taxonomic classification. Zbl. Bakt. Hyg., I. Abt. Orig. A **217,** 176-182.
14. Hajek, V. and Marsalek, E. (1976). Evaluation of classificatory criteria for staphylococci. *In:* J. Jeljaszewicz (ed.). Staphylococci and staphylococcal diseases. pp. 11-21. Gustav Fischer Verlag, Stuttgart.
15. Hajek, V. and Marsalek, E. (1976). Staphylococci outside the hospital. Zbl. Bakt. Hyg., I. Abt. Orig. B **161,** 455-461.
16. Kloos, W. E. (1980). Natural populations of the genus *Staphylococcus.* Ann. Rev. Microbiol. **34,** 559-592.
17. Kloos, W. E. (1982). Coagulase-negative staphylococci. Clin. Microbiol. Newsl. **4,** 75-79.
18. Kloos, W. E., Musselwhite, M. S. and Zimmerman, R. J. (1976). A comparison of the distribution of *Staphylococcus* species on human and animal species. *In:* J. Jeljaszewicz (ed.). Staphylococci and staphylococcal diseases. pp. 967-973. Gustav Fischer Verlag, Stuttgart.
19. Mackie, D. P., Pollock, D. A., Rodgers, S. P. and Logan, E. F. (1987). Phage typing of *Staphylococcus aureus* associated with subclinical bovine mastitis. J. Dairy Res. **54,** 1-5.
20. Menes, I., García, M. L., Moreno, B., Gutiérrez, L. and Francisco, J. J. (1984). Staphylococci isolated from abcesses in slaughtered animals: Characterization and epidemiological studies. Zbl. Bakt. Hyg., I. Abt. Orig. B **178,** 551-561.
21. Meyer, W. (1967). Über die branchbarkeit des Kristallviolett-testes zur differenzierung von *Staphylococcus aureus*-stämmen. Z. Med. Mikrobiol. u. Imunol. **153,** 158-168.
22. Milch, H. and Laszlo, V. G. (1973). Subdivision of *Staphylococcus aureus* for epidemiological purposes. *In:* J. Jeljaszewicz (ed.) Contributions to microbiology and immunology. Vol. I. Staphylococci and staphylococcal infections. pp. 469-497. Karger. Basel.
23. Nakagawa, M. (1958). Studies on staphylococci from the bovine udder. I. Biological characteristics of staphylococci and some observations on the pathogenic strains. Jpn. J. Vet. Res. **6,** 19-34.
24. O'Toole, D. K. (1987). Differences in the egg yolk reaction on Baird-Parker medium between bovine and human strains of coagulase-positive staphylococci. Letters Appl. Microbiol. **4,** 111-112.

24a. Parker, M. T. (1972). Phage typing of *Staphylococcus aureus. In:* J. R. Norris and D. W.. Ribbons (eds.). Methods in microbiology, vol. 7B. pp 1-28. Academic Press, London.

25. Robbins, R., Gould, S. and Bergdoll, M. S. (1974). Detecting the enterotoxigenicity of *Staphylococcus aureus* strains. Appl. Microbiol. **28,** 946-950.
26. De Saxe, M., Coe, A. W. and Wieneke, A. A. (1982). The use of phage typing in the investigation of food poisoning caused by *Staphylococcus aureus* enterotoxin. *In:* J. E. L. Corry, D. Roberts and F. A. Skinner (eds.). Isolation and identification methods for food poisoning organisms. pp. 173-197. Academic Press, London.
27. Suárez, G. (1966). Microflora estafilocócica de leche natural. An. Fac. Vet. León XII, 11-166.
28. Subcommittee on Phage Typing of Staphylococci (1984). Report of the Subcommittee on Phage Typing of Staphylococci to the International Committee on Systematic Bacteriology. Int. J. Syst. Bacteriol. **34,** 260-261.
29. Turner, F. J. and Schwartz, B. S. (1958). The use of a lyophilized human plasma standardized for blood coagulation factors in the coagulase and fibrinolytic tests. J. Lab. Clin. Med. **52,** 888-894.

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Utilization of beet molasse for sterol production by some moulds

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Summary

Different moulds were cultivated in beet molasses (BM)-containing medium. *Penicillium crustosum* Thom was superior to the other moulds in total sterols production (4 % on dry weight basis), efficiency of convertibility of the BM sugars to sterols (2 %), total lipids (19.4 %) and unsaponified lipids (13.4 %). The treatment of BM with H_2SO_4- followed by centrifugation allowed maximum fermentation yields. The highest unsaponified lipids (16.5 %) and total sterols level (7.4 %) were obtained with a medium composed of (g/1): $NaNO_3$, 3; K_2HPO_4, 3; $MgSO_4 \cdot 7H_2O$, 1.5; K_2SO_4, 0.11; $ZnSO_4 \cdot 7H_2O$, 0.05; $FeCl_3 \cdot 6H_2O$, 0.16; H_2SO_4 - treated BM, 60. Maximal sterol yields (8.4 %) and high growth rate were achieved at the accelerated growth phase (8 days old cultures), when the initial pH value of the medium was adjusted to 7.0.

Key words: Sterol production.

Resumen

Se cultivaron diferentes hongos en medio que contenía melaza de remolacha (BM). *Penicillium crustosum* Thom fue el mejor en la producción de esteroles totales (4 % del peso seco), convertibilidad de azúcares de melaza de remolacha en esteroles (2,0 %), lípidos totales (19,4 %) y lípidos insaponificables (13,4 %). El tratamiento de la melaza con H_2SO_4 seguido de centrifugación, permitió el máximo rendimiento en la fermentación. La mayor cantidad de lípidos insaponificables (16,5 %) y de esteroles totales (7,4 %) se obtuvieron con un medio compuesto de (g/l): $NaNO_3$, 3; K_2HPO_4, 3; $MgSO_4 \cdot 7H_2O$, 1,5; K_2SO_4, 0,11; $ZnSO_4 \cdot 7H_2O$, 0,05; $FcCl_3 \cdot 6H_2O$, 0,16; melaza de remolacha tratada con H_2SO_4, 60. La máxima cantidad de esterol (8,4 %) y el mejor crecimiento se consiguieron en la fase de crecimiento rápido (cultivos de 8 días), cuando el pH inicial del medio se ajustó a 7.

Introduction

For many years ago, special impetus was given to the study of the content in fungal cells of sterols, especially ergosterol (the precursor of vitamin D) (4, 5, 6, 10, 22, 23, 25, 26). Fungi were found to be able to synthesize appreciable amounts of sterols when being cultivated on carbohydrate-rich media. Among the by-products which give rise to concern in this field for their suitability for micro-

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bial utilization are the black strap molasses left after crystallization of sugar from sugar cane or beet syrups (6, 16, 18, 20).

In Egypt, sugar industry by-products are not being utilized to their fullest potential. Additional quantities of molasses are now available in our country from the newly operated factory for the production of sugar from sugar-beet plant. In previous communications (7, 11, 12) we explored the potentiality of the Egyptian beet molasses for the production of single cell protein and lipid materials by some yeasts. It is the objective of the present article to evaluate the suitability of this available cheap carbon source for the production of sterols by some moulds.

Materials and methods

Microorganisms

The moulds used comprise the following genera: *Alternaria, Aspergillus, Cladosporium, Drechslera, Fusarium, Mucor, Ulocladium,* and *Penicillium.* The organism, which was extensively used in the present experiments, was identified by CAB International Mycological Institute, Ferry Lane, Kew, Surrey, England, as *Penicillium crustosum* Thorm. All the tested fungi were isolated from different local habitats. The cultures were maintained on glucose-peptone slopes.

Molasses

The crude beet molasses (CBM) used as a sole carbon source throughout the present work, was kindly supplied by the Delta Sugar Company, Egypt.

Cultivation

The moulds were allowed to grow in 100 ml portions of the basal medium dispensed in 250 ml Erlenmeyer flasks. The basal medium has the following composition (g/l): BM, 60 (equivalent to 3346 mg total reducing substances); $(NH_4)_2HPO_4$, 2; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KC1, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; yeast extract, 1. The reaction of the medium was usually adjusted to an initial pH of 7.0. The flasks were sterilized by autoclaving for 15 min at a pressure of 15 1b/in^2 to raise the temperature to 121° C. The sterilized media were inoculated with a spore suspension prepared by adding 10 ml of sterile distilled water to a 5 day old culture tube and agitating the growth with the aid of a sterilized inoculating needle. For each flask 2 ml of the spore suspension were added as standard inoculum.

The culture flasks were incubated at 30 ± 1° C for 10 days under stationary culture conditions. Thereafter, the necessary analyses were made.

Analysis

The surface fungal mats were separated from the culture medium by centrifugation, washed with distilled water and dried at 60° C to constant weight. The mould dry weight was then estimated and the crude lipids were extracted (19), purified (9) and the lipid content was calculated, while the total sterols were estimated (2) in the unsaponified lipid fraction.

TABLE 1
APPROXIMATE CHEMICAL COMPOSITION OF THE CRUDE BEET MOLASSES (7)

Constituents	% to Fresh weight
Moisture	24.91
Total sugars	52.00
Crude proteins	1.83
Total lipids	0.70
Total titrable acidity	1.34
Ash content (sulphated)	11.63
Elements (as % of total ash)	
Sodium	82.750
Calcium	4.710
Potassium	2.210
Magnesium	0.562
Iron	0.407
Zinc	0.410
Copper	0.036
Phosphorus	0.023
Manganese	0.014

The original, as well as the unassimilated, sugar contents of the BM were determined in the medium as glucose (3). Each treatment was carried out in triplicate and the results obtained throughout this work were the arithmetic mean.

Results and discussion

Sterol production by the tested moulds

The CMB used as the sole carbon source, were previously analysed (7). The results of these analyses were however given in Table 1.

Screening studies (Table 2) made on the tested moulds revealed that they manifested wide variations in growth values and in their capacity to produce lipids, unsaponified lipid fractions and total sterols when allowed to grow statically on CBM-containing medium. However, *Penicillium crustosum* Thom was found by far to be the most promising mould from the standpoint of biomass yield and sterols formation. The lipid and its unsaponifiable fraction contributed 19.4 and 13.4 % of the fungus dry weight, respectively. On the other hand, no consistent relationships were observed between the biomass yield and the accumulation of sterols. Thus, *Ulocladium* sp. gave the poorest growth yield while it produced moderate sterol content. In contrast, *Drechslera* sp. exhibited a comparatively high growth value and the lowest sterol yield. *P. crustosum* Thom showed a peculiar effeciency to assimilate CBM's sugars as well as a profound activity to convert these sugars into lipid materials including sterols; this stimulated its selection as the test organism in the subsequent work.

Utilization of clarified BM

Attempts were made to minimize the deleterious effect exerted by the heavy metals present in the CBM. Equally important is the removal of the suspended matter which forms what is called

TABLE 2
SUGAR CONSUMED, GROWTH (D.WT) AND TOTAL LIPIDS AS WELL AS THE UNSAPONIFIED LIPID FRACTION AND TOTAL STEROLS CONTENTS (MG/100 ML MEDIUM) OF THE TESTED FUNGI

Organism	Final pH	Sugar consumed %	Fungus D.wt	Total lipids		Unsaponified lipids		Total sterols		SCC
			mg	mg	% of D.wt	mg	% of D.wt	mg	% of D.wt	
Penicillium crustosum Thom	7.0	96.3	1607	311.7	19.4	215.3	13.4	64.6	4.0	2.00
Fusarium sp.	7.0	77.3	1261	234.5	18.6	167.4	13.3	49.8	3.9	1.93
Ulocladium sp.	7.5	58.9	942	139.4	14.8	76.8	8.2	19.2	2.0	0.97
Cladosporium sp.	6.0	69.3	1093	193.5	17.7	111.1	10.2	21.1	1.9	0.91
Aspergillus sp.	6.5	71.7	1142	167.9	14.7	78.9	6.9	21.3	1.9	0.89
Alternaria sp.	6.0	85.0	1361	223.2	16.4	116.1	8.5	20.9	1.5	0.73
Mucor sp.	6.5	90.2	1430	248.8	17.4	133.9	9.4	22.1	1.5	0.73
Drechslera sp.	7.0	88.2	1400	179.2	12.8	55.4	4.0	13.2	0.9	0.45

$$\text{SCC (Sterol conversion coefficient)} = \frac{\text{mg total sterols}}{\text{mg sugar consumed}} \times 100$$

Initial pH value of the medium = 7.0.
Initial sugar content = 3346 mg/100 ml medium.
Incubation period = 10 days.

TABLE 3
PENICILLIUM CRUSTOSUM THOM, GROWTH (D.WT), TOTAL LIPIDS, THE UNSAPONIFIED LIPID FRACTION AND STEROL CONTENTS (MG/100 ML MEDIUM) AS INFLUENCED WITH THE COMPOSITION OF THE FERMENTATION MEDIUM

Medium*	Final pH	Fungus D.wt	Total lipids			Unsaponified lipids			Total sterols		
		mg	mg		% of D.wt	mg		% of D.wt	mg		% of D.wt
I (basal)	7.0	1638	387.7	(0.12)*	23.7	265.5	(0.12)*	16.2	84.2	(0.24)*	5.1
II	6.7	1143	313.3	(0.19)	27.4	188.4	(0.29)	16.5	84.6	(0.26)	7.4
III	6.4	1404	390.3	(0.23)	27.8	213.8	(0.22)	15.2	70.8	(0.24)	5.0
IV	6.7	1248	339.5	(0.20)	27.2	163.2	(0.18)	13.1	55.8	(0.23)	4.5
V	6.4	293	87.0	(0.18)	29.7	41.9	(0.25)	14.3	14.4	(0.14)	4.9
VI	7.0	1320	314.2	(0.28)	23.8	177.4	(0.18)	13.4	50.5	(0.35)	3.8

Initial pH value of the tested medium = 7.0.
Media (g/l) I: $(NH_4)_2HPO_4$ 2; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; yeast extract, 1.0; Clarified BM, 60. II: $NaNO_3$, 3; K_2HPO_4, 3; $MgSO_4 \cdot 7H_2O$, 1.5; K_2SO_4, 0.11; $ZnSO_4 \cdot 7H_2O$, 0.05; $FeCl_3 \cdot 6H_2O$, 0.16; Clarified BM, 60. III: peptone, 15; yeast extract, 3; clarified BM, 60. IV: $(NH_4)H_2PO_4$, 5; $MgSO_4 \cdot 7H_2O$, 1; $MnSO_4$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.005; $CaSO_4 \cdot 5H_2O$, 0.005; yeast extract, 1.0; clarified BM, 60. V: KNO_3, 2.0; $MgSO_4 \cdot 7H_2O$, 2.5; K_2HPO_4, 0.5; clarified BM, 60. VI: NH_4NO_3, 2; K_2HPO_4, 7; yeast extract, 2; clarified BM, 60.
( )* denote the standard error.

mud. Centrifugation, precipitation or decationization (7) were used to clarify the BM and the clarified BM was used as the sole carbon source in media for fungal growth. Undoubtedly the elements content of the CBM were markedly affected by the tested treatments. H_2SO_4-treatment followed by centrifugation clarified the BM and gave best mold activities (Fig. 1). The H_2SO_4-treated BM lost about 75, 87, 50 and 50 % of their contents of Na, Ca, K and Mg, respectively (7). However, H_2SO_4-treated BM appeared to supply adequate levels of both macro and micro-elements enhancing the different tested metabolic activities by the experimental mould. On the other hand, the most deleterious effect was recorded with the decationized-BM treatment wherein the yields of the lipid, unsaponified lipid fraction and sterols were inferior by about 40, 55, and 60 % respectively. This could be attributed to the complete loss of Zn, Cu, P and Mn, as well as to the marked reduction of the Na, Ca, K, Mg and Fe levels of the decatonized BM sample (7).

Suitability of the fermentation medium

The H_2SO_4-treated BM was separately supplemented with the ingredients necessary for fungal growth in an attempt to select a basal medium most favourable for sterols production. The results (Table 3) revealed that, the formulation of medium II supported the production of the highest unsaponified lipids (16.5 %) and total sterols level (7.4 %), though the tested mold exhibited lower growth yields under the same conditions. In accordance to our results some workers (14, 17) have reported that the decreased growth rate of some yeasts enhance the lipid and sterols production.

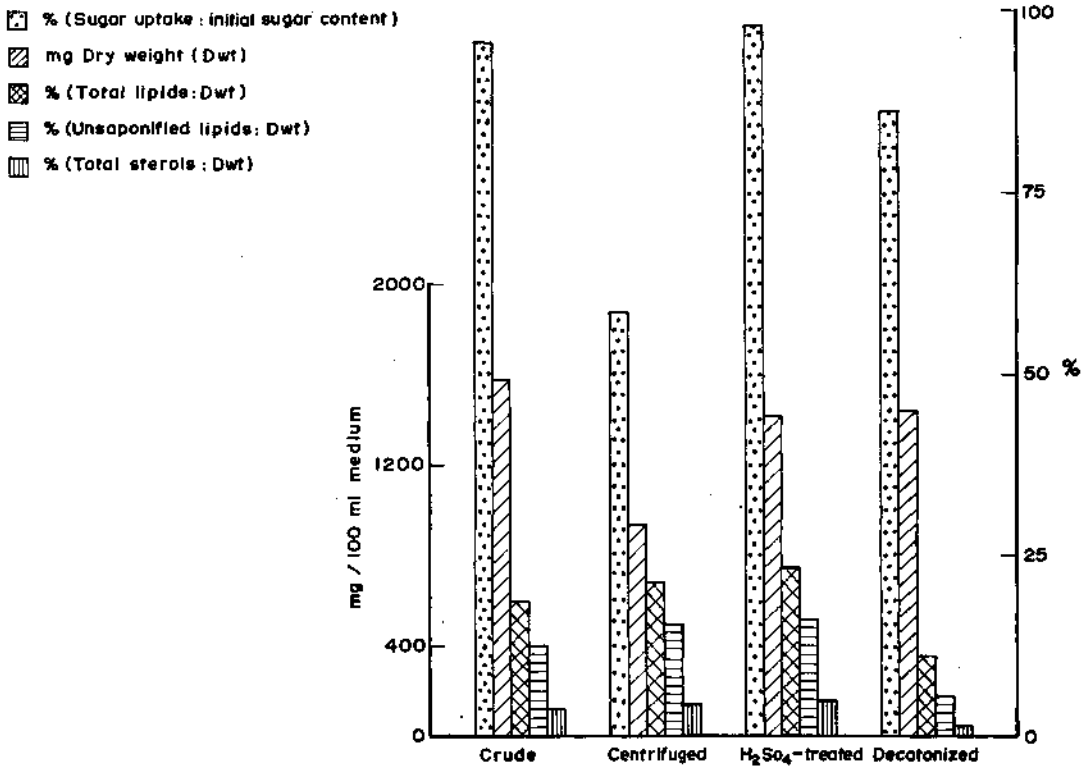

Fig. 1.—Sugar uptake, dry weight, total lipids, the unsaponified lipids fraction and total sterols of *Penicillium crustosum* Thom as affected by the pretreatment of BM.

The analysis of variance indicated that the variations with the different tested media in the content of total lipids, unsaponified lipids and total sterols were significant ($F = 8.15$, 16.95 and 12.19 respectively; $P < 0.05$).

Growth phases-relations

The activities of the tested mould were estimated during the different phases of growth. The results (Fig. 2) indicated that at the end of the 8th day of incubation which representing the end of the accelerated phase, higher growth value correlated with active sugar assimilation and maximal sterol output were reached. However, the late phases of growth (extended from 10-14 days) were accompanied by lower rate of sugar assimilation, lower biomass yields, higher amounts of lipids which were found to embrace relatively higher unsaponified fraction. In this respect, it was reported (8) that the new membrane material and that a subsequent reduction in sterol production occurs when the culture enters the stationary phase. This finding may interpret the reduced sterol production at the end of the 10th day of incubation, which represents the beginning of the stationary phase of growth.

It is of noteworthy that during the active phase of sterol biosynthesis (6-8 days) vigorous mould sporulation was traced visually. Thus, a 2.2 fold increase in the total sterols was attained when the incubation period was extended from 6 to 8 days. In accordance with the present results, many workers (1, 13, 15) found that the lipid production and sterol biosynthesis were enhanced by the sporulation of the fungus used.

TABLE 4
PENICILLIUM CRUSTOSUM THOM, GROWTH (D.WT), TOTAL LIPIDS, THE UNSAPONIFIED LIPID FRACTION AND TOTAL STEROL CONTENTS AS INFLUENCED WITH THE pH VALUE OF THE MEDIUM

	pH value		Fungus D.wt	Total lipids		Unsaponified lipids		Total sterols	
	Initial	Final	mg	mg	% of D.wt	mg	% of D.wt	mg	% of D. Wt
Initially adjusted	3	3.1	no growth						
	4	5.0	616	84.4 (0.26)*	13.7	28.3 (0.17)*	4.6	9.9 (0.06)*	1.6
	5	5.8	918	158.8 (0.20)	17.3	72.5 (0.14)	7.9	26.6 (0.29)	2.9
	6	6.1	1001	204.2 (0.16)	20.4	107.1 (0.28)	10.7	48.0 (0.29)	4.8
	7	6.2	1060	276.7 (0.23)	26.1	183.4 (0.26)	17.3	89.0 (0.23)	8.4
	8	7.8	1004	248.0 (0.18)	24.7	155.6 (0.33)	15.5	75.3 (0.37)	7.5
	9	8.0	980	211.7 (0.23)	21.6	135.2 (0.20)	13.8	60.7 (0.45)	6.2
	10	8.0	791	128.9 (0.30)	16.3	79.1 (0.32)	10.0	31.6 (0.22)	4.0
Phosphate buffer	5.59		1327	301.2 (0.30)	22.7	142.4 (0.70)	10.7	70.3 (0.90)	5.3
	5.91		1345	313.4 (0.26)	23.3	152.5 (0.36)	11.3	86.1 (0.40)	6.4
	6.98		1321	344.8 (0.09)	26.1	189.0 (0.88)	14.3	93.8 (0.55)	7.1
	7.38		1248	300.8 (0.29)	24.1	162.2 (0.23)	13.0	81.1 (0.49)	6.5
	8.04		1197	278.9 (0.32)	23.3	134.8 (0.09)	11.3	67.0 (0.16)	5.6

( )* denote the standard error.

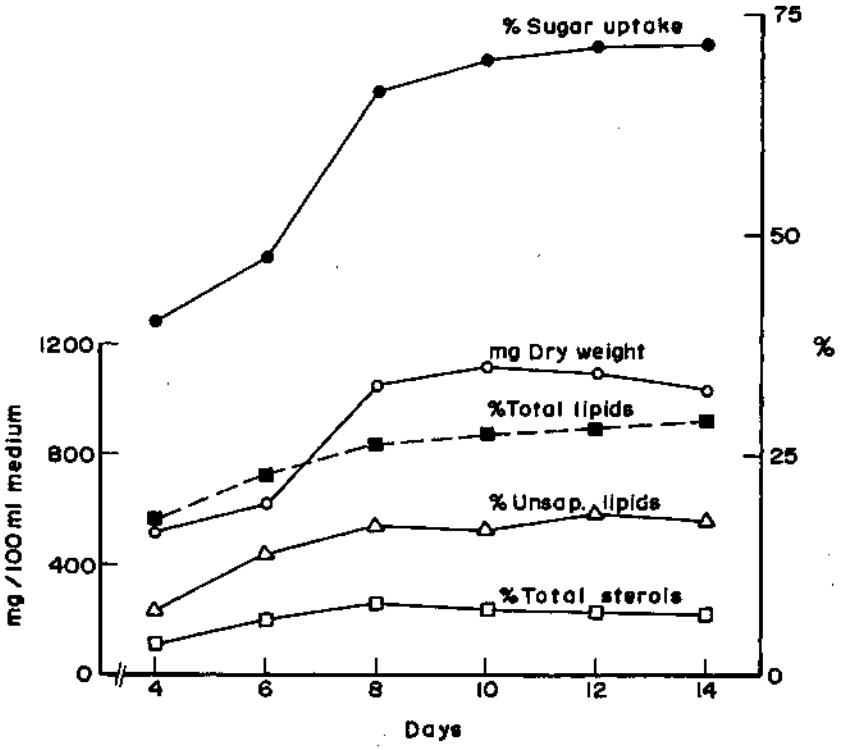

Fig. 2.—*Penicillium crustosum* Thom dry weight, total lipids, the unsaponified lipids fraction and sterols contents (mg/100 ml medium) as well as sugar uptake during 14 days of growth periods.

pH relations

Aliquots of the basal medium were initially adjusted to pH values from 3 to 10 before sterilization. The analyses were carried out after 8 days growth. The results, in Table 4, indicated that, the tested fungus failed to grow when cultivated on a basal medium initially adjusted to pH below 4.0. Similarly, the fungus activities were remarkably restricted at pH 10.0. However, the initial adjustment of the culture medium to pH 7.0, appeared to exert a definite favourable influence on the metabolic processes leading to the production of copious fungal biomass yield rich in total sterols. A noticeable decrease in the fermentation yields was, however, recorded upon adjusting the reaction of the culture medium either to acidity or to alkalinity. In this respect, some workers (21, 24) have reported that neutral or slightly alkaline medium was conducive to vigorous growth and high sterol production by fungi. To overcome the slight shift in the initial pH value during fermentation, a parallel investigation using buffered-adjusted medium was conducted. Evidence had been gained that the adjustment of the culture medium with phosphate buffer to pH 6.98 allowed the recovery of high growth yield and the production of maximal amounts of lipids, unsaponified lipids and total sterols, as compared to the other buffered pHs.

The analysis of variance indicated that the variations with the different tested pHs (initially adjusted or buffered) in the content of total lipids, unsaponified lipids and total sterols were significant ($F = 10.17$, 10.28 and 16.15 for initial pH and 7.30, 7.00, and 11.00 for buffered pH, respectively; $p < 0.05$).

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References

1. Chassang, A., Roger, M., Vezinhet, F. and Galzy, P. (1972). Variation of the lipid content of yeast cells. Folia Microbiol. **18,** 181.
2. Cook, R. P. (1958). Cholesterol (Chemistry, Biochemistry and Pathology). Appendix, pp **484,** Acad. Press, Inc. Pub., New York.
3. Dubois, M., Gilles, K., Hamilton, J. K., Robers, P. A. and Smith, F. (1951). Determination of sugars. Nature, **168,** 167.
4. Dulaney, E. L., Stapley, E. O. and Simpe, K. (1954). Studies on ergosterol production by yeasts. App. Microbiol. **2,** 371.
5. Ellis, W. J. (1945). A note on the sterol production by *Aspergillus oryzae* with special reference to its antirachitic palancy. J. Council. Sci. Ind. Research, **19,** 314.
6. El-Refai, A. H. (1964). Physiological and biochemical studies on the metabolism of fat and sterols in fungi. Ph. D. Thesis, Fac. of Science, Cairo Univ. Egypt.
7. El-Refai, A. H., Ghanem, K. M. and El-Gazaerly, M. (1986). Single cell protein production from beet molasses. Microbios., **64,** 95.
8. Evans, J. L. and Gealt, M. A. (1985). The sterols of growth and stationary phases of *Aspergillus nidulans* cultures. J. Gen. Microbiol., **131,** 279.
9. Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., **226,** 497.
10. Ghanem, K. M. (1982). Physiological studies on steroid transforming microorganisms. Ph. D. Thesis, Fac. of Science, Alex. Univ. Egypt.
11. Ghanem, K. M., El-Refai, A. H. and El-Gazaerly, M. (1986). Some fermentation parameters influencing single cell protein production by *Saccharomyces uvarum* Y-1347. Agricultural Wastes **15,** 113.
12. Ghanem, K. M., Sabry, S. A. and Yusef, H. H. (1989). Some physiological factors influencing lipid production by *Rhodotorula glutinis* from Egyptian beet molasses. Bull. Fac. Sci., Alex. Univ., **29** (4), 14.
13. Henry, S. A. and Halvorson, H. O. (1973). Lipid synthesis during sporulation of *Saccharomyces cerevisia.* J. Bacteriol., **114,** 1158.
14. Hunter, K. and Rose, A. H. (1972). Lipid composition of *Saccharomyces cerevisiae* as influenced by growth temperature. Biochem. Biophys. Acta, **260,** 639.
15. Illingworth, R. F., Rose, A. H. and Beckett, A. (1973). Changes in the lipid composition and fine structure of *Saccharomyces cerevisiae* during ascus formation. J. Bacteriol., **113,** 373.
16. Koch, R. and Bruchmann, E. E. (1953). Studies on the ergosterol formation by yeast. Bronntwein Wirts Chalft, **75,** 361.
17. McMurrough, I. and Rose, A. H. (1971). Effects of temperature variation on the fatty acid composition of *Candida utilis.* J. Bacteriol., **107,** 753.
18. Naim, N., Saad, R. R. and Naim, M. S. (1985). Production of lipids and sterols by *Fusarium oxysporum* (Schlecht). Utilization of some agro-industrial by-products as additives and basal medium. Agricultural Wastes, **14,** 207.
19. Pedersen, T. A. (1962). Lipid formation in *Cryptococcus terricolus* III. Extraction and purification of lipids. Acta. Chem. Scand., **16** (1), 374.
20. Pichova, A., Beran, K., Behalova, B. and Zajicek, J. (1985). Ergosterol synthesis and population analysis of a fedbatch fermentation in *Saccharomyces cerevisiae.* Folia Microbiol., **30,** 134.
21. Prickett, P. S., Massengale, O. N., Cox, W. M. and Bills, C. E. (1930). Factors determining the ergosterol content of fungi. Proc. Soc. Exp. Biol. and Med., **27,** 701.
22. Renauld, R. L., Subden, R. E., Pierce, A. M. and Oehleschlager, A. C. (1978). Sterol composition of *Neurospora crassa.* Lipids, **13** (1), 56.
23. Shapiro, B. E. and Gealt, M. A. (1982). Ergosterol and lanosterol from *Aspergillus nidulans.* J. Gen. Microbiol, **128,** 1053.
24. Stros, F. (1958). Methods of acquisition of yeast with high ergosterol content (In Czech). Final Report, Assignment 04.01. Distillery and Canning Research Institute, Prague.
25. Vanghelovici, M. and Serbon, F. (1940). Biosynthesis of sterols by *Aspergillus niger.* Bull. Sect. Sci. Acad. Roumaine, **22,** 287.
26. Weete, J. D. (1973). Sterols of the fungi distribution and biosynthesis. Phytochemistry, **12,** 1843.

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Plasmid profiles as an epidemiological marker for *Salmonella enterica* serotype Enteritidis foodborne outbreaks

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Summary

The incidence of enteritidis serotype of *Salmonella enterica* in salmonellae infections has steadily increased in Spain from 27.1 % in 1982 up to 63.4 % in 1987. Given this high incidence, we have studied the plasmid profiles of Enteritidis isolates to subclassify them. Different profiles were observed in 50 isolates. In 13 Enteriditis serotype outbreaks, up to 5 different plasmid profiles were found. Each outbreak correlated with a single plasmid profile except in one case where plasmids of two different profiles were observed in strains from the same outbreak.

Key words: Salmonella, *epidemiological marker, plasmids.*

Resumen

La incidencia de infecciones por *Salmonella enterica* serotipo Enteritidis en España ha aumentado desde un 27,1 % en 1982 hasta un 63,4 % en 1987. Dada esta alta incidencia, hemos estudiado el perfil plasmídico de aislamientos de Enteritidis para conseguir una mayor discriminación. En 50 cepas procedentes de casos aislados se observaron 6 perfiles plasmídicos diferentes. En 13 brotes del serotipo Enteritidis encontramos 5 perfiles plasmídicos distintos. Cada brote estaba correlacionado con un solo perfil plasmídico excepto en un caso, donde se encontraron 2 perfiles plasmídicos en cepas de un mismo brote.

Introduction

Salmonella is the main foodborne disease causative agent in Spain. Different epidemiological markers have been developed to characterize *Salmonella* isolates related to outbreaks, including: serotyping, phagetyping, bacteriocintyping, antimicrobial susceptibility, and plasmid profiles.

Serotyping is the most important epidemiological marker for *Salmonella* infections. In Spain, the most common *Salmonella* serotype is Enteritidis, accounting for 63.4 % of total cases in 1987 (6). Hence the interest to introduce complementary epidemiological markers for this serotype.

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In 1973, Christiansen *et al.* (4) isolated different plasmids from *Salmonella.* Subsequently, Barth *et al.* (1) demonstrated that the plasmid content was stable. Different laboratories have studied the electrophoretic plasmid patterns of *Salmonella* serotypes isolates from gastrointestinal infections. Bezanson *et al.* (2) used plasmid profiles to separate 40 isolates of serotype Muenster from diverse sources into four distinct groups. Threlfall *et al.* (15) identified four distinctive plasmid profiles in strains of serotype Gold Coast isolated in Britain. The plasmid profile designated as type 4 caused an extensive outbreak of food-poisoning, and it was confirmed that the vehicle of infection was imported paté. In the Enteritidis serotype, there have been scarce reports on plasmid profiles and, in some instances, surveys were originated by the spread of antibiotic resistant organisms bearing large (120-140 MDa) R-plasmids (8).

We have now applied the same methodology to characterize Enteritidis strains originating from 13 foodborne outbreaks which took place in Spain between 1987 and 1988. Our results indicated the interest of studying plasmid profiles as an epidemiological marker to subclassify Enteritidis isolates.

Materials and methods

Bacterial strains

A total of 139 *Salmonella* strains of the Enteritidis serotype, from our collection, were used for this study. Fifty strains were isolated from individual cases originated from different locations throughout Spain. The other 89 strains were isolated from foodborne outbreaks.

The source material for isolation of the individual cases was faeces. The strains from outbreaks were isolated from faeces of patients and of healthy carriers as well as from food stocks associated to the outbreaks. The infected samples were plated on MacConkey agar for bacterial growth. Biochemical identification was done in the isolated bacteria (7) and serotyping characterization was performed according to the Pasteur Institute guidelines (10).

Isolation of plasmid DNA and agarose gel electrophoresis

Plasmid DNA was prepared by the Kado and Liu method (9), modified by Nakamura *et al.* (12). Bacterial cells were grown overnight in 9 ml of tripticase soy broth at 37° C, harvested by centrifugation and suspended in 1 ml of 40 mM Tris-acetate buffer and 2 mM EDTA. The cells were lysed by addition of 2 ml of 3 % SDS and 50 mM Tris (pH 12.6), and incubated for 1 h at 55° C; afterwards, extraction with 6 ml o phenol/chloroform was carried out. For restriction analysis, the aqueous phase was dialyzed first against 25 % polyethylene glycol 6000, and then, against 0.1 X SSC (prepared from a 20 X stock containing 3 M sodium chloride and 0.3 M sodium citrate) during 16 h; twenty µl samples were analyzed by electrophoresis in horizontal slab gels containing 0.7 % agarose (w/v, Sigma type II). The electrophoresis was run at 60 V for 2.5 h at room temperature. The gels were stained for 30 minutes with 0.5 µg/ml of ethidium bromide in distilled water. Photographs were taken with a short wave UV radiation (Ultraviolet Products Inc.) using Polaroid type 665 films and a Tiffen 15 orange filter.

Molecular sizes were determined using, as standars, the mobility of *Escherichia coli* V517 plasmids ranging from 1.4 to 35.8 MDa (11).

Restriction enzyme analysis

Two µl samples of plasmid DNA isolated as above were digested with *Hind*III, *Eco*RI and *Bam*HI (Pharmacia) following the manufacturers instructions. The digested samples were analyzed by gel electrophoresis as before. Molecular weights were determined using *Hind* III-digested Lambda DNA (Pharmacia), as marker.

Identification of antimicrobial resistance plasmids

Susceptibility to antibiotics was determined by the standard agar disk diffusion test (5). Conjugative transfer of antimicrobial resistance was performed on solid media on nitrocellulose filters (5). The recipient strain used was *E. coli* BM694 (5) which is resistant to nalidixic acid. Selection of transconjugants was carried out on MacConkey agar with nalidixic acid (50 µg/ml) plus ampicillin (50 µg/ml) or kanamycin (10 µg/ml). Transformation of *E. coli* with *Salmonella* plasmid DNA was accomplished by the standard calcium-chloride method (5).

Results

Preliminar plasmid screening

As á first attempt to isolate plasmids from Enteritidis serotype and to evaluate the variability of the plasmid profiles to differentiate strains, we selected 50 *Salmonella* strains (classified as belonging to the Enteritidis serotype) isolated from individual cases. The electrophoretic patterns of the isolated plasmids revealed the presence of 6 different profiles (Table 1). When the plasmids isolated from outbreaks strains (see below) were included, up to 8 different profiles could be detected. The plasmid size ranged from 1.2 to 55 MDa. Eighty per cent of the isolates harboured a single plasmid of 36 MDa. The other isolates yielded complex patterns containing more than one plasmid.

Strain analysis from foodborne outbreaks

Eighty nine *Salmonella* strains, classified within the Enteritidis serotype, were analyzed for their plasmid content. The strains originated from 13 foodborne outbreaks which occurred in Spain during 1987-1988. Table 2 shows the results obtained. Strains from 8 outbreaks gave rise to a single 36 MDa plasmid. In order to see whether this plasmid isolated from different strains was identical, restriction analysis was performed in 20 different isolates containing the 36 MDa plasmid. In all cases, the restriction patterns were identical, as previously described by Nakamura *et al.* (12).

In most outbreaks, all strains generated the same plasmid profile indicating that they were originated from a common source. Up to 5 different profiles were found among the strains, profile I was the most frequent, being present in 61.5 % of all out break strains. Most plasmids were present in strains isolated from food stock, carriers and gastrointestinal infections.

One of the outbreaks (M), however, yielded strains with 2 different plasmid profiles. Three strains implicated in this outbreak showed plasmid profile I; they were isolated from a food handler, a patient and food stock, respectively (Table 2). The other 6 strains had the plasmid profile III and none of them were isolated from a food handler but from patients and food stocks. Profile III was also present in strains isolated from outbreak J which took place in a close geographical area.

Plasmid profiles and antimicrobial resistance

All strains of *Salmonella* serotype Enteritidis tested were fully susceptible to antibiotics, except in the case of those with plasmid profile VI bearing 4 plasmids of 55, 36, 22 and 5.5 MDa (Table 1) which were resistant to ampicillin, streptomycin, kanamycin and sulphonamides.

Ampicillin resistance was independently transferred by conjugation to *E. coli,* being associated to the 22 MDa plasmid in all the transconjugants tested. Transfer of streptomycin-kanamycin-sulphonamides resistance could only be detected by transformation of *E. coli,* as it was linked in the transformants to the 5.5 MDa plasmid (not shown).

TABLE 1
PLASMID PROFILES PERCENTAGES

Plasmid profiles (MDa)		Isolated cases %	Outbreaks %
I	(36)	80	61.5
II	(36; 2.6; 1.2)	10	7.7
III	(36; 3.9; 2.4)	4	7.7
IV	(5.3; 3.3)	0	7.7
V	(36; 1.8; 1.2)	0	7.7
VI	(55; 36; 22; 5.5)	2	0
VII	(36; 1.8)	2	0
VIII	(36; 1.2)	2	0

Discussion

In this report, we describe the plasmid profiles detected in strains of the Enteritidis serotype of *Salmonella*. Eight different profiles were identified, with plasmid sizes ranging from 1.2 to 55 MDa. Profile I was found in 80 % of strains coming from individual cases and 61.5 % of strains from foodborne outbreaks. This indicates that bacteria harbouring the 36 MDa plasmid are prevalent in Spain. A 36 MDa plasmid has been isolated by Popoff *et al.* (13) and Nakamura *et al.* (12) from Enteritidis strains. The latter authors describe a high incidence, wide distribution, and stability of restriction pattern for the 36 MDa plasmid (12). This suggested that the plasmid might confer some advantage for colonization of intestinal cells and participate to the spreading among strains infecting the human population. The same authors also correlated the presence of the 36 MDa plasmid with virulence in mice. Recently, Woodward *et al.* (16) have described different plasmid profiles in Enteritidis isolates of animal origin for each of 8 different phagetypes. As they were looking for virulence regions presumably located in large plasmids by means of a DNA probe, they only indicated that

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plasmids of 35, 40, 55 and 60 MDa were found to hybridize. Plasmids of aproximately 35-36 MDa were confined to a single phagetype in these Enteritidis isolates from animals (16).

Our results also confirm the high incidence of bacteria harbouring the 36 MDa plasmid in the Enteritidis isolates of human origin. This plasmid was present in all profiles found, either alone or with other plasmids, except in profile IV where the 36 MDa was not present. However, profile IV had 2 other plasmids. Whether any of these other plasmids might provide a selective advantage is not known at present.

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In conclusion, plasmid profile analysis can be a useful complementary marker for serotyping of the Enteritidis strains. In addition, plasmid analysis might be useful to study the prevalence of strains and the molecular mechanisms underlying this advantageous selection.

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References

1. Barth, P. T., Richards, R. and Datta, N. (1978). Copy number of coexisting plasmids in *Escherichia coli* K-12. J. Bacteriol. **135,** 760-765.
2. Bezanson, G. S., Khakhria, R. and Pagnutti, D. (1983). Plasmid profiles of value differentiating *Salmonella muenster* isolates. J. Clin. Microbiol. **17,** 1159-1160.
3. Bezanson, G. S., Khakhria, R., Duck, D. and Lior, H. (1985). Molecular analysis confirms food source and simultaneous involvement of two distinct but related subgroups of *Salmonella typhimurium* bacteriophage type 10 in major interprovincial *Salmonella* outbreak. Appl. Environ. Microbiol. **50,** 1279-1284.
4. Christiansen, C., Christiansen, G., Back, A. L. and Stenderup, A. (1973). Extrachromosomal DNA in different Enterobacteriaceae. J. Bacteriol. **144,** 367-377.
5. Courvalin, P., Goldstein, F., Philippon, A. and Sirot, J. (1985). L'antibiogramme. MPC-VIDEOM. París.
6. Echeita, A. and Usera, M. A. (1989). Prevalence of *Salmonella* serotypes isolated in Spain from human and non human sources (1983-1987). Microbiología SEM. **5,** 95-103.
7. Ewing, W. H. (1986). The genus *Salmonella. In:* Edwards, P. R. and Ewing, W. H. (eds.). Identification of Enterobacteriaceae, pp. 182-245. Elsevier Science Pub. B. V. Amsterdam.

8. Hadfield, T. L., Monson, M. H. and Wachsmuth, I. K. (1985). An outbreak of antibiotic resistance *Salmonella enteritidis* in Liberia, West Africa, J. Infect. Dis. **151,** 790-795.
9. Kado, C. I. and Liu, S. T. (1981). Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145,** 1365-1373.
10. Le Minor, L., Veron, M. and Popoff, M. (1982). Proposition pour une nomenclature des *Salmonella.* Ann. Microbiol. (Inst. Pasteur). **133B,** 245-254.
11. Macrina, F. L., Kopecko, D. J., Jones, K. R., Ayers, D. J. and McCowen, S. M. (1978). A multiple plasmid containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1,** 417-420.
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13. Popoff, M., Miras, I., Coynault, C., Lasselin, C. and Paradon, P. (1984). Molecular relationship between virulence plasmids of *Salmonella* serotypes Typhimurium and Dublin and large plasmids of other *Salmonella* serotypes. Ann. Microbiol. (Inst. Pasteur), **135A,** 389-398.
14. Taylor, D. N., Wachsmuth, I. K. and Shankusn, Y-H. (1982). Salmonellosis associated with marijuana. A multistate outbreak traced by plasmid fingerprinting. N. Engl. J. Med. **306,** 1249-1253.
15. Threlfall, E. J., Hall, M. L. M. and Rowe, B. (1986). *Salmonella gold-coast* from outbreaks of food-poisoning in the British Isles can be differentiated by plasmid profiles. J. Hyg. (Camb). **97,** 115-122.
16. Woodward, M. J., McLaren, I. and Wray, C. (1989). Distribution of virulence plasmids within Salmonellae. J. Gen. Microbiol. **135,** 503-511.

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Plasmid profiles as an epidemiological marker for *Salmonella enterica* serotype Enteritidis foodborne outbreaks

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Summary

The incidence of enteritidis serotype of *Salmonella enterica* in salmonellae infections has steadily increased in Spain from 27.1 % in 1982 up to 63.4 % in 1987. Given this high incidence, we have studied the plasmid profiles of Enteritidis isolates to subclassify them. Different profiles were observed in 50 isolates. In 13 Enteriditis serotype outbreaks, up to 5 different plasmid profiles were found. Each outbreak correlated with a single plasmid profile except in one case where plasmids of two different profiles were observed in strains from the same outbreak.

Key words: Salmonella, *epidemiological marker, plasmids.*

Resumen

La incidencia de infecciones por *Salmonella enterica* serotipo Enteritidis en España ha aumentado desde un 27,1 % en 1982 hasta un 63,4 % en 1987. Dada esta alta incidencia, hemos estudiado el perfil plasmídico de aislamientos de Enteritidis para conseguir una mayor discriminación. En 50 cepas procedentes de casos aislados se observaron 6 perfiles plasmídicos diferentes. En 13 brotes del serotipo Enteritidis encontramos 5 perfiles plasmídicos distintos. Cada brote estaba correlacionado con un solo perfil plasmídico excepto en un caso, donde se encontraron 2 perfiles plasmídicos en cepas de un mismo brote.

Introduction

Salmonella is the main foodborne disease causative agent in Spain. Different epidemiological markers have been developed to characterize *Salmonella* isolates related to outbreaks, including: serotyping, phagetyping, bacteriocintyping, antimicrobial susceptibility, and plasmid profiles.

Serotyping is the most important epidemiological marker for *Salmonella* infections. In Spain, the most common *Salmonella* serotype is Enteritidis, accounting for 63.4 % of total cases in 1987 (6). Hence the interest to introduce complementary epidemiological markers for this serotype.

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In 1973, Christiansen *et al.* (4) isolated different plasmids from *Salmonella.* Subsequently, Barth *et al.* (1) demonstrated that the plasmid content was stable. Different laboratories have studied the electrophoretic plasmid patterns of *Salmonella* serotypes isolates from gastrointestinal infections. Bezanson *et al.* (2) used plasmid profiles to separate 40 isolates of serotype Muenster from diverse sources into four distinct groups. Threlfall *et al.* (15) identified four distinctive plasmid profiles in strains of serotype Gold Coast isolated in Britain. The plasmid profile designated as type 4 caused an extensive outbreak of food-poisoning, and it was confirmed that the vehicle of infection was imported paté. In the Enteritidis serotype, there have been scarce reports on plasmid profiles and, in some instances, surveys were originated by the spread of antibiotic resistant organisms bearing large (120-140 MDa) R-plasmids (8).

We have now applied the same methodology to characterize Enteritidis strains originating from 13 foodborne outbreaks which took place in Spain between 1987 and 1988. Our results indicated the interest of studying plasmid profiles as an epidemiological marker to subclassify Enteritidis isolates.

Materials and methods

Bacterial strains

A total of 139 *Salmonella* strains of the Enteritidis serotype, from our collection, were used for this study. Fifty strains were isolated from individual cases originated from different locations throughout Spain. The other 89 strains were isolated from foodborne outbreaks.

The source material for isolation of the individual cases was faeces. The strains from outbreaks were isolated from faeces of patients and of healthy carriers as well as from food stocks associated to the outbreaks. The infected samples were plated on MacConkey agar for bacterial growth. Biochemical identification was done in the isolated bacteria (7) and serotyping characterization was performed according to the Pasteur Institute guidelines (10).

Isolation of plasmid DNA and agarose gel electrophoresis

Plasmid DNA was prepared by the Kado and Liu method (9), modified by Nakamura *et al.* (12). Bacterial cells were grown overnight in 9 ml of tripticase soy broth at 37° C, harvested by centrifugation and suspended in 1 ml of 40 mM Tris-acetate buffer and 2 mM EDTA. The cells were lysed by addition of 2 ml of 3 % SDS and 50 mM Tris (pH 12.6), and incubated for 1 h at 55° C; afterwards, extraction with 6 ml o phenol/chloroform was carried out. For restriction analysis, the aqueous phase was dialyzed first against 25 % polyethylene glycol 6000, and then, against 0.1 X SSC (prepared from a 20 X stock containing 3 M sodium chloride and 0.3 M sodium citrate) during 16 h; twenty µl samples were analyzed by electrophoresis in horizontal slab gels containing 0.7 % agarose (w/v, Sigma type II). The electrophoresis was run at 60 V for 2.5 h at room temperature. The gels were stained for 30 minutes with 0.5 µg/ml of ethidium bromide in distilled water. Photographs were taken with a short wave UV radiation (Ultraviolet Products Inc.) using Polaroid type 665 films and a Tiffen 15 orange filter.

Molecular sizes were determined using, as standars, the mobility of *Escherichia coli* V517 plasmids ranging from 1.4 to 35.8 MDa (11).

Restriction enzyme analysis

Two µl samples of plasmid DNA isolated as above were digested with *Hind*III, *Eco*RI and *Bam*HI (Pharmacia) following the manufacturers instructions. The digested samples were analyzed by gel electrophoresis as before. Molecular weights were determined using *Hind* III-digested Lambda DNA (Pharmacia), as marker.

Identification of antimicrobial resistance plasmids

Susceptibility to antibiotics was determined by the standard agar disk diffusion test (5). Conjugative transfer of antimicrobial resistance was performed on solid media on nitrocellulose filters (5). The recipient strain used was *E. coli* BM694 (5) which is resistant to nalidixic acid. Selection of transconjugants was carried out on MacConkey agar with nalidixic acid (50 µg/ml) plus ampicillin (50 µg/ml) or kanamycin (10 µg/ml). Transformation of *E. coli* with *Salmonella* plasmid DNA was accomplished by the standard calcium-chloride method (5).

Results

Preliminar plasmid screening

As á first attempt to isolate plasmids from Enteritidis serotype and to evaluate the variability of the plasmid profiles to differentiate strains, we selected 50 *Salmonella* strains (classified as belonging to the Enteritidis serotype) isolated from individual cases. The electrophoretic patterns of the isolated plasmids revealed the presence of 6 different profiles (Table 1). When the plasmids isolated from outbreaks strains (see below) were included, up to 8 different profiles could be detected. The plasmid size ranged from 1.2 to 55 MDa. Eighty per cent of the isolates harboured a single plasmid of 36 MDa. The other isolates yielded complex patterns containing more than one plasmid.

Strain analysis from foodborne outbreaks

Eighty nine *Salmonella* strains, classified within the Enteritidis serotype, were analyzed for their plasmid content. The strains originated from 13 foodborne outbreaks which occurred in Spain during 1987-1988. Table 2 shows the results obtained. Strains from 8 outbreaks gave rise to a single 36 MDa plasmid. In order to see whether this plasmid isolated from different strains was identical, restriction analysis was performed in 20 different isolates containing the 36 MDa plasmid. In all cases, the restriction patterns were identical, as previously described by Nakamura *et al.* (12).

In most outbreaks, all strains generated the same plasmid profile indicating that they were originated from a common source. Up to 5 different profiles were found among the strains, profile I was the most frequent, being present in 61.5 % of all out break strains. Most plasmids were present in strains isolated from food stock, carriers and gastrointestinal infections.

One of the outbreaks (M), however, yielded strains with 2 different plasmid profiles. Three strains implicated in this outbreak showed plasmid profile I; they were isolated from a food handler, a patient and food stock, respectively (Table 2). The other 6 strains had the plasmid profile III and none of them were isolated from a food handler but from patients and food stocks. Profile III was also present in strains isolated from outbreak J which took place in a close geographical area.

Plasmid profiles and antimicrobial resistance

All strains of *Salmonella* serotype Enteritidis tested were fully susceptible to antibiotics, except in the case of those with plasmid profile VI bearing 4 plasmids of 55, 36, 22 and 5.5 MDa (Table 1) which were resistant to ampicillin, streptomycin, kanamycin and sulphonamides.

Ampicillin resistance was independently transferred by conjugation to *E. coli,* being associated to the 22 MDa plasmid in all the transconjugants tested. Transfer of streptomycin-kanamycin-sulphonamides resistance could only be detected by transformation of *E. coli,* as it was linked in the transformants to the 5.5 MDa plasmid (not shown).

TABLE 1
PLASMID PROFILES PERCENTAGES

Plasmid profiles (MDa)		Isolated cases %	Outbreaks %
I	(36)	80	61.5
II	(36; 2.6; 1.2)	10	7.7
III	(36; 3.9; 2.4)	4	7.7
IV	(5.3; 3.3)	0	7.7
V	(36; 1.8; 1.2)	0	7.7
VI	(55; 36; 22; 5.5)	2	0
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Discussion

In this report, we describe the plasmid profiles detected in strains of the Enteritidis serotype of *Salmonella*. Eight different profiles were identified, with plasmid sizes ranging from 1.2 to 55 MDa. Profile I was found in 80 % of strains coming from individual cases and 61.5 % of strains from foodborne outbreaks. This indicates that bacteria harbouring the 36 MDa plasmid are prevalent in Spain. A 36 MDa plasmid has been isolated by Popoff *et al.* (13) and Nakamura *et al.* (12) from Enteritidis strains. The latter authors describe a high incidence, wide distribution, and stability of restriction pattern for the 36 MDa plasmid (12). This suggested that the plasmid might confer some advantage for colonization of intestinal cells and participate to the spreading among strains infecting the human population. The same authors also correlated the presence of the 36 MDa plasmid with virulence in mice. Recently, Woodward *et al.* (16) have described different plasmid profiles in Enteritidis isolates of animal origin for each of 8 different phagetypes. As they were looking for virulence regions presumably located in large plasmids by means of a DNA probe, they only indicated that

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References

1. Barth, P. T., Richards, R. and Datta, N. (1978). Copy number of coexisting plasmids in *Escherichia coli* K-12. J. Bacteriol. **135,** 760-765.
2. Bezanson, G. S., Khakhria, R. and Pagnutti, D. (1983). Plasmid profiles of value differentiating *Salmonella muenster* isolates. J. Clin. Microbiol. **17,** 1159-1160.
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4. Christiansen, C., Christiansen, G., Back, A. L. and Stenderup, A. (1973). Extrachromosomal DNA in different Enterobacteriaceae. J. Bacteriol. **144,** 367-377.
5. Courvalin, P., Goldstein, F., Philippon, A. and Sirot, J. (1985). L'antibiogramme. MPC-VIDEOM. París.
6. Echeita, A. and Usera, M. A. (1989). Prevalence of *Salmonella* serotypes isolated in Spain from human and non human sources (1983-1987). Microbiología SEM. **5,** 95-103.
7. Ewing, W. H. (1986). The genus *Salmonella. In:* Edwards, P. R. and Ewing, W. H. (eds.). Identification of Enterobacteriaceae, pp. 182-245. Elsevier Science Pub. B. V. Amsterdam.

8. Hadfield, T. L., Monson, M. H. and Wachsmuth, I. K. (1985). An outbreak of antibiotic resistance *Salmonella enteritidis* in Liberia, West Africa, J. Infect. Dis. **151,** 790-795.
9. Kado, C. I. and Liu, S. T. (1981). Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145,** 1365-1373.
10. Le Minor, L., Veron, M. and Popoff, M. (1982). Proposition pour une nomenclature des *Salmonella.* Ann. Microbiol. (Inst. Pasteur). **133B,** 245-254.
11. Macrina, F. L., Kopecko, D. J., Jones, K. R., Ayers, D. J. and McCowen, S. M. (1978). A multiple plasmid containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1,** 417-420.
12. Nakamura, M., Sato, S., Ohyo, T., Suzuki, S. and Ikeda, S. (1985). Possible relationship of a 36 megadalton *Salmonella enteritidis* plasmid virulence in mice. Infect. Immun. **47,** 831-833.
13. Popoff, M., Miras, I., Coynault, C., Lasselin, C. and Paradon, P. (1984). Molecular relationship between virulence plasmids of *Salmonella* serotypes Typhimurium and Dublin and large plasmids of other *Salmonella* serotypes. Ann. Microbiol. (Inst. Pasteur), **135A,** 389-398.
14. Taylor, D. N., Wachsmuth, I. K. and Shankusn, Y-H. (1982). Salmonellosis associated with marijuana. A multistate outbreak traced by plasmid fingerprinting. N. Engl. J. Med. **306,** 1249-1253.
15. Threlfall, E. J., Hall, M. L. M. and Rowe, B. (1986). *Salmonella gold-coast* from outbreaks of food-poisoning in the British Isles can be differentiated by plasmid profiles. J. Hyg. (Camb). **97,** 115-122.
16. Woodward, M. J., McLaren, I. and Wray, C. (1989). Distribution of virulence plasmids within Salmonellae. J. Gen. Microbiol. **135,** 503-511.