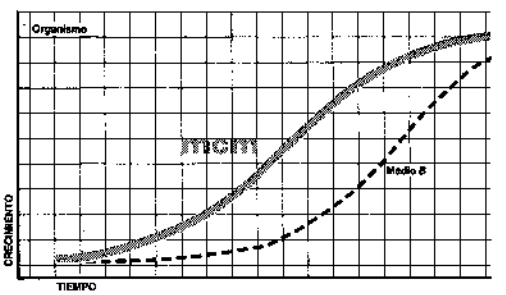
Volumen 7. N.º 1
Junio 1991
ISSN 02 13-4101

PUBLICACION DE LA SOCIEDAD
ESPAÑOLA DE MICROBIOLOGIA

Microbiología

mcm

Medios de cultivo microbiológicos



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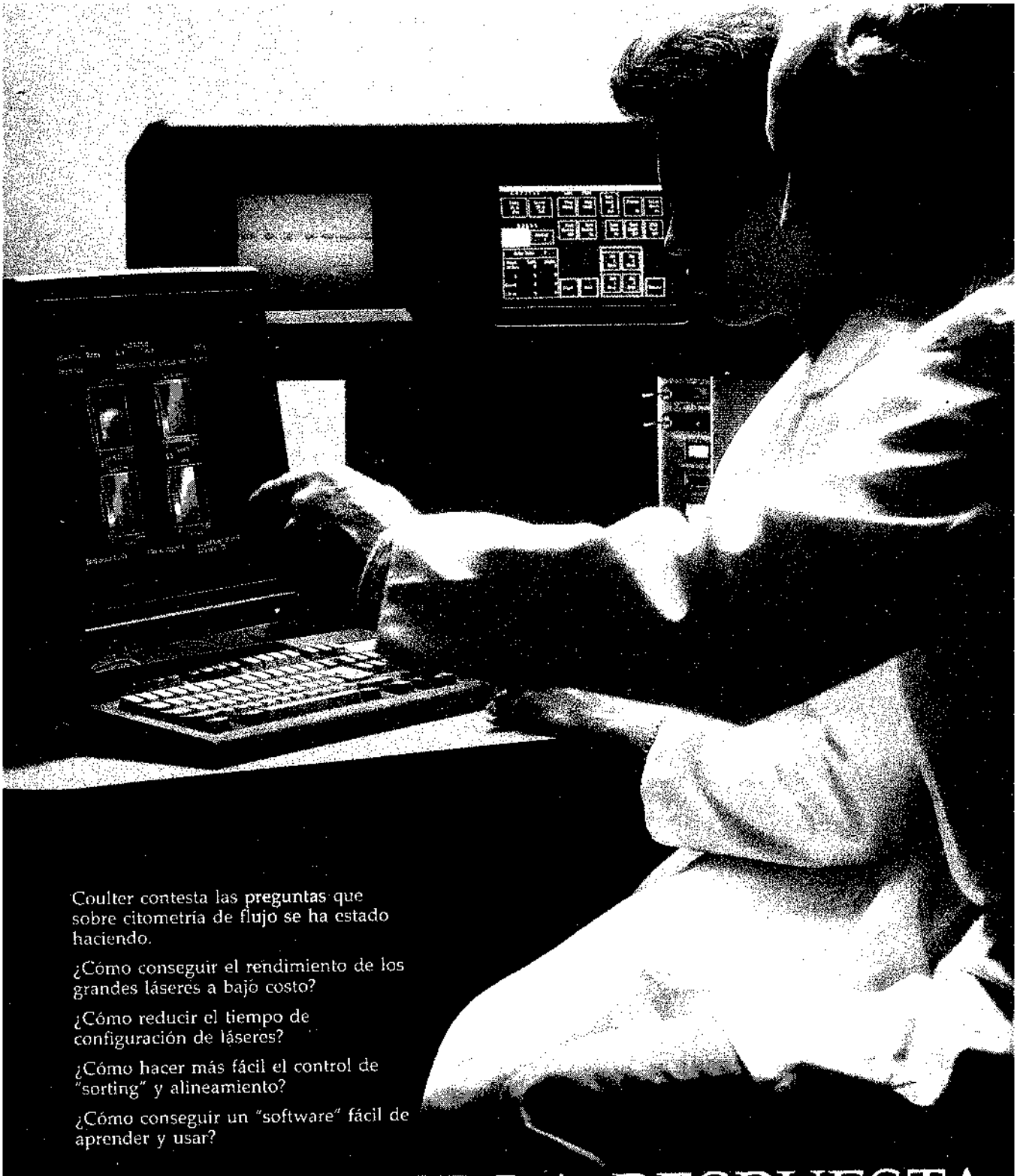

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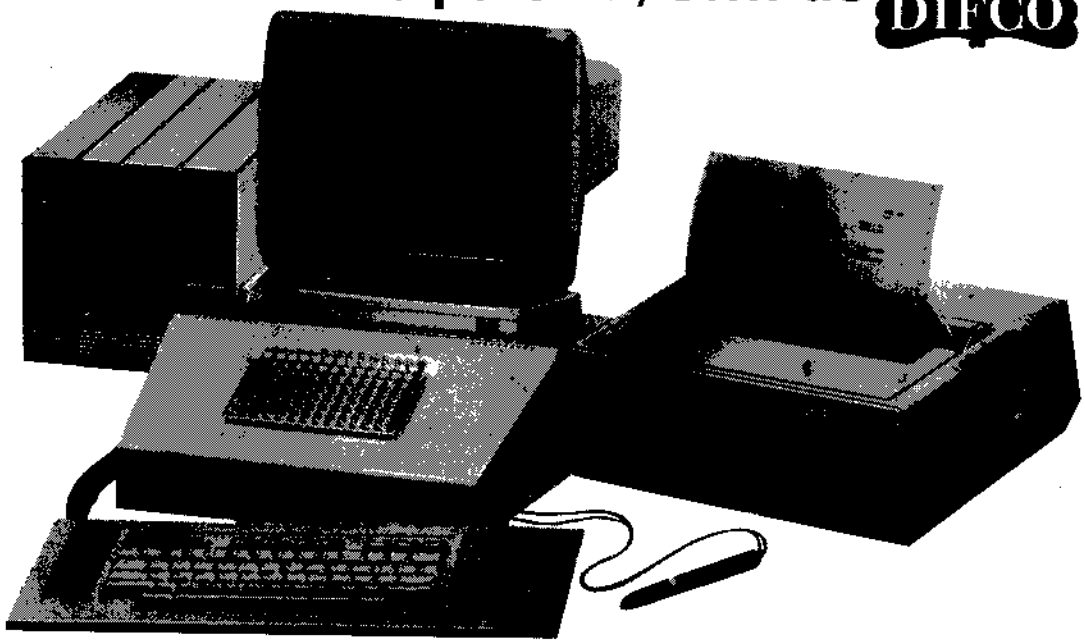

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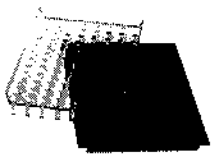

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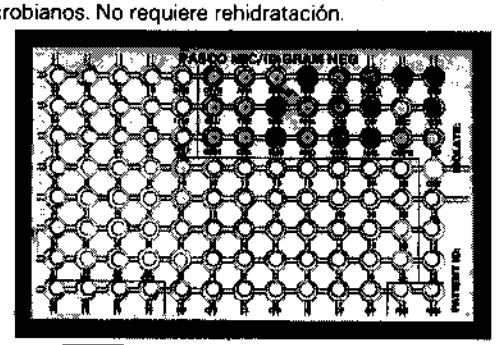

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Dirección: Sociedad Española de Microbiología. Vitrubio, 8. 28006 Madrid (España). Tel. (91) 261 98 00. Ext. 211.
Aparecen dos números al año (1990), que se integran en un volumen.
Precio de suscripción anual. Año 1991: España, 5.900 ptas. (IVA incluido); Europa, 60 $; resto países, 72 $.

FOTOCOMPOSICION: Lasercom, S. A.
IMPRIME: Tave, S. A.
DEPOSITO LEGAL: M-30455-1985.

EDITORIAL GARSI, S.A.

Edita: EDITORIAL GARSI, S. A. Publicidad: Sociedad para la Publicidad Especializada (SPE, S. L.). Londres, 17. 28028 Madrid. Teléfono: 256 08 00. Delegación Barcelona: Numancia, 85-87. 08029 Barcelona. Teléfono (93) 322 99 11. Teléfono para cambios de domicilio y suscripciones: 255 55 87.

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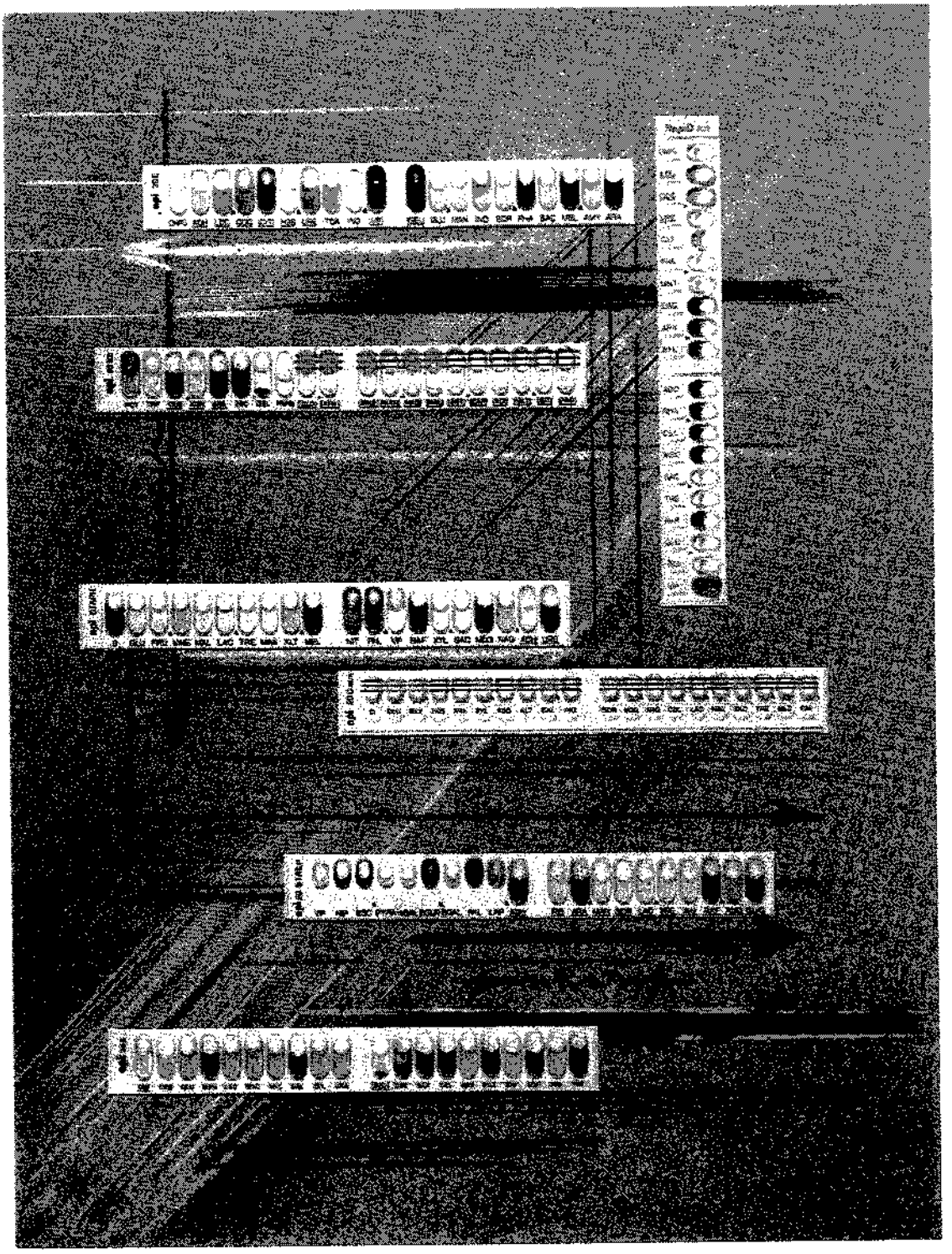

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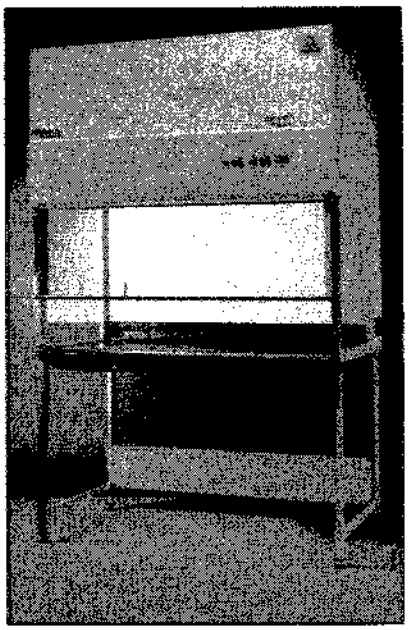

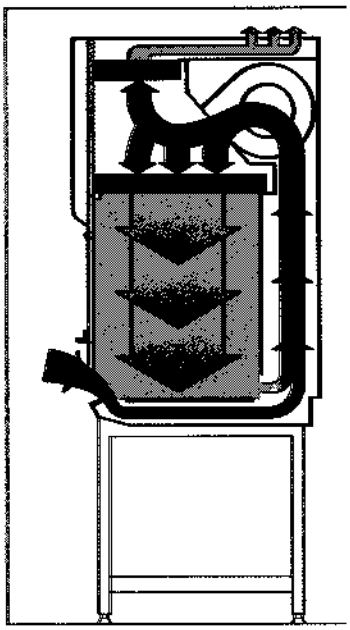

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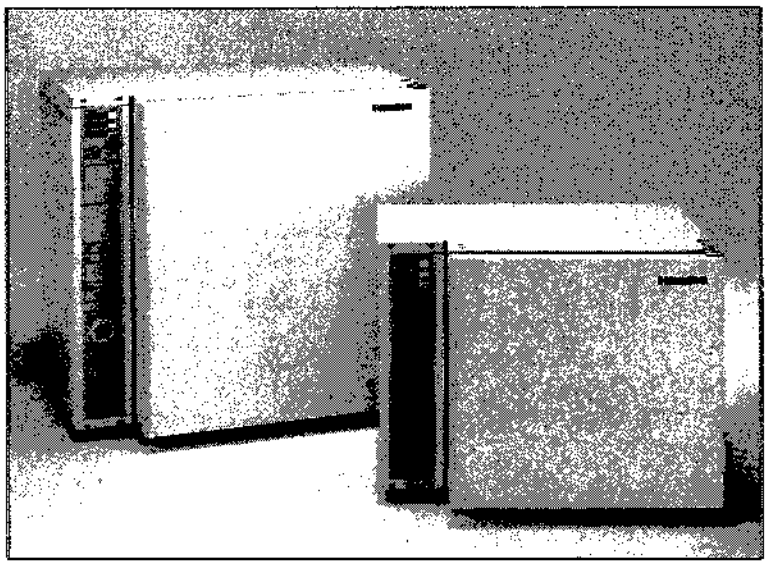

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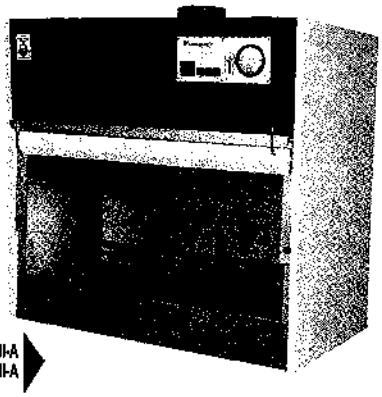

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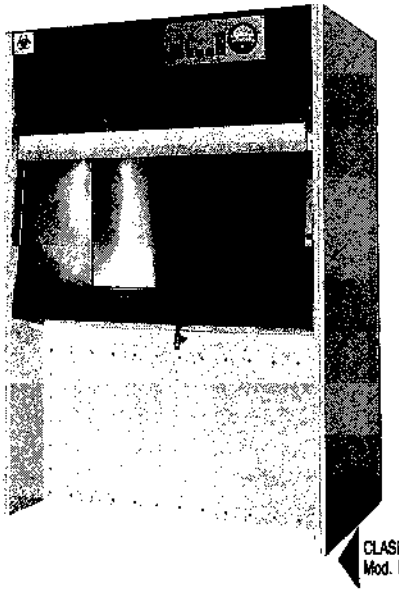

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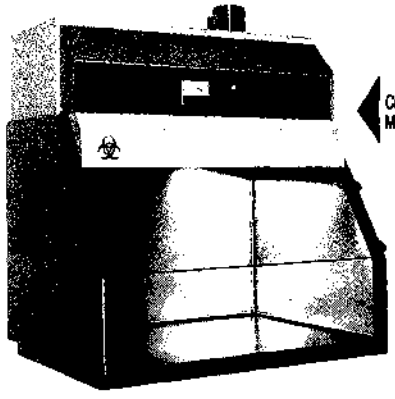

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MICROBIOLOGIA SEM 7 (1991), 1-12 **MINIREVIEW**

Genes directly involved in the biosynthesis of ß-lactam antibiotics

José Luis Barredo and Juan Francisco Martín*

Area de Microbiología, Departamento de Ecología, Genética y Microbiología.
Universidad de León. 24071 León (Spain).

Summary

Several genes encoding enzymatic activities involved in penicillin and cephalosporin biosynthesis have been identified. The first two steps in the biosynthesis of both antibiotics are common in penicillin, cephalosporin and cephamycin producers: condensation of the three precursor amino acids to form the tripeptide ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine, and oxidative cyclization of the tripeptide to form isopenicillin N. The genes involved in the two steps are *pcb*AB and *pcb*C respectively. The conversion of isopenicillin N to penicillin G is carried out by the enzyme isopenicillin N:6-APA acyltransferase encoded by the gene *pen*DE.

The biosynthesis of cephalosporin diverges from that of penicillin G at the isopenicillin N level. The isopenicillin N is first isomerized to penicillin N by an epimerase that is encoded by the gene *cef*D. The penicillin N is converted in deacetoxycephalosporin C by an expansion of the five-membered thiazolidine ring to the six-membered dihydrothiazine ring. The deacetoxycephalosporin C is finally converted into cephalosporin C by a hydroxylation and O-acetylation. The enzymes which catalyze these last three steps are encoded by the genes *cef*E, *cef*F and *cef*G. The penicillin, cephalosporin and cephamycin biosynthetic genes are organized in clusters (and subclusters) of genes.

Key words: ß-Lactam biosynthesis, penicillin, cephalosporin, enzymatic activities, gene identification.

Resumen

Gran parte de los genes implicados en la biosíntesis de penicilina y cefalosporina han sido identificados. Los dos primeros pasos biosintéticos de ambos antibióticos son compartidos por los microorganismos productores de penicilina, cefalosporina y cefamicina: condensación de los tres aminoácidos precursores para formar el tripéptido ∂-(L-α-aminoadipil)-L-cisteinil-D-valina y ciclación oxidativa del tripéptido originando isopenicilina N. Los genes implicados en estos dos pasos son *pcb*AB y *pcb*C respectivamente. La conversión de isopenicilina N a penicilina G es llevada a cabo por la enzima isopenicilina N:6-APA aciltransferasa codificada por el gen *pen*DE.

La biosíntesis de cefalosporinas diverge de la de penicilina G al nivel de isopenicilina N. La isopenicilina N es primero isomerizada a penicilina N por la actividad enzimática epimerasa, codificada por el gen *cef*D. Seguidamente el anillo tiazolidínico de 5 miembros de la penicilina N es expan-

(*) Corresponding author.

dido generando un anillo de 6 miembros denominado dihidrotiazínico. La desacetoxicefalosporina C así formada es convertida en cefalosporina C tras su hidroxilación y posterior acetilación. Estos tres últimos pasos biosintéticos están codificados por los genes *cef*E, *cef*F y *cef*G. Los genes biosintéticos de penicilina, cefalosporina y cefamicina generalmente se encuentran ligados en el genoma del microorganismo productor.

Introduction

Numerous gaps still exist in our knowledge of the genes involved in the biosynthesis of ß-lactam antibiotics despite the fact that information about the nature of these genes has grown steadily in the last few years. Initial studies dealt with the characterization of the enzymes involved in ß-lactam biosynthesis. These studies served as the basic ground for the later isolation and characterization of the genes encoding those enzymes.

The biosynthesis of penicillins and cephalosporins starts with the condensation of the three precursor amino acids L-α-aminoadipic acid, L-cysteine and L-valine to form the intermediate tripeptide ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine (so called LLD-ACV) (39). Initially, it was proposed that formation of the tripeptide proceeds *via* the synthesis of the dipeptide ∂-(L-α-aminoadipyl)-L-cysteine (1, 8) but recent data indicate that the synthesis of the complete tripeptide takes place by a single multidomain enzyme named ACV synthetase (7, 32, 65, 67, 68). The ACV synthetase is encoded by the gene *pcb*AB [see proposal of nomenclature in the review by Martín et al. (43)].

The linear ACV tripeptide is oxidatively cyclized by the isopenicillin N synthase (IPNS) (14, 26, 31, 50, 53) to form isopenicillin N (IPN). This compound which is a precursor of both penicillins and cephalosporins possesses a weak antibiotic activity. The gene encoding IPNS has been named *pcb*C.

The conversion of isopenicillin N to penicillin G takes place by the exchange of a side chain of α-aminoadipic acid (which exists in isopenicillin N) by a phenylacetic acid residue previously activated as phenylacetyl-CoA. This reaction is carried out by the enzyme isopenicillin N: acyl-CoA acyltransferase (IAT) (3, 40, 51). It was previously thought that two enzyme activities isopenicillin N amidolyase (releasing 6-APA) and acyl-CoA:6-APA acyltransferase were involved in the conversion of IPN to penicillin G. However recent studies (4) have proved that both activities are located in the IAT which is encoded by a single gene named *pen*DE.

The biosynthesis of cephalosporins and cephamycins diverges from that of penicillin G at the IPN level. The branch of the cephalosporins and cephamycins pathway starts with the isomerization of IPN to penicillin N by the enzyme isopenicillin N epimerase (IPNE), encoded by the gene *cef*D (30, 33, 36, 57). In this reaction the L-α-aminoadipic side chain of IPN is converted to the D-configuration.

The penicillin N is converted into deacetoxycephalosporin C (DAOC) by the enzyme deacetoxycephalosporin C synthase (DAOCS) (18, 29, 34). This enzyme expands the five-membered thiazolidine ring of penicillin into the six-membered dihidrothiazine ring of cephalosporins and cephamycins. This enzyme (so-called expandase) is encoded by the gene *cef*E.

The following reaction consists in the incorporation of an oxygen atom resulting in the hydroxylation of the molecule of DAOC to form deacetylcephalosporin C (DAC). This step is catalyzed by the enzyme deacetoxycephalosporin C hydroxylase also called deacetylcephalosporin C synthase (DACS) (18, 23, 64) encoded by the gene *cef*F.

The last step of the cephalosporin biosynthetic pathway in cephalosporin-producing fungi is the acetylation of DAC to form cephalosporin C. This reaction is catalyzed by the enzyme DAC acetyltransferase (22).

TABLE 1
GENES AND ENZYMES INVOLVED IN PENICILLIN AND CEPHALOSPORIN BIOSYNTHESIS. THE DESIGNATION OF THE GENES IS MADE ACCORDING TO THE PROPOSAL OF MARTIN et al. (43)

GENE	PROTEIN
Penicillin and cephalosporin biosynthesis:	
*pcb*AB	α-aminoadipyl-cysteinyl-valine synthetase (ACVS)
*pcb*C	Isopenicillin N synthase (IPNS)
Penicillin biosynthesis:	
*pen*DE	Isopenicillin N:6-APA acyltransferase (IAT)
Cephalosporin biosynthesis:	
*cef*D	Isopenicillin N epimerase (IPNE)
*cef*E	Deacetoxycephalosporin C synthase (DAOCS)
*cef*F	Deacetylcephalosporin C synthase (DACS)
*cef*G	Deacetylcephalosporin C acetyltransferase (DACA)

As proposed by Ingolia and Queener (28), and Martín et al. (43) the genes common to the penicillin and cephalosporin biosynthetic pathways are named *pcb;* the genes which are involved only in penicillin biosynthesis are named *pen* whereas those involved in cephalosporin biosynthesis are named *cef.* A list of those genes with their corresponding gene products is in Table 1.

At least four different methods may be used to clone genes involved in the biosynthesis of ß-lactam antibiotics, i) complementation of mutants altered in a particular gene, what requires the previous isolation of blocked mutants and an efficient system of transformation of the producer microorganisms; ii) gene disruption, that may be achieved by the mutational cloning procedure, iii) cloning of antibiotic resistance genes which are easily selectable and are frequently linked to antibiotic biosynthetic genes (see review by Martín and Liras, 42), and iv) «reverse genetics». In this approach a series of oligonucleotide probes are constructed from the amino acid sequence of the amino terminal end of the corresponding enzyme; this requires a previous purification to homogeneity of the desired enzyme.

*pcb*AB, A Gene Involved in the Biosynthesis of ∂-(L-α-Aminoadipyl)-L-Cysteinyl-D-Valine

The condensation of the three precursor amino acids is carried by a single enzyme, the ACV synthetase, in *A. nidulans* (65), *S. clavuligerus* (68), and *A. chrysogenum* (7, 8).

The *pcb*AB gene encoding the ACV synthetase of *P. chrysogenum* has been cloned and characterized recently (20). This gene was identified by complementation of mutants deficient in ACV synthetase activity with DNA fragments carrying the region upstream of the *pcb*C gene. The *pcb*AB gene complements the deficiency in ACVS activity of mutants *npe*5 and *npe*10 which are blocked in the biosynthesis of penicillin and restored penicillin production to mutant *npe*5.

The *pcb*AB gene encodes a protein of about 250 kDa in *P. chrysogenum* which is of similar size as the ACVS of *A. nidulans* (220 kDa) (65). However these numbers are probably lower than the real molecular weight since the determination of the molecular weight of proteins of this size in SDS-PAGE is not precise (H. von Dohren, personal communication).

Transcriptional studies have shown the presence in *P. chrysogenum* of a single transcript of about 11.5 kb in *P. chrysogenum* (20) and slightly smaller (11.4 kb) in *C. acremonium* (25). A similar

result has been described by MacCabe and coworkers (41) in *A. nidulans*. The size reported there was 9.0 kb, which is too small to correspond to a transcript of the entire gene. In the three filamentous fungi the orientation of the transcription of the *pcb*AB gene is opposite to that of the genes *pcb*C and *pen*DE. In contrast, studies carried out in *Nocardia lactamdurans* (16), *Streptomyces clavuligerus* (Hong, Y., Coque, J. J. R., Piret, J., Liras, P. and Martín, J. F., unpublished results) and *Lysobacter lactamgenus* YK90 (Kimura et al., 6th International Symposium on Genetics of Industrial Microorganisms, Strasbourg, 1990) indicate that in bacteria the direction of transcription of the *pcb*AB gene is the same as that of the *pcb*C gene.

In *P. chrysogenum* the sequence of a 12.36 kb DNA fragment that contains the *pcb*AB gene has been determined (20). The ORF of this gene (11376 nucleotides starts in an ATG located 1 kb upstream of the *pcb*C gene and shows a G+C content of 54% with a codon usage similar to that of the *pcb*C gene. The ORF encodes a protein of 3719 amino acids with a deduced Mr of 426 kDa.

The ACVS of *P. chrysogenum* contains three domains with conserved amino acid sequences. These domains show regions of high similarity with amino acid sequences of the enzymes tyrocidine synthetase I and gramicidin synthetase I of *Bacillus brevis*. These amino acid sequences which are conserved in the three peptide synthetases may represent active centers involved in ATP-mediated amino acid activation. The presence of three amino acid activating domains in the ACV synthetase is consistent with the organization existing in tyrocidine synthetase II and III, which activate 3 and 6 amino acids respectively during tyrocidine biosynthesis.

We have observed similar findings in the ACV synthetases of *C. acremonium* (25) and *N. lactamdurans* (16).

*pcb*C Gene Encodes Isopenicillin N Synthase

Isopenicillin N synthase carries out a complex reaction; it removes four hydrogen atoms using an O_2 molecule as acceptor, and introduces two C-N and C-S bonds in the tripeptide to form the ß-lactam and thiazolidine rings of penicillin. It was initially proposed that formation of isopenicillin N might take place in two steps (47) but recent biochemical studies showed that the theoretical intermediate does not exist in the free form (1, 5). Recently, molecular biology studies have proved that the IPNS activity is present in a single polypeptide encoded by the *pcb*C gene.

The *pcb*C gene was first cloned from the DNA of *C. acremonium* by Samson et al. (54). These scientists purified the IPNS protein, sequenced the amino terminal end and constructed a series of probes based on the amino acid sequence. The DNA probes were used to screen a genomic library of *C. acremonium* DNA constructed in a cosmid and amplified in *E. coli*. Once the positive clones were isolated an ORF was found that encoded a protein of 38416 Da, a very similar value to that determined previously by purification of the enzyme. The gene was expressed in *E. coli* and formed a protein of the expected size with IPNS activity. The protein was purified from *E. coli* and compared to the protein purified from *C. acremonium* (6). The enzyme kinetics and substrate specificity of the fungal enzyme were identical to that of the recombinant protein produced in *E. coli*. In this way it is easy to obtain enough amount of the enzyme since about 10% of the proteins synthetized by *E. coli* was IPNS (54). A similar strategy was used in our laboratory to clone the *pcb*C gene of *P. chrysogenum* (10).

The *pcb*C genes of several other microorganisms which produce ß-lactam antibiotics have been cloned and characterized. The *pcb*C gene of *C. acremonium* was used as a probe to hybridize the corresponding gene of *P. chrysogenum* (13) and these two genes were later used to clone the corresponding gene from *A. nidulans* (52, 66).

Similarly, the *pcb*C genes of *S. clavuligerus* (37), *S. lipmanii* (59, 66), *S. griseus* (24), *S. jumonjinensis* (59) and *N. lactamdurans* (16) have been cloned. The strategy used to clone the *pcb*C genes

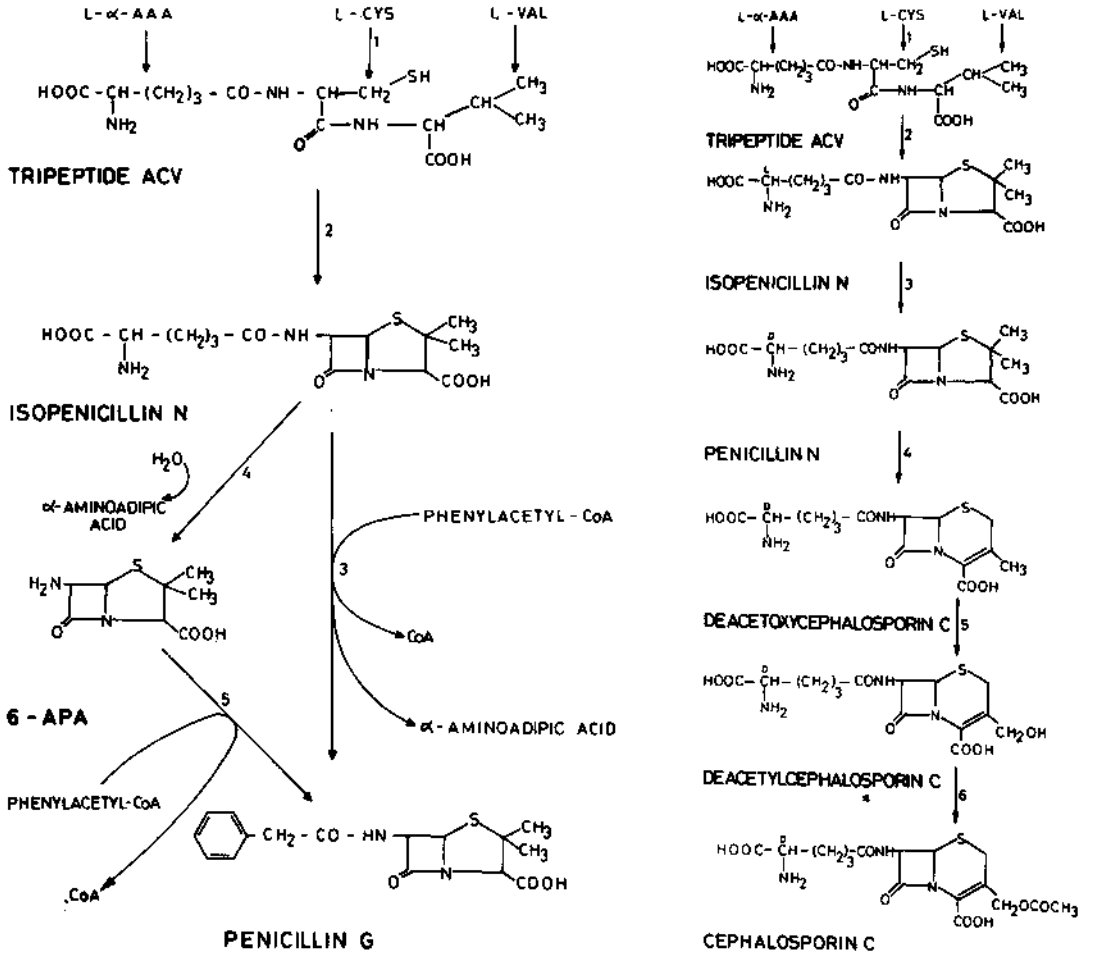

Fig. 1. *Left:* Biosynthetic pathway of penicillin G from the amino acids L-α-aminoadipic, L-cysteine and L-valine. 1, ACV synthetase. 2. Isopenicillin N synthase. 3. Isopenicillin N acyltransferase. 4. Isopenicillin N amidase (6-APA forming). 5. 6-APA acyltransferase. *Right:* Biosynthetic pathway of cephalosporin C from the same component amino acids. 1. ACV synthetase. 2. Isopenicillin N synthase. 3. Isopenicillin N epimerase. 4. Deacetoxycephalosporin C synthase. 5. Deacetoxycephalosporin C hydroxylase. 6. Deacetylcephalosporin C acetyltransferase. Note that the two initial steps are identical in both biosynthetic pathways. From Martín and Liras (42a).

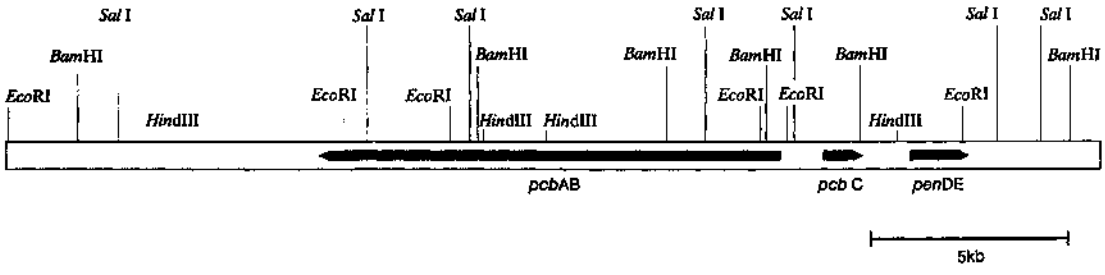

Fig. 2. Organization of the cluster of penicillin biosynthetic genes in *P. chrysogenum*. The thick arrows correspond to the *pcb*AB, *pcb*C and *pen*DE genes. The orientation of the genes is indicated by the arrowheads. The bar makers, corresponds, to 5 kb.

of *S. clavuligerus* (37), and *S. lipmanii* (66) was similar to that used in *C. acremonium*, i.e. the IPNS was purified to near homogeneity and oligonucleotide probes were prepared from the amino terminal sequence. On the other hand, the *pcb*C genes of *S. jumonjinensis* and *S. lipmanii* (59) were cloned using the *pcb*C gene of *C. clavuligerus* as probe. Furthermore, the *pcb*C of *P. chrysogenum* (10) was used as probe to clone the homologous gene of *S. griseus* (24) which in turn served as probe to clone the *pcb*C gene of *N. lactamdurans*.

The genes that encode the IPNS in different actinomycetes are very similar among themselves (78.8-84.8 % at the nucleotide level) and also in the proteins encoded by them (69.7-81.5 %). They also show a high homology with the fungal *pcb*C genes both at the nucleotide (63.0-70.9 %) and at the amino acid levels (53.6-59.1 %). Finally, the fungal *pcb*C genes show a strong homology among themselves (71.5-76.2 %) at the nucleotide level and 74.0-81.3 % at the amino acid level.

The high similarity between the different *pcb*C genes indicates a close evolutive relationship between them. Since the biosynthetic routes of ß-lactam antibiotics in actinomycetes are longer and the fungal routes may be considered truncated pathways, it has been suggested that the genes that encode the enzymes involved in the biosynthesis of ß-lactam antibiotics were formed first in *Streptomyces* or other bacteria, and were later transfered to filamentous fungi (13). The same evolutive relationship was proposed by Ramón and coworkers (52) based on the following assumptions: i) the *pcb*C gene of *C. acremonium* has a higher G+C content when compared with the genome of filamentous fungi, and ii) *Streptomyces* contains a high G+C content in the genome. However, this hypothesis assumes that if the genes were transfered to the fungi in a single transfer event, a) the *pcb*C genes of *P. chrysogenum* and *A. nidulans* should show a high G+C content and b) the organization and orientation of the different ß-lactam genes in actinomycetes *(S. clavuligerus* and *N. lactamdurans)* and filamentous fungi *(P. chrysogenum, C. acremonium* and *A. nidulans)* should be the same. None of these two assumptions turned out to be true.

A comparison of the DNA sequences of the different cloned *pcb*C genes, suggests that this gene moved from *Streptomyces* to fungi (66). The time of the transfer has been estimated [assuming a nucleotide mutation rate of 1×10^{-9} nucleotides per site and year (38)] to take place 370 million years ago, whereas the divergence of prokaryots and eucaryots took place two billion years ago. The evolutionary relationships between different ß-lactam producing fungi are the same using genes that encode the 5S RNA (27) that using the similarity between the *pcb*C genes existing in these fungi.

More information on the IPNS activity has been obtained by mutagenesis of the cloned *pcb*C genes and expression in *E. coli* of the mutated proteins. Biochemical experiments showed that alkylating agents which modify the thiol groups inactivate the IPNS, what suggested the involvement of a thiol group (28). The fungal *pcb*C genes contain two conserved cysteine residues. These two cysteines are conserved in all known bacterial IPNS's except in the enzyme of *N. lactamdurans* (16).

The cysteine residues in the IPNS of *C. acremonium* were mutated *in vitro* to serine residues. The IPNS lacking the cysteine-106 showed a 5 % specific activity as compared to the enzyme derived from the native gene, and the Km value increased five-fold (55). However the mutation in the cysteine-225 produced only a small decrease of enzyme activity. This second cysteine corresponds to the *cys* lacking in the *N. lactamdurans* enzyme. The finding that the IPNS is active even in absence of both cysteine residues suggests that a free thiol group is important but not essential for IPNS activity.

*pen*DE Gene Encoding Isopenicillin N:6-APA Acyltransferase

The purification of a *P. chrysogenum* protein having acyl-CoA:6-APA acyltransferase (AAT) (2, 3) showed that such activity was located in a polypeptide of molecular weight 29.000.

The enzyme is capable of forming penicillin G from phenylacetyl-CoA and 6-APA or isopenicillin N; when isopenicillin N was used as substrate the enzyme showed 1/5 of the activity (3). The conversion of isopenicillin N to penicillin G is consistent with the presence of two separate active centers in the same enzyme: one carrying the isopenicillin N amidolyase reaction and the second the acyl-CoA:6-APA acyltransferase activity. Researchers at Eli Lilly described that purified or semipurified preparations of acyl-CoA:6-APA acyltransferase were unable to convert isopenicillin N to penicillin G in presence of phenylacetyl-CoA (28) and suggested the involvement of two proteins each one carrying one activity: isopenicillin N amidolyase or acyl-CoA:6-APA acyltransferase. Both proteins might be subunits of the same enzyme.

Results obtained by Barredo et al. (11) are consistent with the presence of a single gene that encodes both isopenicillin N amidolyase and acyl-CoA:6-APA acyltransferase. Therefore, the best designation for that gene is *pen*DE. The gene encoding the isopenicillin N acyltransferase was cloned by screening a library of *P. chrysogenum* DNA in vector EMBL3 using a combination of oligonucleotide probes based on the amino terminal sequence of the 29 kDa protein with acyl-CoA:6-APA acyltransferase activity (3).

The *pen*DE gene of *A. nidulans* was identified and isolated by Montenegro et al. (49) after screening a gene library of *A. nidulans* DNA with the *pen*DE gene of *P. chrysogenum* as a probe. The *pen*DE genes of *P. chrysogenum* (1241 nt) and *A. nidulans* (1274 nt) encode proteins of 39943 and 39420 Da, respectively. Both of them have three introns in the 5' region.

As indicated above, the size of the active AAT protein of *P. chrysogenum* is 29 kDa. Since this size is smaller than that of the polypeptide encoded by the gene, it is likely that a preacyltransferase protein of 40 kDa initially formed that will be later processed originating a polypeptide of 11 kDa and the mature acyltransferase of 29 kDa (11). This hypothesis is supported by the following fundings: i) in partially purified preparations of acyltransferase the 29 kDa protein copurifies always with a polypeptide of 11 kDa. ii) The amino terminal sequences of the 11 and 40 kDa proteins correspond to the deduced amino acid sequence from the nucleotide sequence of the *pen*DE gene. iii) There are examples of processing of related enzymes such as the penicillin acylases from a variety of bacteria (9, 44, 58) and cephalosporin acylases (45, 46).

A phenylacetyl-CoA ligase activity which activates phenylacetic acid using CoA and ATP is supposed to occur in *P. chrysogenum*. This activity does not seem to occur as part of the isopenicillin N acyltransferase. In fact, it seems unlikely that the isopenicillin N acyltransferase may contain additional enzymatic activities to those reported above. If the phenylacetyl-CoA ligase is encoded by a separate gene this might be named *pen*F.

The genes *pcb*C and *pen*DE were found to be linked in the genome of *P. chrysogenum* (19). Later, we have found that they are also linked to the *pcb*AB gene (20, 63).

The DNA region which contains the genes *pcb*AB, *pcb*C and *pen*DE in *P. chrysogenum* was found to be amplified in penicillin overproducing strains (12, 62).

*cef*D Encoding Isopenicillin N Epimerase (IPNE)

In the cephalosporin-producing microorganisms *(C. acremonium, S. clavuligerus, S. lipmanii, N. lactamdurans)* conversion of isopenicillin N to cephalosporin starts with the isomerization of isopenicillin N to penicillin N catalyzed by the isopenicillin N epimerase. This protein has been purified to homogeneity as a monomer in *S. clavuligerus* (28) and *N. lactamdurans* (36) but it is not present in microorganisms which are producers of penicillin such as *P. chrysogenum*.

The amino terminal sequence of the isopenicillin N epimerase was determined and used to construct a nucleotide probe to clone the *cef*D gene that was also expressed in *E. coli* (28).

*cef*EF, A Gene Encoding the Bifunctional Deacetoxycephalosporin C Synthase (DAOCS) and Deacetylcephalosporin C synthase (DACS) in *C. acremonium*. *cef*E and *cef*F Separate Genes in *Streptomyces clavuligerus*

The expansion of the five membered thiazolidine ring of penicillin N to the six-membered dihidrothiazine ring of deacetoxycephalosporin C is carried out by the enzyme DAOC synthase. The deacetylcephalosporin C synthase (DACS) carries out the conversion of deacetoxycephalosporin C into deacetylcephalosporin C by means of an hydroxylation reaction.

In *C. acremonium* both acivities DAOCS and DACS are located in a single protein (21). Sequencing of the amino terminal region could not be carried out due to the blocking of the amino terminal end of the protein. The purified protein was digested enzymatically and the fragments formed were purified and sequenced. The amino acid sequences were used to construct probes which served to clone the *cef*EF gene (56). The open reading frame of the *cef*EF gene encoded a protein of molecular weight 36,460 Da, that contained the amino sequences of the internal peptides. When the *cef*EF gene was expressed in *E. coli* crude extracts were obtained that contained both activities DAOCS and DACS, what proved that the cloned gene encoded a bifunctional polypeptide.

The bifunctionality is particularly interesting when compared with the arrangement in *S. clavuligerus* where the two activities (DAOCS and DACS) are easily separated by ion exchange chromatography. A gene encoding the DAOCS but not the DACS has been cloned recently from the DNA of *S. clavuligerus* (35, 48). This gene named *cef*E was cloned using a probe constructed from the amino terminal amino acid sequence of the purified *S. clavuligerus* DAOCS. The *cef*E gene has been expressed in *E. coli* and the polypeptide formed showed DAOCS activity but not DACS. It has been suggested that a separate gene *cef*F should occur in *S. clavuligerus* but it has not been cloned so far. Cloning and expression in *E. coli* will provide the definitive proof for the existence of such a separate gene.

A comparative analysis of the fungal and bacterial genes that encode DAOCS and DACS is interesting since i) it may lead to elucidate if the fungal *cef*EF gene derives by fusion of the *cef*E and *cef*F genes of *Streptomyces sp.*, and ii) may help to identify potential active centers in the protein. Comparison of the *cef*E and *cef*EF genes shows that both genes have an homology of 65 % at the DNA level and 56.7 % at the amino acid level (48) which are close to the similarity found in the sequenced *pcb*C genes, what supports the hypothesis that all genes were transfered simultaneously. It is likely that the *cef*EF gene of *C. acremonium* ATCC 11550 arose by deletion of one of the two identical regions of a fused ancestral gene in which both regions carried out the same function (28).

The genes *cef*EF and *pcb*C encoding DAOCS/DACS and IPNS respectively in *C. acremonium* show a very limited similarity (11 % of the amino acids are conserved in both proteins). There are, however, some common motifs in both genes: i) both of them contain a cysteine residue in position 106 *(pcb*C) or in position 100 *(cef*EF) which seems to be important for the enzyme activity, and ii) a region of 10 amino acids that includes those cysteine residues shows a 50 % homology (56). This might be due to a functional evolutive convergence, or might indicate that both genes derive from an ancestral progenitor gene.

In *S. clavuligerus* the genes *pcb*C and *cef*E are physically linked in the genome, whereas in *C. acremonium* they are located in different chromosomes (60, 61).

*cef*G Gene Encoding Deacetylcephalosporin C acetyltransferase. Linkage of the Biosynthetic Genes

The *cef*G gene of *C. acremonium* appears to be located close to the expandase/hydroxylase gene *(cef*EF) of *C. acremonium* (S. Gutiérez, S. Velasco and J. F. Martín, unpublished; Ramsden et al., 6th International Symposium on Genetics of Industrial Microorganisms, Strasbourg, 1990).

There is evidence suggesting that the genes involved in the biosynthesis of cephamycins are linked in *Streptomyces* (28) as occurs with other antibiotic biosynthetic genes (42). Kovacevick and Miller used the *pcb*C gene of *S. lipmanii* as a probe to isolate three cosmids with *S. clavuligerus* DNA, one of which contained also the gene encoding DAOCS (*cef*E) (28). DNA sequences that stimulate the synthesis of deacetylcephalosporin C synthase, isopenicillin N epimerase, and O-carbamoyldeacetylcephalosporin C hydroxylase are linked in the chromosome (European Patent Application O-223-715-AZ, 1987). It is likely that these DNA sequences carry biosynthetic and/or regulatory genes. Chen and coworkers (15) described that the transformation of *S. lividans* (which usually does not synthesize ß-lactam antibiotics) with a DNA fragment of *Streptomyces cattleya* (a producer of cephamycin and thienamycin) conferred to the first strain the ability to synthesize cephamycin. Very recently in our group Coque et al. (16) have cloned and characterized the genes *pcb*AB and *pcb*C encoding respectively the ACV synthetase and the isopenicillin N synthase of *N. lactamdurans* that are closely linked. A third gene *lat* encoding lysine-6-aminotransferase that forms α-aminoadipic acid from lysine is also clustered in the cluster of cephamycin-biosynthetic genes (17).

Future Outlook

In summary, DNA fragments of *Streptomyces* or filamentous fungi that contain regulatory and/or structural genes that affect the biosynthesis of ß-lactams are becoming an increasingly important source of information on the genetics and molecular biology of the producers of ß-lactam antibiotics.

The linkage of genes involved in the biosynthesis of ß-lactam antibiotics may lead to the isolation of other, still unknown, cephalosporin or cephamycin biosynthetic genes.

References

1. Adlington, R. M., Aplin, R. T., Baldwin, J. E., Chakravarti, B., Field, L. D., John. E. M. M., Abraham, E. P. and White, R. L. (1983). Conversion of $^{17}O/^{18}O$-labelled ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine to $^{17}O/^{18}O$-labelled isopenicillin N in a cell free extract of *Cephalosporium acremonium*. A study by ^{17}O-NMR spectroscopy and mass spectroscopy. Tetrahedron **39,** 1061-1068.
2. Alonso, M. J., Bermejo, F., Reglero, A., Fernández-Cañón, J. M., González de Buitrago, G. and Luengo, J. M. (1988). Enzymatic synthesis of penicillins. J. Antibiot. **41,** 1074-1084.
3. Alvarez, E., Cantoral, J. M., Barredo, J. L., Díez, B. and Martín, J. F. (1987). Purification to homogeneity and characterization of acylcoenzyme A:6-amino penicillanic acid acyltransferase of *Penicillium chrysogenum*. Antimicrob. Agents Chemother. **31,** 1675-1682.
4. Alvarez, E., Meesschaert, B., Montenegro, E., Gutiérrez, S., Díez, B., Barredo, J. L. and Martín, J. F. (1991). The isopenicillin N acyltransferase system of *Penicillium chrysogenum* has isopenicillin N amidohydrolase, 6-APA acyltransferase, penicillin transacylase and penicillin amidase activities, all of which are encoded by the single *pen*DE gene. J. Biol. Chem. (in press).
5. Bahadur, G. A., Baldwin, J. E., Field, L. D., Lehtonen, E. M. M., Usher, J. J. and Vallejo, C. A. (1981). Direct HMNR observation of the cell-free conversion of ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine and ∂-(L-α-aminoadipyl)-L-cysteinyl-D-(-)-isoleucine into penicillins. J. Chem. Soc. Chem. Commun. pp. 917.
6. Baldwin, J. E., Killin, S. J., Pratt, A. J., Sutherland, J. D., Turner, N. J., Crabbe, M. J. C., Abraham, E. P. and Willis, A. C. (1987). Purification and characterization of cloned isopenicillin N synthetase. J. Antibiot. **40,** 652-659.
7. Baldwin, J. E., Bird, J. W., Field, R. A., O'Callaghan, N. M., Schofield, C. J. and Willis, A. C. (1991). Isolation and partial characterization of ACV synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus*. Evidence of the presence of phosphopantothenate in ACV synthetase. J. Antibiot. **44,** 241-248.
8. Banko, G., Demain, A. L. and Wolfe, S. (1987). ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACV synthetase): a multifunctional enzyme with broad substrate specificity for the synthesis of penicillin and cephalosporin precursors. J. Am. Chem. Soc. **109,** 2858-2860.

9. Barbero, J. L., Buesa, J. M., González de Buitrago, G., Méndez, E., Pérez-Aranda, A. and García, J. L. (1986). Complete nucleotide sequence of the penicillin acylase gene from *Kluyvera citrophila*. Gene **49,** 69-80.
10. Barredo, J. L., Cantoral, J. M., Alvarez, E., Díez, B. and Martín, J. F. (1989a). Cloning, sequence analysis and transcriptional study of the isopenicillin N synthase of *Penicillium chrysogenum* AS-P-78. Mol. Gen. Genet. **216,** 91-98.
11. Barredo, J. L., van Solingen, P., Díez, B., Alvarez, E., Cantoral, J. M., Kattevilder, A., Smaal, E. B., Groenen, M. A. M., Veenstra, A. E. and Martín, J. F. (1989b). Cloning and characterization of the acyl-Coenzyme A:6-aminopenicillanic acid acyltransferase gene of *Penicillium chrysogenum*. Gene. **83,** 291-300.
12. Barredo, J. L., Díez, B., Alvarez, E. and Martín, J. F. (1989c). Large amplification of a 35 kb DNA fragment carrying two penicillin biosynthetic genes in high penicillin producing strains of *Penicillium chrysogenum*. Curr. Genet. **16,** 453-459.
13. Carr, L. G., Skatrud, P. L., Scheetz III, M. E., Queener, S. W. and Ingolia, T. D. (1986). Cloning and expression of the isopenicillin N synthetase gene from *Penicillium chrysogenum*. Gene **48,** 257-266.
14. Castro, J. M., Liras, P., Láiz, L., Cortés, J. and Martín, J. F. (1988). Purification and characterization of the isopenicillin N synthase of *Streptomyces lactamdurans*. J. Gen. Microbiol. **134,** 133-141.
15. Chen, C. W., Lin, H. F., Kuo, C. L., Tsai, H. L. and Tsai, J. F. Y. (1988). Cloning and expression of a DNA sequence conferring cephamycin C production. Biotechnology **6,** 1222-1224.
16. Coque, J. J. R., Martín, J. F., Calzada, J. G. and Liras, P. (1991a). The cephamycin biosynthetic genes *pcb*AB, encoding a large multidomain peptide synthetase, and *pcb*C of *Nocardia lactamdurans* are clustered together in an organization different from the same gene in *Acremonium chrysogenum* and *Penicillium chrysogenum* Mol. Microbiol. (in press).
17. Coque, J. J. R., Liras, P., Láiz, L. and Martín, J. F. (1991b). A gene encoding lysine 6-aminotransferase which forms α-aminoadipic acid, a precursor of ß-lactam antibiotics, is located in the cluster of cephamycin biosynthetic genes in *Nocardia lactamdurans*. J. Bacteriol. (in press).
18. Cortés, J., Martín, J. F., Castro, J. M., Láiz, L. and Liras, P. (1987). Purification and characterization of a 2-oxoglutarate-linked ATP-independent deacetoxicephalosporin C synthase of *Streptomyces lactamdurans*. J. Gen. Microbiol. **133,** 3165-3174.
19. Díez, B., Barredo, J. L., Alvarez, E., Cantoral, J. M., van Solingen, P., Groenen, M. A. M., Veenstra, A. E. and Martín, J. F. (1989). Two genes involved in penicillin biosynthesis are linked in a 5.1 kb fragment in the genome of *Penicillium chrysogenum*. Mol. Gen. Genet. **218,** 572-576.
20. Díez, B., Gutiérrez, S., Barredo, J. L. van Solingen, P., van der Voort, L. H. M. and Martín, J. F. (1990). The cluster of penicillin biosynthetic genes. J. Biol. Chem. **265,** 16358-16365.
21. Dotzlaf, J. E. and Yeh, W. K. (1987). Copurification and characterization of deacetoxicephalosporin C synthetase/hydroxylase from *Cephalosporium acremonium*. J. Bacteriol. **169,** 1611-1618.
22. Fujisawa, Y., Shirafuji, H., Hida, M., Nara, K. L., Yoneda, M. and Kanzaki, T. (1973). New findings on cephalosporin C biosynthesis. Nature **246,** 154.
23. Fujisawa, Y., Kikuchi, M. and Kanzaki, T. (1977). Deacetylcephalosporin C synthesis by cell-free extracts of *Cephalosporium acremonium*. J. Antibiot. **30,** 775-777.
24. García-Domínguez, M., Liras, P. and Martín, J. F. (1991). Cloning and characterization of the isopenicillin N synthase gene of *Streptomyces griseus* NRRL 3851 and studies of expression and complementation of the cephamycin pathway in *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **35,** 44-52.
25. Gutiérrez, S., Díez, B., Montenegro, E. and Martín, J. F. (1991). Characterization of the *Cephalosporium acremonium pcb*AB gene encoding α-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: Linkage to the *pcb*C gene as a cluster of early cephalosporin-biosynthetic genes and evidence of multiple functional domains. J. Bacteriol. **173,** 2354-2365.
26. Hollander, I. J., Shen, Y. Q., Heim, J. and Demain, A. L. (1984). A pure enzyme catalyzing penicillin biosyntesis. Science **2245,** 610-612.
27. Hori, H. and Osawa, S. (1979). Evolutionary change in 5S RNA secondary structure and a phylogenic tree of 54 5S RNA species. Proc. Natl. Acad. Sci. USA **76,** 381-385.
28. Ingolia, T. D. and Queener, S. W. (1989). Beta-lactam biosynthetic genes. Medicinal Res. Rev. **9,** 245-264.
29. Jensen, S. E., Westlake, D. W. S., Bowers, R. J. and Wolfe, S. (1982). Cephalosporin formation by cell-free extracts from *Streptomyces clavuligerus*. J. Antibiot. **34,** 1351-1360.
30. Jensen, S. E., Westlake, D. W. S. and Wolfe, S. (1983). Partial purification and characterization of isopenicillin N epimerase activity from *Streptomyces clavuligerus*. Can. J. Microbiol. **29,** 1526-1531.
31. Jensen, S. E., Leskiw, B. K., Vining, L. C., Aharanowitz, Y., Westlake, D. W. S. and Wolfe, S. (1986). Purification of isopenicillin N synthetase from *Streptomyces clavuligerus*. Can. J. Microbiol. **32,** 953-958.
32. Jensen, S. E., Westlake, D. W. S. and Wolfe, S. (1988). Production of the penicillin precursor ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by cell-free extracts from *Streptomyces clavuligerus*. FEMS Microbiol. Lett. **49,** 213-218.
33. Konomi, T., Herchen, D., Baldwin, J. E., Yoshida, M., Hunt, N. A. and Demain, A. L. (1979). Cell-free conversion of ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine into *Cephalosporium acremonium*. Biochem. J. **184,** 427-430.
34. Koshaka, M. and Demain, A. L. (1976). Conversion of penicillin N to deacetoxicephalosporin(s) by cell-free extracts of *Cephalosporium acremonium*. Biochim. Biophys. Res. Commun. **70,** 465-473.

35. Kovacevic, S., Weigel, B. J., Tobin. M. B., Ingolia, T. D. and Miller, J. R. (1989). Cloning, characterization and expression in *E. coli* of the *Streptomyces clavuligerus* gene encoding deacetoxicephalosporin C synthetase. J. Bacteriol. **171,** 754-760.
36. Láiz, L., Liras, P., Castro, J. M. and Martín, J. F. (1990). Purification and characterization of the isopenicillin N epimerase from *Nocardia lactamdurans*. J. Gen. Microbiol. **136,** 663-671.
37. Leskiw, B. K., Aharanowitz, Y., Mevarech, M. Wolfe, S., Vining, L. C., Westlake, D. W. S. and Jensen, S. E. (1988). Cloning and nucleotide sequence determination of the isopenicillin N synthetase gene from *Streptomyces clavuligerus*. Gene **62,** 187-196.
38. Li, W. H., Luo, C. C. and Wu, C. I. (1985). Evolution of DNA sequences. In: R. J. MacIntyre (ed.). Molecular Evolutionary Genetics. pp. 1-94. Plenum Press. New York.
39. López-Nieto, M. J., Ramos, F. R., Luengo, J. M. and Martín, J. F. (1985). Characterization of the biosynthesis in vivo of α-aminoadipyl-L-cysteinyl-D-valine in *Penicillium chrysogenum*. Appl. Microbiol. Biotechnol. **22,** 343-351.
40. Luengo, J. M., Iriso, J. L. and López-Niego, M. J. (1986). Direct evaluation of phenylacetyl-CoA:6-aminopenicillanic acid acyltransferase of *Penicillium chrysogenum* by bioassay. J. Antibiot. **39,** 1565-1573.
41. MacCabe, A. P., Riach, M. B. R., Unkles, S. E. and Kinghorn, J. R. (1990). The *Aspergillus nidulans npe*A locus consists of the three contiguous genes required for penicillin biosynthesis. EMBO J. **9,** 279-287.
42. Martín, J. F. and Liras, P. (1989a). Organization and expression of genes involved in the biosynthesis of antibiotics and other secondary metabolites. Ann. Rev. Microbiol. **43,** 173-206.
42a. Martín, J. F. and Liras, P. (1989b). Enzymes involved in penicillin, cephalosporin and cephamycin biosynthesis. *In:* A. Fiechter (ed.). Advances in Biochemical Engineering/Biotechnology, Vol. **39,** pp. 153-197. Springer-Verlag, Heidelberg, Berlin.
43. Martín, J. F., Ingolia, T. D. and Queener, S. W. (1991). Molecular genetics of penicillin and cephalosporin antibiotic biosynthesis. *In:* S. A. Leong and R. M. Berka (eds.). Molecular Industrial Mycology. pp. 149-196. Marcel Dekker, Inc. New York.
44. Matsuda, A. and Komatsu, K. I. (1985). Molecular cloning and structure of the gene for 7ß-(4-carboxybutanamido) cephalosporanic acid acylase from a *Pseudomonas* strain. J. Bacteriolo. **163,** 1222-1228.
45. Matsuda, A., Matsuyama, K. I. Yamamoto, K., Ichikawa, S. and Komatsu, K. I. (1987a). Cloning and characterization of the gene for two distinct cephalosporin acylases from a *Pseudomonas* strain. J. Bacteriol. **169,** 5815-5820.
46. Matsuda, A., Toma, K. and Komatsu, K. I. (1987b). Nucleotide sequences of the genes for two distinct cephalosporin acylases from a *Pseudomonas* strain. J. Bacteriol. **169,** 5821-5826.
47. Meeschaert, B., Adriaens, P. and Eyssen, H. (1980). Studies on the biosynthesis of isopenicillin N with a cell-free preparation of *Penicillium chrysogenum*. J. Antibiot. **33,** 722-730.
48. Miller, J. R. and Ingolia, T. D. (1989). Cloning and characterization of beta-lactam biosynthetic genes. Mol. Microbiol. **3,** 689-695.
49. Montenegro, E., Barredo, J. L., Gutiérrez, S., Díez, B., Alvarez, E. and Martín, J. F. (1990). Cloning, characterization of the acyl-CoA:6-APA acyltransferase gene of *Aspergillus nidulans* and linkage to the isopenicillin N synthase gene. Mol. Gen. Genet. **221,** 322-330.
50. Pang, C. P., Chakravarti, B., Adlington, R. M., Ting, H. H., White, R. L., Jayatilake, G. S., Baldwin, J. E. and Abraham, E. P. (1984). Purification of isopenicillin N synthetase. Biochem. J. **222,** 789-795.
51. Pruess, D. L. and Johnson, M. J. (1967). Penicillin acyltransferase in *Penicillium chrysogenum*. J. Bacteriol. **94,** 1502-1508.
52. Ramón, D., Carramolino, L., Patiño, C., Sánchez, F. and Peñalva, M. A. (1987). Cloning and characterization of the isopenicillin N synthetase gene mediating the formation of the ß-lactam ring in *Aspergillus nidulans*. Gene **57,** 171-181.
53. Ramos, F. R., López-Nieto, M. J. and Martín, J. F. (1985). Isopenicillin N synthetase of *Penicillium chrysogenum* an enzyme that converts ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine to isopenicillin N. Antimicrob. Agents Chemother. **27,** 380-387.
54. Samson, S. M., Belagaje, R., Blankenship, D. T., Chapman, J. L., Perry, D., Skatrud, P. L., Vankfrank, R. M., Abraham, E. P., Baldwin, J. E., Queener, S. W. and Ingolia, T. D. (1985). Isolation, sequence determination and expression in *Escherichia coli* of the isopenicillin N synthetase gene from *Cephalosporium acremonium*. Nature **318,** 191-194.
55. Samson, S. M., Chapman, J. L. Belagaje, R., Queener, S. W. and Ingolia, T. D. (1987a). Analysis of the role of cysteine residues in isopenicillin N synthetase activity by site-directed mutagenesis. Proc. Natl. Acad. Sci. USA **84,** 5705-5709.
56. Samson, S. M., Dotzlaf, J. E., Slisz, M. L., Becker, G. W., Van Frank, R. M., Veal, L. E., Yeh, W. K., Miller, J. R., Queener, S. W. and Ingolia, T. D. (1987b). Cloning and expression of the fungal expandase-hydroxylase gene involved in cephalosporin biosynthesis. Biotechnology **5,** 1207-1214.
57. Sawada, Y., Konomi, T., Solomon, N. and Demain, A. L. (1980). Increase in activity of ß-lactam synthetases after growth of *Cephalosporium acremonium* with methionine or norleucine. FEMS Microbiol. Lett. **9,** 281-284.
58. Schumacher, G., Sizmann, D., Buckel, P. and Bock, A. (1986). Penicillin acylase from *E. coli:* unique gene-protein relation. Nucl. Acids Res. **14,** 5713-5727.
59. Shiffman, D., Mevarech, M., Jensen, S. E., Cohen, G. and Aharonowitz, Y. (1988). Cloning and comparative sequence analysis of the gene coding for isopenicillin N synthase in *Streptomyces*. Mol. Gen. Genet. **214,** 562-569.
60. Skatrud, P. L., Tietz, A. J., Ingolia, T. D., Cantwell, C. A., Fisher, D. L., Chapman, J. L. and Queenr, S. W. (1989). Use of

recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. Biotechnology **7,** 477-485.

61. Skatrud, P. L. and Queener, S. W. (1989). An electrophoretic molecular karyotype for an industrial strain of *Cephalosporium acremonium*. Gene **78,** 331-338.
62. Smith, D. J., Bull, J. H., Edwards, J. and Turner, G. (1989). Amplification of the isopenicillin N synthetase gene in a strain of *Penicillium chrysogenum* producing high levels of penicillin. Mol. Gen. Genet. **216,** 492-497.
63. Smith, D. J., Burnham, M. K. R., Edwards, J., Earl, A. M. and Turner, G. (1990). Cloning and heterologous expression of the penicillin biosynthetic gene cluster from *Penicillium chrysogenum*. Biotechnology **8,** 39-41.
64. Turner, M. K., Farthing, J. E. and Brewer, S. J. (1978). The oxygenation of (3-methyl-^{3}H) deacetoxycephalosporin C [7B-(5-D-aminoadipamido)-3-methylceph-3-em-4-carboxylic acid] to (3-hydroxymethyl-3-H) deacetylcephalosporin C by 2-oxoglutarate-linked dioxygenases from *Acremonium chrysogenum* and *Streptomyces clavuligerus*. Biochem. J. **173,** 839-850.
65. Van Liempt, H., von Dohren, H. and Kleinkauf, H. (1989). ∂-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*. J. Biol. Chem. **264,** 3680-3684.
66. Weigel, B. J., Burgett, S. G., Chen. V. J., Skatrud, P. L., Frolik, C. A., Queener, S. W. and Ingolia, T. D. (1988). Cloning and expression in *Escherichia coli* of isopenicillin N synthetase gene from *Streptomyces lipmanii* and *Aspergillus nidulans*. J. Bacteriol. **170,** 3817-3826.
67. Zhang, J. Y., Wolfe, S. and Demain, A. L. (1989). Phosphate regulation of ACV synthetase and cephalosporin biosynthesis in *Streptomyces clavuligerus*. FEMS Microbiol. Lett. **57,** 145-150.
68. Zhang, J. and Demain, A. L. (1990). Purification of ACV synthetase from *Streptomyces clavuligerus*. Biotechnol. Lett. **12,** 649-654.

MICROBIOLOGIA SEM 7 (1991), 13-22

Isolation and characterization of *Rhizobium meliloti* mutants affected in exopolysaccharide production

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(Received September 6/November 8, 1990)

Summary

Rhizobium meliloti mutants affected in the production of exopolysaccharide (EPS) were isolated after N-methyl-N'-nitro-N-nitrosoguanidine mutagenesis. The mutants were classified into three phenotypic classes: (I) Exo$^-$, rough mutants lacking exopolysaccharide; (II) Exo^s (for «small») which form tiny, compact colonies and synthesize reduced amounts of EPS; and (III) Exo^c (for «constitutive»), hypermucoid mutants which overproduce EPS. Hypermucoid strains showed increased resistance to desiccation. All the mutants were able to nodulate, although a significant decrease in infectivity degree and/or competitiveness was found in rough and compact strains. Two mutants proved to be deficient in nitrogen fixation. Complementation analysis with cloned *R. meliloti exo* genes could not be applied to the study of these Fix$^-$ mutants because introduction of plasmids derived from cosmid vector pLAFR1 caused loss of nodulating ability. However, complementation of calcofluor staining and EPS production was observed. Complementation with certain *exo* genes also caused a marked increase in motility.

Key words: Extracellular polysaccharide, nodule formation, resistante to desiccation, motility.

Resumen

Mediante mutagénesis con N-metil-N'-nitro-N-nitrosoguanidina se aislaron mutantes de *Rhizobium meliloti* afectados en la producción de polisacárido extracelular (EPS). Los mutantes se clasificaron en 3 clases fenotípicas: (I) Exo$^-$, mutantes rugosos que no producen EPS; (II) Exo^s (de «small»), que forman colonias pequeñas y compactas y sintetizan cantidades reducidas de EPS; (III) Exo^c (de «constitutivos»), mutantes hipermucosos que superproducen EPS. Los mutantes hipermucosos mostraron una mayor resistencia a la desecación. Todos los mutantes fueron capaces de nodular, pero algunos mostraron diferencias significativas en su grado de infectividad. Dos mutantes resultaron incapaces de fijar nitrógeno. Al intentar complementar estos mutantes Fix$^-$ con genes *exo* clonados de *R. meliloti*, se observó que la introducción de plásmidos derivados de pLAFR1 originaba la pérdida de la capacidad nodular. Sin embargo, se observó complementación de la tinción con calcoflúor y la producción de EPS. La complementación con algunos genes *exo* también produjo un notable incremento de la movilidad.

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Introduction

The biological significance of exopolysaccharide production by Rhizobia has been the subject of a long controversy. Early studies correlating EPS production with symbiotic specificity (9, 20) were later contradicted by the isolation of mutants lacking EPS but exhibiting a wild-type phenotype (22). Both views were tentatively conciliated by the suggestion that EPS might be to some extent dispensable but necessary for optimal infection (19). Moreover, a number of mutants deficient in both exopolysaccharide production and formation of nitrogen-fixing nodules have been described (4, 12, 15, 16, 17), unambiguously proving that EPS plays a role in nodule development. One interesting observation is the existence of EPS mutants that induce nodule formation but are unable to fix nitrogen (4). Thus EPS seems to play a role not only in the early steps of the symbiosis, but also in the process of bacteroid differentiation within the nodule.

This paper reports the isolation of several classes of mutants affected in EPS synthesis and the examination of their properties with a broad outlook: besides of analyzing their symbiotic behavior, we explored hypothetical relationships between EPS production and UV sensitivity, motility, sensitivity to bacteriophages and resistance of desiccation. Our results support the idea that EPS production affects the degree of infectivity, even though EPS production may not be an absolute requirement for infection. We also report the isolation of hypermucoid strains which are likely constitutive for EPS synthesis. Overproduction of EPS does not increase infectivity degreee, but confers increased resistance to desiccation, a feature that might be of practical importance. During the study of two Fix$^-$ mutans by complementation with cloned *exo* genes, we were surprised to find that cosmid pLAFR1 and its derivatives cause loss of nodulation ability in *R. meliloti* GR4.

Materials and Methods

Bacterial strains, plasmids and bacteriophages. All the *Rhizobium meliloti* strains mentioned in this paper derive from the wild-type strain GR4 (6). Plasmids pD34, pD2, pD15 and pD5 derive from cosmid vector pLAFR1 (Tc^r) and respectively contain genes *exoA*, *exoB*, *exoC* and *exoD* from *R. meliloti* (16). Cosmid pLAFR1 is described in ref. 13. Plasmid pRK2013 (Km^r) is a ColE1 derivative containing transfer funcions of broad-range plasmid RK2 (11). The *E. coli* host strain for all these plasmids was DH5α (14). Bacteriophages DF2, AL1, LO0, f2D and FAR (Corral, E. 1980. Ph. D. Thesis, University of Granada, Spain) were kindly provided by J. Olivares (CSIC, Granada).

Culture media and growth conditions. M-79 broth was prepared according to Vincent (24). For solid medium, Difco agar was added at a final concentration of 1.5%. In certain experiments, the usual carbon source of M-79 (mannitol) was replaced by one of the following: sucrose, maltose, lactose, glutamate, glycerol, glucose or mannose (see the section Results). Cultures were always grown at 30° C. Experiments involving *R. meliloti* phages were carried out in yeast-sucrose agar and yeast-sucrose broth, as described by Vincent (24). Assays of phage sensitivity were performed using the double layer method. For motility assays, soft (0.4%) TY agar (3) was used. Calcofluor (Sigma) was used at a concentration of 200 mg/l. Antibiotics (all from Sigma) were used at the following concentrations: tetracycline, 10 mg/l; kanamycin, 50 mg/l; streptomycin, 100 mg/l.

Nitrosoguanidine mutagenesis. An exponential culture of *R. meliloti* GR4 (O.D. = 0.5) was centrifuged, washed with cold tris-maleate buffer (TM) and resuspended in TM containing 150 mg/l N-methyl-N'-nitro-N-nitrosoguanidine (Sigma). After a 30 min incubation at 37° C without shaking, the culture was centrifuged, washed twice with TM, resuspended in M-79 broth and incubated at 30° (with shaking) during 6-8 h (or 3-4 generations). Then the mutagenized culture was plated on M-79 agar.

Extraction and analysis of extracellular polysaccharide. Supernatants from *R. meliloti* batch cul-

TABLE 1
SUMMARY OF PHENOTYPES OF EPS MUTANTS

Strain	Colonial morphology	EPS production (mg/100 ml)	Calcofluor staining	Nitrogen fixation[a]
GR4	Wild-type	115	Bright	+
JC4004	Hypermucoid	306	Bright, haloed	+
ISM101	Hypermucoid	157	Bright	–
ISM103	Hypermucoid	162	Dull	+
ISM104	Hypermucoid	126	Bright, haloed	+
ISM105	Compact	73	Bright, haloed	+
ISM106	Compact	83	Dull	+
ISM107	Rough	NM[b]	Dark	–
ISM109	Rough	NM	Bright	+
ISM110	Rough	NM	Dull	+

[a] Nitrogen fixation data are presented unified since Kjeldhal measurements and acetylene reduction provided coincident data. For acetylene reduction, (+) means 19-31 C_2H_2 mols/h/mg nodules (dry weight); (—) is less than 7 C_2H_2 mols/h/mg dry nodules. For Kjeldahl, (+) is a N content over 3%, while (—) is a N content below 2%.

[b] NM: Not measurable.

tures were used as the source of extracellular polysaccharide. Extraction was carried out by acetone precipitation (Palomares A. 1975. Ph. D. Thesis, University of Granada, Spain). For qualitative analysis, dry extracts were rehydrated with distilled H_2O and hydrolyzed with H_2SO_4. After $BaCO_3$ neutralization, the hydrolysates were filtered and concentrated by evaporation. Sugar identification was carried out by HPLC, using a Millipore Lambda Max chromatograph, mod. 481.

UV sensitivity assays. Aliquots from *R. meliloti* liquid cultures containing 2-3 $\times$ 10^8 cells/ml (O. D. 0.7) were transferred to sterile, empty Petri dishes. Irradiation was achieved by opening the plates under a 15W Sylvania lamp, at a distance of 30 cm, in the absence of daylight illumination. Cultures were stirred during irradiation. After serial dilution in foil-covered tubes, irradiated cultures were plated on M-79.

Desiccation assays. Strips of Whatman #4 paper, each of 1 cm^2, were soaked in *R. meliloti* saturated cultures and placed on sterile, empty Petri dishes. The strips were incubated a 30° C in the dark. Rehydration and viable counts on M-79 plates were carried at different times (3, 10, 15 hours after soaking).

Assessment of symbiotic behavior. Nodulation assays were carried out on *Medicago sativa* cv. Aragón, using Leonard jars with 2 plants/jar. Plants were grown under axenic conditions, using a nitrogen-free mineral salt solution (21), supplemented with NO_3K 30 mg/l. Each jar was inoculated with 3-5 $\times$ 10^9 Rhizobia. Infectivity coefficients were calculated according to Olivares et al. (18). Competitiveness was estimated according to Amarger (2). Nitrogen fixation was assessed by two methods: (1) Kjeldahl analysis of nitrogen content; (2) acetylene reduction in a Variant Aerograph gas chromatograph.

Genetic complementation. E. coli DH5α derivatives carrying plasmids pD34, pD2, pD15 and pD5 were mated with Str^r derivatives of *R. meliloti exo* mutants in triparental crosses using pRK2073 as a helper. Tc^r Str^r transconjugants of *R. meliloti* were selected on M-79 plates supplemented with tetracycline and streptomycin. After purification, plasmid-carrying isolates were screened for complementation of staining properties, colonial morphology and symbiotic nitrogen fixation (see the section Results).

Motility assays. Soft TY agar plates were inoculated at their centers, each with 5 ml of an overnight culture of the strain to be tested. Growth diameters were measured after 48 h of incubation.

Results

Isolation of R. meliloti *mutants with altered colonial morphology.* When mutagenized cultures of *R. meliloti* were plated on M-79 agar, three types of anomalous colonies were observed; (I) hypermucoid, light colored colonies; (II) compact, small colonies; (III) rough, slightly wrinkled colonies. Each type occurred at a frequency of about one per 3,000 colonies. Independent isolates were purified and propagated as the following strains:

I. Hypermucoid: JC4004, ISM101, ISM102, ISM103, ISM104.
II. Compact: ISM105, ISM106.
III. Rough: ISM107, ISM108, ISM109.

Calcofluor staining showed that mutants belonging to a certain group were often heterogeneous (see Table 1), suggesting that each phenotypic group includes various mutant types. A corollary is that calcofluor staining may not be the most appropriate method to pinpoint EPS mutants, since certain hypermucoid (e. g. ISM101) and rough (e. g. ISM109) strains show wild-type staining (see Table 1).

Exopolysaccharide production by R. meliloti *mutants with altered colonial morphology.* In the wild-type strain *R. meliloti* GR4, EPS production greatly varies in different media. One major factor is the carbon source used: growth on mannitol leads to the synthesis of 0.09-0.12 g/l, whereas glucose, sucrose, maltose or glutamate give intermediate levels (0.04-0.07 g/l). Glycerol, galactose and lactose are extremely poor sources for EPS synthesis. The pattern of EPS production on different carbon sources was conserved in the hypermucoid and compact strains, indicating that they are actual EPS mutants (and not, for instance, mutants affected in sugar utilization). The rough mutants did not produce EPS with any of the carbon sources tested. A summary of the EPS levels produced with mannitol as the sole carbon source is shown in Table 1. From these results, correlations between EPS production and colonial morphology are easily established: (I) all the hypermucoid strains are overproducers of EPS; (II) the production of EPS by compact strains is well below the wild-type; and (III) the rough mutants do not produce detectable amounts of EPS.

In the wild-type strain, accumulation of EPS mainly occurs in the late exponential and the stationary stage of growth. This timing was clearly altered in all the hypermucoid strains: these produced significative amounts of EPS in early stages of the growth cycle, thus resembling deregulated mutants (hence the symbol Exo^c, for «constitutive»). Compact mutants are designated Exo^s (for «small»). Roungh mutants are designated Exo^-.

Further characterization of EPS mutants

1. *Chemical analysis.* Semiquantitative analysis of hydrolyzed EPS led to the identification of uronic acids, fructose, galactose and glucose as the major components of wild-type EPS (Rodríguez-Navarro, D. 1986. Ph. D. Thesis, University of Granada. Granada, Spain). Wild-type EPS composition was also found in the compact strains ISM105 and ISM106 and in the Exo^c mutants ISM101, ISM102 and ISM104. Thus all these strains seem to synthesize abnormal amounts of wild-type EPS. However, this conclusion only refers to overall EPS composition, leaving open the possibility that their extracellular polysaccharides may exhibit structural abnormalities. Altered EPS's lacking fructose were found in two Exo^c mutants (JC4004 and ISM103). Thus the term «constitutive», albeit suitable to describe the phenotype of overproducers of wild-type EPS, may be somewhat simplistic when it is applied to strains such as JC4004 and ISM103. Genetic analysis to determine whether a single mutation can modify both the composition of EPS and its level of production has not been carried out.

2. *UV sensitivity.* UV sensitivity was assayed on the basis of the following speculation: in enteric bacteria, the isolation of hypermucoid mutants that are UV^s usually allows the isolation of Lon^- mutants (7). The study of *lon* mutations in Rhizobia would be exceedingly interesting, since they might hypothetically affect bacteroid differentiation, a process where permanent arrest of cell division occurs. Unfortunately, all the Exo^- mutants described in this paper showed wild-type levels of UV sensitivity, indicating that none of the strains studied harbored a *lon*-like mutation.

3. *Phage sensitivity.* Although EPS's are not the usual receptors for phage adsorption (5), there is one precedent relating phage sensitivity to the presence of a *R. meliloti* plasmid that affects EPS synthesis (8). Hence we analyzed the patterns of phage sensitivity in all the mutants. The results can be summarized as follows: (I) All the Exo^- and the Exo^s mutants exhibited a wild-type pattern of phage sensitivity, suggesting that EPS does not contain receptors for bacteriophages DF2, AL1, LO0, F2D and FAR. (II) With one exception, wild-type patterns of phage sensitivity were also found among Exo^c mutants, indicating that overproduction of EPS does not result in the occlusion of bacteriophage receptors. The exception was JC4004 which was resistant to bacteriophage DF2. However hypermucoid isolates were not found among 16 independent, spontaneous DF2-resistant mutants, suggesting that in strain JC4004 the Exo^c phenotype and the resistance to phage DF2 are unrelated. For practical purposes, lack of correlation between phage sensitivity and EPS synthesis hampers the design of straightforward methods for the isolation and handling of EPS mutants.

4. *Resistance to desiccation.* Survival in desiccation experiments is shown in Figure 1. All the Exo^c strains showed increased resistance to desiccation when compared to the wild-type strain and to Exo^- and Exo^s mutants. This result may suggest that rhizobial EPS's behave as a protective barrier against desiccation, as described for other soil bacteria (see ref. 10 for a review). However, the rought mutants ISM107 and ISM110 proved to be more resistant to desiccation that the wild-type (but less than the Exo^c strains), indicating that factors other than the rate of EPS synthesis may be involved in desiccation protection. Anyway, Exo^c mutants showed the highest survival rates, thereby suggesting that EPS's can indeed play a role as a dehydration barrier. This idea is supported by the observation that the same Exo^c mutants show increased survival in both sterile and non-sterile peat inoculants, compared to the wild type strain (data not shown).

5. *Motility.* All the mutans were motile (for further details about motility, see the section «Complementation with cloned *exo* genes»).

Symbiotic behavior of EPS mutants

1. *Nodulating ability.* All the mutants (Exo^-, Exo^c and Exo^s) were able to induce nodule formation on *Medicago sativa* cv. Aragón. This result suggests that EPS's can be altered in a number of ways with no effect on the nodulating ability of the mutant. It does not mean, however, that EPS is not essential for infection; it is still possible that only a «critical» fraction of EPS is required. If such is the case, none of the mutants described here was affected in the critical fraction.

2. *Infectivity degree and competitiveness.* Using the test for infectivity degree of Olivares et al. (18) and the assay for competitiveness of Amarger (2), we made quantitative estimations of the infecting ability of the mutants. Results obtained by both methods were largely coincident: the Exo^- strains ISM107, ISM108, ISM109 and ISM110 and the compact strains ISM105 and ISM106 proved to be less infective and less competitive than the parental strain. These data, summarized in Table 2, clearly support the idea that EPS is involved in the infection process. The importance of EPS is confirmed by a second observation: all the Exo^c mutants were more infective and more competitive than the Exo^- and the Exo^s mutants, seemingly fitting in the commonplace logic that overabundance is better tolerated than scarcity. However, the results obtained for Exo^c mutants were less uniform: for instance, ISM102 is quite similar to the wildtype strain in both infectivity degree and

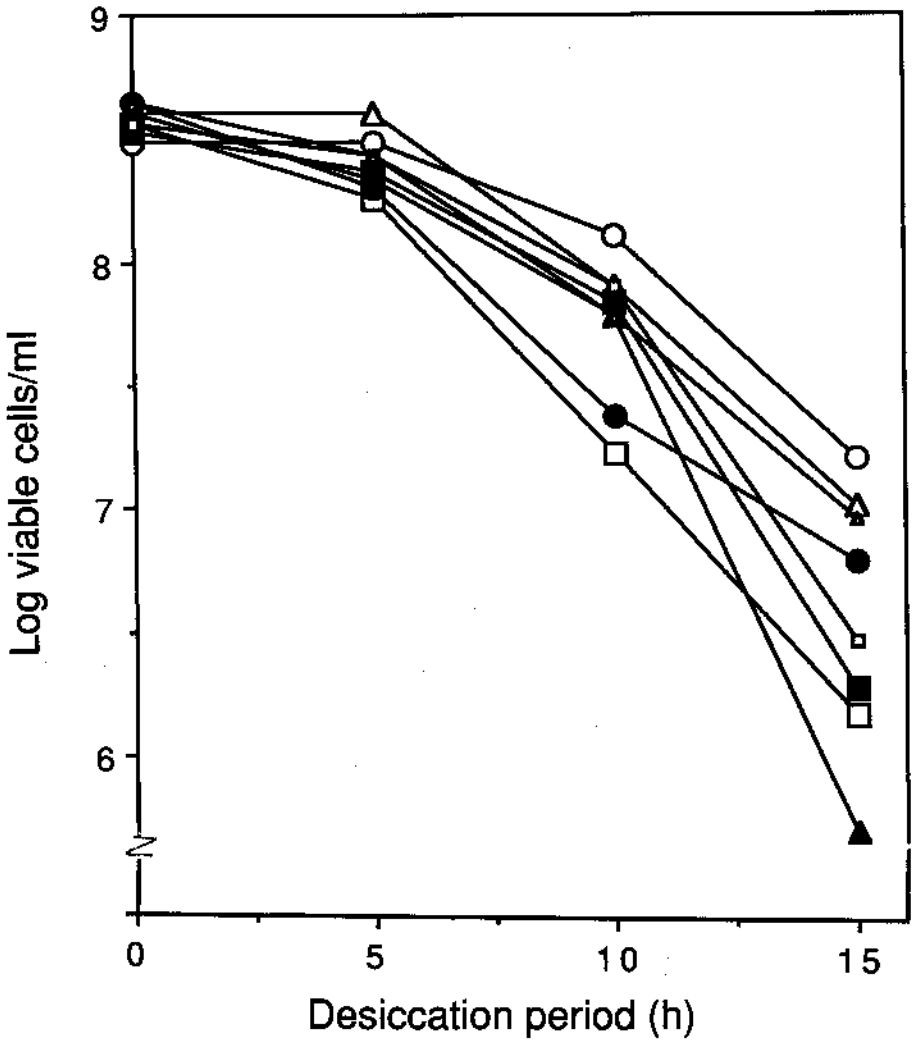

Fig. 1. Survival to desiccation of wild-type strain GR4 and several EPS mutants. Symbols are as follows: GR4 (□), JC4004 (△), ISM102 (○), ISM107 (■), ISM109 (▲), ISM110 (●), ISM105(▫) and ISM106 (▵). Data are mean of three replicates.

competitiveness, while ISM103 and JC4004 are more infective but less competitive than the wild-type. ISM101 shows poor infectivity degree and ISM104 has low competitiveness. Given such variations, general conclusions on the effect of *exo*^{c} mutations on infectivity degree and competitiveness can hardly be reached.

3. *Nitrogen fixation.* Two strains, ISM107 (Exo^{-}) and ISM101 (Exo^{c}) proved to be Fix^{-}; the remaining mutants were fully efficient for nitrogen fixation in alfalfa nodules. Both ISM101 and ISM107 had megaplasmid profiles identical to wild-type (data not shown). Thus they probably carry either chromosomal mutations or plasmidborne point mutations or small deletions. The usual criterion to determine whether a single mutation is responsible for two or more phenotypic traits (the study of revertants) could not be applied to this case, because of the difficulty to score reversion. Complementation analysis was hampered by loss of nodulating ability (see below).

TABLE 2
INFECTIVITY DEGREE AND COMPETITIVE ABILITY OF EXOPOLYSACCHARIDE MUTANTS

Strain	Relevant characteristics	Infectivity degree[a]	Competitiveness[b]
GR4	wild-type	38	79
ISM107	Exo$^-$	27	ND[c]
ISM108	Exo$^-$	26	13
ISM109	Exo$^-$	34	29
ISM110	Exo$^-$	30	25
ISM105	Exo^s	36	75
ISM106	Exo^s	8	20
ISM101	Exo^c	9	ND
ISM102	Exo^c	37	76
ISM103	Exo^c	51	60
ISM104	Exo^c	38	40
JC4004	Exo^c	42	70

[a] Calculated as described in reference 18.
[b] Calculated as described in reference 2.
[c] Not determined.

Complementation analysis with cloned exo genes

Derivatives of the exo mutants ISM101, ISM103, ISM106, ISM107 and ISM110 carrying plasmids pD2, pD5, pD15 and pD34 were examined for colonial morphology, calcofluor staining and nitrogen fixation. A summary of complementation results, including only the cases where complementation was oberved, is shown in Table 3. With one exception, mutants were complemented by more than one plasmid. This fact hampers the classification of mutations into known types. For instance, ISM103 is complemented by both *exo*B and *exo*D clones and ISM110 is complemented by *exo*A, *exo*B and *exo*D. The exception is strain ISM106 which is only complemented by *exo*D. A case of partial complementation is also observed: partial recovery of EPS production level is observed in strain ISM107 upon introduction of plasmids pD2 and pD5. A summary of complementation results is shown in Table 3. The most relevant data are as follows:

1. *Colonial morphology, calcofluor staining and EPS production.* Complementation clearly restored the wild-type colonial morphology in the rough mutants ISM106 (by pD5) and ISM110 (by pD2, pD5 and pD34) and, to some extent, in the compact mutant ISM107 (by pD2 and pD5). Complementation of staining characteristics could not be scored in strain ISM101 since this strain shows wild-type staining. However, plasmids pD34, pD2, pD5 and pD15 were introduced in this strain in an attempt to complement the Fix$^-$ phenotype (see below). Introduction of these plasmids did not alter colonial or staining phenotypes of ISM101. Likewise, derivatives of ISM103 containing either plasmid pD2 or pD5 were hypermucoid like the parental strain; in addition, complementation of the staining phenotype was observed. Complementation of colonial and staining phenotypes was always accompanied by an increase in the production of EPS (sometimes above wild-type level: see Table 1).

2. *Motility.* Although all the mutants were motile, introduction of plasmids carrying cloned genes from *R. meliloti* caused a marked, sometimes spectacular increase in motility. For instance, complementation of ISM106 with plasmid pD5 caused spreading of the strain all over the plate in 48 h, while at the same time the plasmid-free parental strain reached only an area of 19 mm diameter. This effect was observed not only in the mutants but also in the wild-type strain and was most

TABLE 3
COMPLEMENTATION OF EPS MUTANTS WITH CLONED *R. MELILOTI EXO GENES*

Strain	Calcofluor staining	Complementing plasmids[a]	Calcofluor staining	Phenotype of merodiploids EPS production (% respect to wt)
ISM103	Dull	pD2, pD5	Bright, haloed	100-130
ISM106	Dull	pD5	Bright, haloed	90-110
ISM107	Dark	pD2, pD5	Bright	50-80
ISM110	Dull	pD2, pD5, pD34	Bright, haloed	100-130

[a] Only complementation-proficient plasmids are listed.

significant with plasmid pD5, although it could also be observed with pD2, pD15 and pD34 (but not with cosmid pLAFR1). The relationship between introduction of cloned *exo* genes (mostly *exo*B) and motility is first described here; its molecular basis is unknown.

3. *Nitrogen fixation.* Complementation of strains ISM101 and ISM107 was intended to examine whether any of the cloned *exo* genes would complement the Fix$^-$ phenotype (in other words, whether any of these EPS mutants was similar to those described in ref. 16). Complementation of ISM107 by pD2 and pD5 was easily seen for colonial phenotype, calcofluor staining and EPS production (see Table 3). However, when ISM107 derivatives carrying pD2 or pD5 and ISM101 derivatives carrying pD34, pD2, pD5 or pD15 were used to infect *Medicago sativa* plants, they were found to be nodulation-deficient, thus hampering the analysis of Fix complementation. Further experiments showed: (I) that loss of nodulation ability occurs not only in the mutants but also in the parental strain GR4; (II) that this loss is caused not only by plasmids carrying cloned *exo genes,* but also by the cosmid vector pLAFR1; (III) that nodulation ability is not regained by clones having spontaneously lost the plasmid. These data suggest that introduction of pLAFR1 derivatives in strain GR4 may cause a genomic rearrangement (perhaps a deletion or curing of an endogenous plasmid) resulting in the irreversible loss of nodulating ability. This phenomenon is certainly intriguing since pLAFR1 was designed as a vector for library construction in *R. meliloti.* Hence, crucial strain differences may exist. However, it is interesting to point out that a plasmid closely related to pLAFR1, pRK290, has proved useful for library construction in *R. meliloti* GR4 (23). Thus pLAFR1 —associated loss of nodulating ability could be attributed to specific experimental procedures employed in this study. Any insight on the causes of the phenomenon will requiere further studies.

Discussion

Rhizobium meliloti mutants affected in EPS production are easily isolated after chemical mutagenesis. Odd colony shapes or colors often pinpoint EPS mutants. Many (but not all) the mutants also show altered calcofluor staining. Correlations between EPS production and UV sensitivity or susceptibility to bacteriophage infection (which might facilitate isolation and handling of EPS mutants) were not found.

All mutants studied (Exo$^-$, Exo^c and Ex^s) were able to nodulate, suggesting that the extracellular polysaccharide of *R. meliloti* can be altered in a number of ways without causing loss of nodulating ability. However, it is still possible that a «critical» fraction of EPS, intact in these mutants, is absolutely requiered for nodulation (22). However, this paper also shows that wild-types EPS is necessary for optimal infection, as previously suggested (19): all rough strains showed lowered degrees

of both infectivity and competitiveness, not only when compared to the wild-type strain but also to hypermucoid mutants.

Overproduction of EPS can slightly decrease infectivity degree, competitiveness or both. However, hypermucoid strains with infectivity degree and competitiveness similar to the wild-type are also found. These might be of practical interest because of their increased resistance to desiccation and their improved survival in peat inoculants (two traits that might be related). However, data on the behavior of Exo^c mutants in field trials are not yet available.

Genetic analysis of *exo* mutants turned out to be difficult because of (I) the lack of a selection procedure to score reversion and (II) the unexpected loss of nodulating ability of strains carrying pLAFR1-derived plasmids. However, complementation of colonial morphology, calcofluor staining and EPS production by cloned genes was observed in a number of cases. A spectacular increase in motility was also observed, mainly in strains carrying a cloned *exoB* gene. It is interesting to point out that complementation by more than one gene was often found, suggesting that alternative pathways of EPS synthesis may exist in *R. meliloti*. If such is the case and suppression of certain mutations by overexpression of other genes occurs, the system may be extremely complex. This might be a reasonable explanation for the variety of phenotypes found.

References

1. Abe, M., Sherwood, J. E., Hollingsworth, R. I. and Dazzo, F. B. (1984). Stimulation of clover root hair infection by lectin-binding oligosaccharides from the capsular and extracellular polysaccharides of *Rhizobium trifolii*. J. Bacteriol. **60**, 517-520.
2. Amarger, N. (1981). Selection of *Rhizobium* strains on their competitive ability for nodulation. Soil Biol. Biochem. **13**, 481-487.
3. Beringer, J. E. (1974). R-factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**, 178-189.
4. Borthakur, D. Downie, J. A., Johnston, A. W. B. and Lamb, J. W. (1985). *psi*, a plasmid-linked *Rhizobium phaseoli* gene that inhibits exopolysaccharide production and which is required for symbiotic nitrogen fixation. Mol. Gen. Genet. **200**, 278-282.
5. Calendar, R., editor. (1988). The bacteriophages. Plenum Press, New York and London.
6. Casadesús, J. and Olivares, J. (1979). Rough and fine linkage mapping of the *Rhizobium meliloti* chromosome. Mol. Gen. Genet. **174**, 203-209.
7. Chung, C. H. and Goldberg, A. L. (1981). The product of the *lon (capR)* gene in *Escherichia coli* is the ATP-dependent protease, protease La. Proc. Natl. Acad. Sci. USA **78**, 4931-4935.
8. Corral, E., Montoya, E. and Olivares. J. (1978). Sensitivity to phages in *Rhizobium meliloti* as a plasmid consequence. Microbios Lett. **5**, 77-80.
9. Dazzo, F. B. and Hubbell, D. H. (1975). Cross-reactive antigens and lectin as determinants of symbiotic specificity in the *Rhizobium*-clover association. Appl. Microbiol. **30**, 1017-1033.
10. Dudman, W. F. (1977). The role of surface polysaccharides in natural environments. *In:* I. W. Sutherland (ed.) Surface carbohydrates of the prokaryotic cell, pp. 357-414. Academis Press, New York.
11. Figurski, D. H. and Helinski, D. R. (1979). Replications of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76**, 1648-1652.
12. Finan, T. M., Kunkel, B, de Vos, G. F. and Signer, E. R. (1986). Second symbiotic plasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167**, 66-72.
13. Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J. and Ausubel, F. M. (1982). Construction of a broad range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. Gene **18**, 289-296.
14. Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**, 557-580.
15. Hynes, M. F., Simon, R. and Pühler, A. (1985). The development of plasmid-free strains of *Agrobacterium tumefaciens* by using incompatibility with *Rhizobium meliloti* plasmid to eliminate pAtC48. Plasmid **13**, 99-105.
16. Leigh, J. A., Signer, E. R. and Walker, G. C. (1985). Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. USA **82**, 6231-6235.
17. Müller, P., Hynes, M., Kapp, D., Niehaus, K. and Pühler, A. (1988). Two classes of *Rhizobium meliloti* infection mutants differ in exopolysaccharide production and coinoculation properties with nodulation mutants. Mol. Gen. Genet. **211**, 17-26.
18. Olivares, J. Casadesús, J. and Bedmar, E. J. (1980). Method for testing degree of infectivity of *Rhizobium meliloti* strains. Appl. Environ. Microbiol. **39**, 967-970.

19. Olivares, J., Bedmar, E. and Martínez-Molina. E. (1984). Infectivity of *Rhizobium meliloti* as affected by extracellular polysaccharides. J. Appl. Bacteriol. **56**, 389-393.
20. Planqué, K. and Kijne, J. W. (1977). Binding of pea lectins to a glycan type polysaccharide in cell walls of *Rhizobium leguminosarum*. FEBS Lett. **73**, 64-66.
21. Rigaud, J. and Puppo, A. (1975) Indole-3-acetic acid catabolism by soybean bacteroides. J. Gen. Microbiol. **88**, 223-228.
22. Sanders, R. E., Raleigh, E. and Signer, E. (1981). Lack of correlation between extracellular polysaccharide and nodulating ability in *Rhizobium*. Nature **292**, 148-149.
23. Toro, N. and Olivares, J. (1986). Characterization of a large plasmid of *Rhizobium meliloti* involved in enhancing nodulation. Mol. Gen. Genet. **202**, 331-335.
24. Vincent, J. M. (1970). A manual for the practical study of the root nodule bacteria. Blackwell, Oxford.

MICROBIOLOGIA SEM 7 (1991), 23-34

Serotipos de *Salmonella enterica* en aguas residuales de Zaragoza. Comparación con aislamientos clínicos. 1982-1989

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(Recibido septiembre 7/diciembre 3, 1990)

Summary

Between 1982 and 1989, 560 *Salmonella enterica* strains belonging to 63 serovars, were isolated from Zaragoza urban sewage. During the same period of time there were 45 different serovars isolated from faeces of patients from a hospital (in the same city). Only a reduced number of serovars comprised the majority of the human and environmental isolates. An approximately rectilinear relationship is shown between the isolated strains number and the number of different serovars, in non human *Salmonella* strains. In clinical isolates, we have not found this relation. We have compared the local results with those reported in other different Spanish regions.

Key words: Salmonella *serotypes, sewage, clinical isolates.*

Resumen

Entre 1982 y 1989 se aislaron 560 cepas de *Salmonella entérica* a partir de aguas residuales de la ciudad de Zaragoza, identificando 63 serovariedades. En el mismo período se aislaron 45 serovariedades diferentes en heces de pacientes asistidos en un hospital de la misma ciudad. Un número reducido de serovariedades incluye la mayoría de los aislamientos humanos y medioambientales. Encontramos una relación directamente proporcional entre el número de cepas aisladas y serovariedades identificadas en cepas de *Salmonella* de origen no humano. Para los aislamientos clínicos esta relación no es proporcional. Los resultados locales se comparan con los publicados para cepas que proceden de diferentes zonas de España.

Introducción

El género *Salmonella* consta de una sola especie, *S. entérica* (12), lo que hace indispensable la utilización de marcadores intraespecíficos para la realización de estudios epidemiológicos. Desde la instauración del esquema de serotipado por White en 1926, ampliado y perfeccionado por Kauff-

(*) A quien debe dirigirse la correspondencia.

TABLA 1
SEROTIPOS DE *SALMONELLA* SPP. EVOLUCION EN AGUAS RESIDUALES. ZARAGOZA, 1982-1989

	82	83	84	85	86	87	88	89	Total	
									N.°	%
SALMONELLA:										
Ohio	1	5	8	21	9	7	12	2	65	11,6
Enteritidis		3	2	6	12	2	9	13	47	8,4
Infantis	2	4	10	7	11	3	6		43	7,7
Panama			1	14	10	2	13	2	42	7,5
Typhimurium		2	2	12	3	3	13	3	38	6,8
Virchow			1	12	5	2	6	9	35	6,3
Bredeney				8	14		6		28	5,0
Muenchen			1	4	3	1	10	2	21	3,8
Agona	1			2	4	3	3		13	2,3
Paratyphi B.				8	5				13	2,3
Derby	1	1	1	3	1	1	3	1	12	2,1
Newington	2	1	6	2			1		12	2,1
Newport			1	1	5	3	2		12	2,1
Goldcoast	2		1	3	1	1	2	2	12	2,1
Brandenburg				1	1	1	3	5	11	2,0
Tilburg		1		3	1	3	2		10	1,8
London					1		4	3	8	1,4
Blockley		1	1	4			1	1	8	1,4
Blegdam					8				8	1,4
Hadar			1			1		5	7	1,3
Bovismorbf.			1	1	5				7	1,3
Anatum						1	1	5	7	1,3
Mikawasima				6					6	1,1
Mbandaka			2	1	1			1	5	0,9
Montevideo				1	1		2	1	5	0,9
Newbrunswick		1		1				2	4	0,7
Senftemberg					2		1	1	4	0,7
Give							3		3	0,5
Stanley					2			1	3	0,5
Javiana		2	1						3	0,5
Otras*	2	0	7	11	6	2	5	7	40	7,1
No tipables	1	2	1	4	14	2	2	2	28	5,0
TOTAL	12	23	48	135	126	38	110	68	560	100
N.° de serovars diferentes	8	10	22	30	29	17	26	24	63	

* Figuran con 2 cepas en el período de estudio: *dessau, drypool, fyris, salinatis, heidelberg, tennessee, tournay.* Y con 1 cepa: *limete, mendoza, bournemouth, duesseldorf, miyazaki, essen, nchanga, bareilly, bradford, norton, preston, hartfort, sekondi, tananarive, binza, indiana, teddington, thompson, togo, bukuru, irumu, tokoin, adabraka, amager, typhi, uppsala.*

TABLA 2
COMPARACION DE PORCENTAJES DE SEROTIPOS DE *SALMONELLA SPP.* EN AGUAS RESIDUALES Y EN AISLAMIENTOS HUMANOS. ZARAGOZA, 1982-1989

	Aguas residuales (IMSP)	Muestras de heces (HCU)
N.° total de cepas estudiadas	560	1.472
N.° de serovariedades diferentes	64	45
SALMONELLA:	%	%
*Ohio**	11,61	2,31
*Enteritidis**	8,39	59,31
*Infantis**	7,68	2,65
*Panama**	7,50	1,29
*Typhimurium**	6,79	16,51
*Virchow**	6,25	1,97
*Bredeney**	5,00	0,41
*Muenchen**	3,75	0,54
*Agona**	2,32	0,27
*Paratyphi B**	2,32	0,29
*Derby**	2,14	0,14
*Newport**	2,14	1,15
*Goldcoast**	2,14	0,34
*Newington**	2,14	0,14
Otras**	32,15	12,97

* Diferencias de frecuencia significativas entre las 2 series ($p < 0,01$).
** Otras serovariedades presentes en < 2 % en aguas residuales.
HCU: Hospital Clínico Universitario.
IMSP: Instituto Municipal de Salud Pública.

man en 1972, esta técnica ha sido la herramienta epidemiológica más utilizada, ya que permite la diferenciación de más de 2.000 tipos o serovariedades distintas. La necesidad de establecer diferencias que permitan la identificación de clonos dentro de los serotipos más frecuentes ha potenciado el estudio de otros marcadores, como biotipo, fagotipo, colicinotipo, perfil de resistencia a antimicrobianos y más recientemente técnicas de biología molecular que permiten el estudio del ADN plasmídico y cromosómico (20, 22).

La virulencia y el rango de huéspedes de diferentes serotipos presenta variaciones. Así, algunos están muy adaptados a su huésped y dotados de determinantes específicos de patogenicidad, como en el caso de *Salmonella typhi.* Sin embargo, la mayoría tienen una considerable ubicuidad y se pueden encontrar en una gran variedad de especies animales y en muestras medioambientales, donde pueden presentar un alto grado de supervivencia. Debido a esta capacidad para mantenerse, e incluso en algunos casos multiplicarse en ambientes naturales, la vehiculación de salmonelas por aguas residuales no depuradas podría desempeñar un papel importante en el mantenimiento de los reservorios naturales y, por tanto, en los ciclos biológicos de estos microorganismos (10, 15, 18). En cuanto a la importancia del medio hídrico en la cadena epidemiológica de la salmonelosis humana, hay que tener presente que, al menos en países en desarrollo, los abastecimientos de agua no están sometidos con la deseable frecuencia a procesos de higienización.

TABLA 3
COMPARACION DE DISTRIBUCION SEROTIPOS DE *SALMONELLA SPP.* AISLAMIENTOS HUMANOS DE ZARAGOZA (HCU) Y DEL CNMVIS 1983-1988

	HCU	CNMVIS
N.° total de cepas estudiadas	1.117	18.830
N.° de serovariedades diferentes	41	72
SALMONELLA:	%	%
Enteritidis	59,98	61,29
*Typhimurium**	15,40	12,03
Ohio	2,59	2,31
Infantis	2,05	1,93
*Virchow**	1,97	4,54
*Typhi**	1,79	3,22
Heidelberg	1,70	1,06
Panama	1,34	0,78
Newport	1,07	0,52
Otras*	12,11	12,32

* Diferencias de frecuencias significativas ($p < 0,01$).
** Figuran sólo las serovariedades con una presencia > 1 % en el HCU.
HCU: Hospital Clínico Universitario.
CNMVIS: Centro Nacional de Microbiología, Virología e Inmunología Sanitarias (3, 7).

La contaminación de los efluentes urbanos por *Salmonella* es bien conocida. En el origen de esta presencia ha de desempeñar un papel importante el reservorio humano, constituido por enfermos agudos o convalecientes y portadores sanos. En este sentido, el número de salmonelas por gramo de heces excretadas por enfermos alcanza cifras de 1×10^{10}, en tanto que los portadores eliminan cantidades inferiores, pero durante más tiempo (10, 15).

El propósito de este estudio fue conocer la presencia, circulación y fluctuaciones de las serovariedades de *Salmonella* en las aguas residuales de la ciudad de Zaragoza y analizar su relación con las cepas aisladas en coprocultivos de enfermos asistidos en un hospital general de la misma ciudad (HCU), durante un período amplio de tiempo (8 años). También hemos comparado nuestros resultados con los publicados por el Centro Nacional de Microbiología, Virología e Inmunología Sanitarias (CNMVIS) del Instituto de Salud Carlos III (2, 3, 7) para establecer las posibles analogías y analizar las diferencias.

Material y métodos

Durante un período de 8 años (1982-1989) investigamos la presencia de *Salmonella* spp. en aguas residuales de Zaragoza y en las heces de enfermos asistidos en el Hospital Clínico Universitario (HCU) de la misma ciudad. Las cepas de aguas residuales se obtuvieron a partir de muestras de diferentes colectores, clasificados como urbanos, urbano-industriales o rurales, según recogieran preferentemente vertidos domésticos, de polígonos industriales o de barrios rurales, respectivamen-

TABLA 4
COMPARACION DE LA DISTRIBUCION DE SEROTIPOS DE *SALMONELLA SPP.* EN AGUAS RESIDUALES. RESULTADOS IMSP/CNMVIS 1987-1988

	IMSP	CNMVIS
N.° de cepas estudiadas	148	224
SALMONELLA:	%	%
Ohio	12,84	7,14
*Panama**	10,13	3,57
Enteritidis	7,43	9,82
Muenchen	7,43	3,12
Infantis	6,08	1,78
Virchow	5,40	9,37
Bredeney	4,05	8,03
Tilburg	3,38	7,14
London	2,70	4,46
Brandenburg	2,70	2,67
Give	2,03	3,57
Goldcoast	2,03	2,23
Anatum	1,35	4,46
Montevideo	1,35	2,67
Thyphimurium	1,08	5,36
Blockley	0,68	2,67
Otras*	29,34	21,94

* Diferencias de frecuencias significativas ($p < 0,01$).

** Sólo figuran los serotipos de los que se han publicado datos del Centro Nacional.

IMSP: Instituto Municipal de Salud Pública.
CNMVIS: Centro Nacional de Microbiología, Virología e Inmunología Sanitarias (2, 3).

te. Se contabilizó una sola cepa por muestra de agua cuando se obtuvieron aislamientos repetidos de la misma serovariedad. De modo similar se han excluido los aislamientos repetidos de un mismo enfermo, de modo que cada cepa representa un paciente diferente.

Para el aislamiento e identificación bioquímica de las cepas se han utilizado las técnicas habituales (1, 21, 22), si bien en el curso del estudio se han introducido algunas modificaciones en la metodología, en función de la experiencia adquirida (11, 16) y de la incorporación de mejoras recomendadas en la literatura (4, 15, 19).

El serotipado se realizó por medio de aglutinación en porta para la identificación de los antígenos somáticos y por aglutinación en tubo y/o microaglutinación en porta para el estudio de los antígenos flagelares. Cuando fue necesario se realizó la inversión de fase por el método de Sven Gard. Se utilizaron antisueros comerciales de Difco y del Instituto Pasteur.

Se ha realizado la prueba estadística de chi cuadrado, en la comparación de frecuencias de serovariedades, aplicando la corrección de Yates, cuando fue necesario por el tamaño de la muestra, y el análisis de correlación entre número de cepas tipables y número de serovariedades diferentes, utilizando el programa estadístico Microsta de Microsoft.

TABLA 5
DISTRIBUCION DE CEPAS DE *SALMONELLA* SPP. EN AGUAS RESIDUALES, SEGUN SEROGRUPO «0». COMPARACION DE RESULTADOS ZARAGOZA/SORIA

	Zaragoza (IMSP) 1982-1989	Soria (CU) 1985
N.° total de cepas estudiadas	560	291
	%	%
Serogrupo B	23,7	25,5
Serogrupo C	42,1	47,4
Serogrupo D	18,6	16,8
Serogrupo E*	10,54	4,5
Serogrupo I*	0	6,9

* Diferencias de frecuencias significativas ($p < 0,01$).
IMSP: Instituto Municipal de Salud Pública.
CU: Colegio Universitario (5).

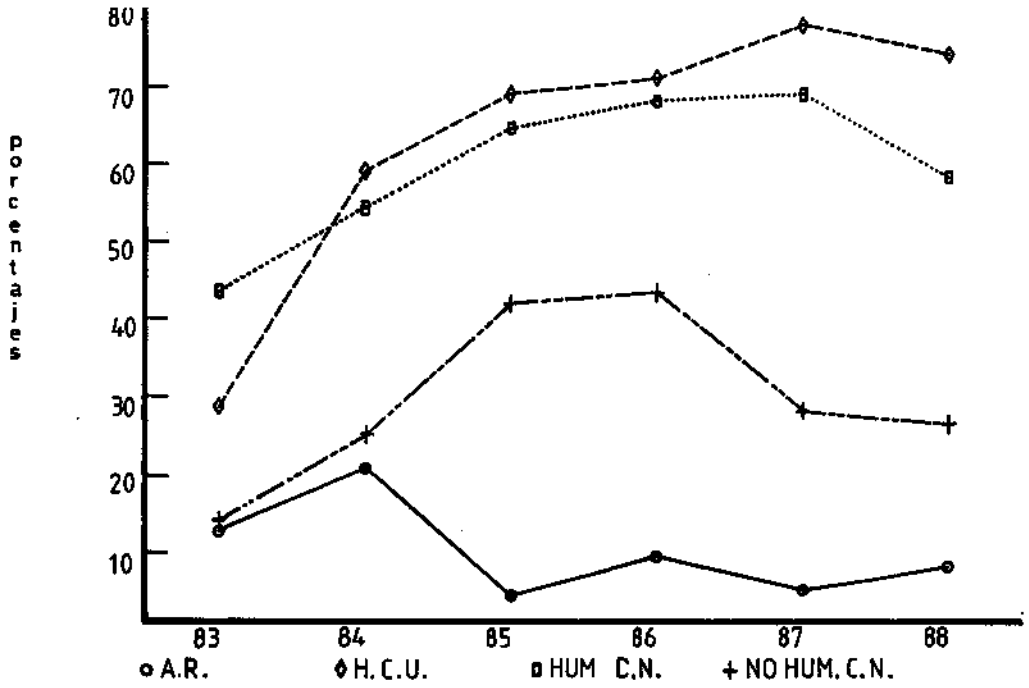

Fig. 1. Evolución de *S. enteritidis*, 1983-1988. Comparación de cepas de diferentes orígenes.

AR: Cepas aisladas a partir de aguas residuales de Zaragoza.
HCU: Cepas aisladas a partir de pacientes de un hospital de Zaragoza.
HUM CN: Cepas de origen humano tipadas en el Centro Nacional.
NO HUM CN: Cepas de origen no humano tipadas en el Centro Nacional.

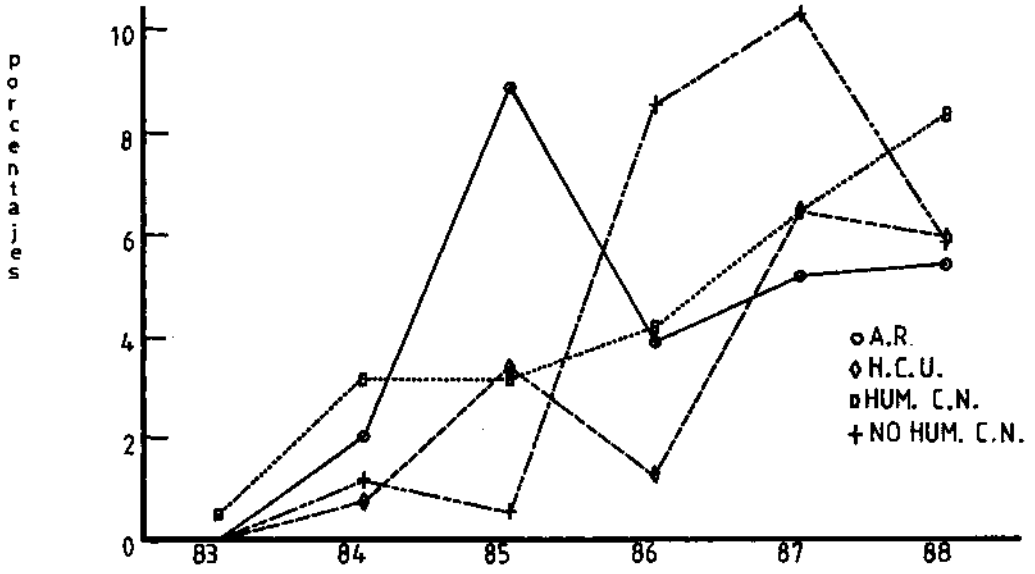

Fig. 2. Evolución de *S. typhimurium*, 1983-1988. Comparación de cepas de diferentes orígenes.

AR: Cepas aisladas a partir de aguas residuales de Zaragoza.
HCU: Cepas aisladas a partir de pacientes de un hospital de Zaragoza.
HUM CN: Cepas de origen humano tipadas en el Centro Nacional.
NO HUM CN: Cepas de origen no humano tipadas en el Centro Nacional.

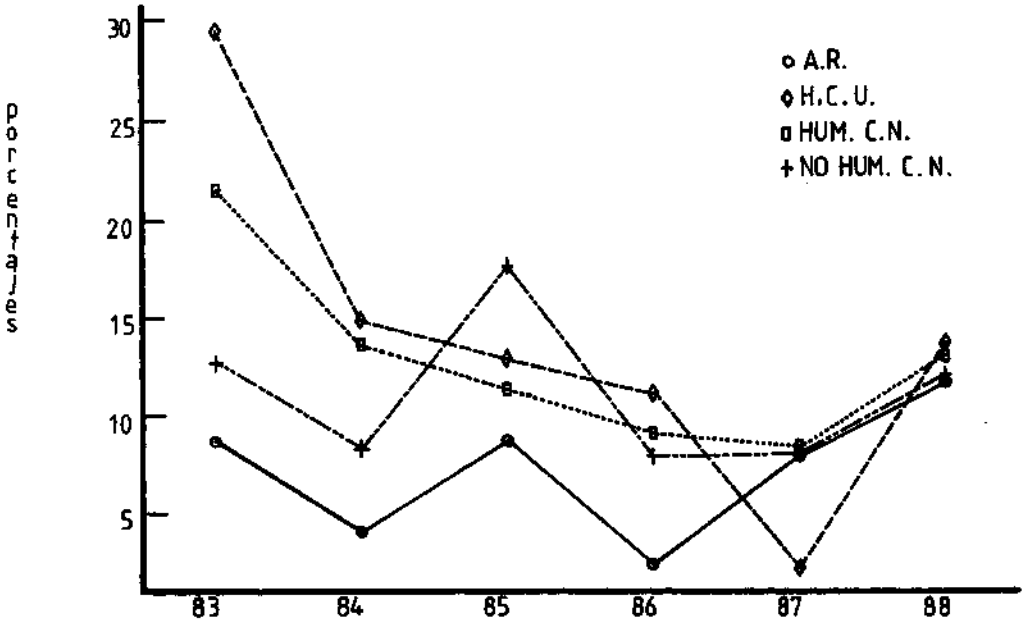

Fig. 3. Evolución de *S. virchow*, 1983-1988. Comparación de cepas de diferentes orígenes.

AR: Cepas aisladas a partir de aguas residuales de Zaragoza.
HCU: Cepas aisladas a partir de pacientes de un hospital de Zaragoza.
HUM CN: Cepas de origen humano tipadas en el Centro Nacional.
NO HUM CN: Cepas de origen no humano tipadas en el Centro Nacional.

TABLA 6
DISTRIBUCION TRIMESTRAL DE ALGUNOS SEROTIPOS DE *SALMONELLA* SPP. EN AGUAS RESIDUALES

	Primer trimestre		Segundo trimestre		Tercer trimestre		Cuarto trimestre	
	N.° (+)	N.° (-)	N.° (+)	N.° (-)	N.° (+)	N.° (-)	N.° (+)	N.° (-)
SALMONELLA:								
Ohio	21	122	11	121	11	113	23	138
*Enteritidis***	11	132	8	124	12	112	20	141
Infantis	14	129	12	120	6	118	11	150
Panama	9	134	12	120	10	114	11	150
*Typhimurium***	4	139	9	123	9	115	16	145
*Virchow**	8	135	3	129	16	118	8	153

* Frecuencias significativas mayores en el tercer trimestre.
** Frecuencias significativas mayores en el cuarto trimestre.

Resultados

Entre 1982 y 1989 se han aislado 560 cepas de *Salmonella enterica* a partir de aguas residuales de la ciudad de Zaragoza. Identificamos 63 serovariedades, siendo las más frecuentes: *S. ohio, S. enteritidis, S. infantis, S. panama, S. typhimurium, S. virchow, S. bredeney* y *S. muenchen.* La distribución por serotipos y años de los aislamientos aparece recogida en la Tabla 1. El número de serovariedades diferentes encontradas cada año está relacionado con el número de cepas identificadas, y osciló entre 8 y 30.

En la Tabla 2 se comparan los porcentajes de presentación de las serovariedades de *Salmonella* aisladas con mayor frecuencia en aguas residuales, con los valores obtenidos para las mismas en pacientes. Todas las serovariedades aparecen representadas en las 2 muestras, si bien su incidencia porcentual es marcadamente diferente, de modo que prácticamente todas muestran diferencias con una probabilidad estadística superior al 99 %.

El número de serovariedades encontradas exclusivamente en aguas residuales fue de 32, con una representación porcentual del 10,71 % de todas las cepas medioambientales. El número de serovariedades recuperadas en aislamientos clínicos y no identificadas en los vertidos de la ciudad fue de 24, aunque en este caso su aportación al conjunto de los casos clínicos fue muy escasa, ya que sólo se identificaron en el 2,31 % de los pacientes.

En la Tabla 3 se establece la relación entre nuestros aislamientos clínicos y las cepas de origen humano tipadas en el CNMVIS entre 1983 y 1988. En esta relación sólo se han incluido los serotipos que en aquel período tuvieron una presencia superior al 1 % en el HCU. Hemos encontrado diferencias significativas en las frecuencias comparadas para 3 de las serovariedades: *S. typhimurium, S. virchow* y *S. typhi.*

En la Tabla 4 se comparan los resultados de aguas residuales de Zaragoza con los publicados para cepas del mismo origen por el CNMVIS (2, 3). Sólo hemos incluido los 2 años (1987-1988) para los que el CNMVIS proporciona datos pormenorizados según el origen de la muestra. Todos los serotipos relacionados en la muestra del CNMVIS están representados en las aguas residuales de nuestra ciudad y tan sólo en el caso de *S. panama* la diferencia porcentual entre ambos estudios alcanza significación estadística.

La Tabla 5 presenta la distribución por serogrupos de las cepas procedentes de aguas residua-

TABLA 7
EVOLUCION EN EL NUMERO DE SEROTIPOS DE *SALMONELLA*, 1982-1989
(COMPARACION EN CUATRO ESTUDIOS)

Años	1982	1983	1984	1985	1986	1987	1988	1989
IMSP								
— N.° de tipos distintos	8	10	22	30	29	17	26	24
— $\bar{X}$ de cepas que corresponden a cada tipo	1,50	2,30	2,18	4,50	4,34	2,23	4,23	2,83
HCU								
— N.° de tipos distintos	17	16	16	15	16	14	12	12
— $\bar{X}$ de cepas que corresponden a cada tipo	7,12	11,94	7,94	7,73	9,5	13,21	19,5	6,09
CN (humanos)								
— N.° de tipos distintos	–	43	35	47	50	40	48	–
— $\bar{X}$ de cepas que corresponden a cada tipo	–	54,48	72,8	66,51	75,8	108,2	46,39	–
CN (no humanos)								
— N.° de tipos distintos	–	23	16	21	38	40	47	–
— $\bar{X}$ de cepas que corresponden a cada tipo	–	7,48	5,25	8,09	10,42	16,42	17,06	–

IMSP: Instituto Municipal de Salud Pública. Zaragoza.
HCU: Hospital Clínico Universitario.
CN (humano): Cepas de *Salmonella* de origen humano tipadas en el Centro Nacional de Microbiología, Virología e Inmunología Sanitarias (2, 7).
CN (no humano): Cepas de origen no humano tipadas en el Centro Nacional (2, 7).

les. Como puede observarse, las cepas se distribuyeron en 4 serogrupos, siendo prevalente el serogrupo C (42,1 %). En la misma Tabla figuran los resultados obtenidos en muestras del mismo origen de la ciudad de Soria (5). En esta población, próxima a la nuestra, se aíslan cepas de 5 serogrupos, si bien los 3 más frecuentes coinciden con los encontrados en Zaragoza.

Para estudiar comparativamente la tendencia evolutiva en cuanto a frecuencias de las serovariedades más importantes, hemos elaborado los gráficos 1, 2 y 3. Se ha abarcado el período 1983-1988 para el que disponemos de datos de las 4 series comparadas: cepas de aguas residuales y heces de enfermos de Zaragoza y cepas tipadas en el CNMVIS de origen humano y no humano (3, 7).

No hemos encontrado diferencias estacionales en la presencia en aguas residuales de las serovariedades consideradas globalmente. Unicamente cuando comparamos las serovariedades con mayor representación clínica y agrupando los resultados por trimestres, encontramos una presencia significativamente mayor de *S. virchow* en el tercer trimestre y de *S. enteritidis* y *S. typhimurium* en el cuarto (Tabla 6).

Discusión

Al igual que otros autores que han comparado aislamientos de origen clínico y medioambiental (9, 16, 17), hemos encontrado una desigual distribución de las serovariedades identificadas en personas y aguas residuales, con la presencia de algunas de ellas en una serie y no en la otra. Sin embargo, parece destacable que mientras las 24 serovariedades aisladas exclusivamente en pacien-

tes representan sólo el 2,31 % de los casos clínicos, en aguas residuales hemos encontrado un 10,71 % de cepas que no tienen representación clínica. Pensamos que entre estos aislamientos no humanos se encontrarían cepas procedentes de contaminación por deyecciones de animales urbanos que son recogidos por el alcantarillado, así como salmonelas vehiculadas por vertidos de distintos orígenes (agrícolas, mataderos, animales domésticos y de granjas, etc.). Al intentar comprobar este último punto, separando los serotipos encontrados de acuerdo con los diferentes tipos de colectores estudiados, no hemos encontrado diferencias estadísticamente significativas, si bien hay que tener en cuenta que el número de cepas distribuido por serovariedades y colectores fue pequeño, por lo que este resultado no puede considerarse definitivo.

Las serovariedades más frecuentes en aguas residuales se han aislado también en pacientes de nuestro hospital (Tabla 2), si bien hemos encontrado diferencias notables en sus respectivas frecuencias de presentación, alcanzando significación estadística en casi todas las que contaban con una presencia superior al 1 %. Es posible que a este resultado contribuya la diferente selectividad de los medios de cultivo utilizados en cada tipo de muestra (18). También podría estar relacionado con diferencias en la capacidad de supervivencia de las distintas serovariedades en ambientes naturales. En ellos las salmonelas están sometidas a la acción de bacteriófagos específicos, predadores, cambios en la temperatura y el pH, acción lesiva de la luz solar, fenómenos de absorción y sedimentación, competición por nutrientes y con la flora autóctona, acción de antibióticos e iones metálicos (10, 21). De forma que, como ya se ha demostrado para algunos serotipos (13) y para otros patógenos entéricos (14), los más adaptados al hombre decrecen en número rápidamente, mientras que otros pueden llegar incluso a multiplicarse (15). Por último, y a pesar de ser aguas de origen urbano, la contribución del reservorio humano puede ser menos importante de lo que cabría esperar.

Los resultados locales son bastante equiparables a los publicados para cepas que proceden de diferentes puntos de España. Así, la distribución de cepas de origen humano es semejante en el HCU y en el CNMVIS (3, 7), aunque en los datos de este último, y en relación con el mayor número de cepas y zonas estudiadas, el número de serovariedades diferentes es, lógicamente, mucho mayor (Tabla 3). También son resaltables las diferencias existentes entre las frecuencias que alcanzan significación estadística en serovariedades importantes, como *S. typhimurium* y *S. virchow.* Sin embargo, como puede apreciarse en los gráficos 2 y 3, la tendencia ya informada (6) es similar en ambos estudios, decreciente para *S. typhimurium* y creciente para *S. virchow.* Para valorar adecuadamente estos resultados, y especialmente la mayor frecuencia de *S. typhi* en los resultados nacionales (Tabla 3), hay que tener en cuenta que los mismos incluyen, además de cepas aisladas de heces, cepas de otros orígenes: hemocultivos, urinocultivos, etc.

Hemos comprobado que la distribución de serovariedades en los vertidos domésticos de Zaragoza es bastante similar a la informada en los años 1987 y 1988 a nivel nacional (2, 3); únicamente *S. panama* tiene una frecuencia significativamente mayor en nuestra zona (Tabla 4). Si la comparación se hace a nivel de serogrupos, nuestros resultados son bastantes semejantes a los encontrados por Arribas y cols. (5) en Soria (Tabla 5).

Respecto a la distribución estacional de los aislamientos, no encontramos justificación para la mayor presencia en el cuarto trimestre de *S. enteritidis* y *S. typhimurium* y de *S. virchow* en el tercero.

En relación con el objetivo básico de nuestro estudio, nos parece muy significativo que en tan sólo 23 de las 63 serovariedades identificadas en aguas residuales de Zaragoza se incluye el 83 % de todos los aislamientos de dicho origen (Tabla 1); además, estas mismas serovariedades se identifican en el 89,5 % de las cepas aisladas en pacientes de nuestro hospital. Estas mismas serovariedades se identifican a nivel nacional (7) en un porcentaje muy similar al encontrado en nuestro hospital en el caso de los aislamientos clínicos (88,9 %), mientras que su representación en muestras de origen no humano es algo menor (74,1 %). Este resultado demuestra la existencia de un número limitado de serovariedades de *Salmonella* que, con fluctuaciones, se mantienen con carácter endémi-

co en nuestra ciudad y que coincide con las que se identifican a nivel nacional. Lógicamente la situación es dinámica y pueden observarse modificaciones en función del período de tiempo que se considere.

Por último, encontramos que el número de serovariedades diferentes que se identifican está en relación con el número de cepas aisladas (Tabla 7), de una forma directamente proporcional en las aguas residuales de Zaragoza (r = 0,9330) y en aislamientos no humanos del CNMVIS (r = 0,9620). En el caso de los aislamientos clínicos, y aunque hay un mayor número de serovariedades cuando se estudia un número mayor de cepas, la relación no es proporcional (Tabla 7). Este resultado parece indicar que el número de serovariedades que se mantienen en circulación cada año entre la población no experimenta grandes oscilaciones. Si bien, es lógico, cuando el estudio se refiere a un menor número de aislamientos y a un menor espacio geográfico, el número de serotipos distintos que se aíslan en años diferentes experimenta también menores fluctuaciones (de 12 a 17 en el HCU) que cuando el estudio abarca un campo mayor (de 35 a 50 en CNMVIS).

Bibliografía

1. Anon (1985). Standard methods for the examination of water and wastewater. 16th ed. APHA, AWWA and WPCF. Washington D.C.
2. Anon (1988). Análisis de los resultados de las cepas de *Salmonella* recibidas para su estudio en el laboratorio de enterobacterias del Servicio de Bacteriología del CNMVIS. Año 1987. Boletín Microbiológico Semanal. Ministerio de Sanidad y Consumo. España **42-43,** 1-5.
3. Anon (1989). Estudio de las cepas de *Salmonella* recibidas en el Centro Nacional de Referencia del CNMVIS (Instituto de Salud Carlos III) durante el año 1988. Boletín Microbiológico Semanal. Ministerio de Sanidad y Consumo. España **51-52,** 1-5.
4. Alcaide, E., Martínez, J. P. and Garay, E. (1984). Comparative study on *Salmonella* isolation from sewage contaminated natural waters. J. Appl. Bacteriol. **56,** 365-371.
5. Arribas, R. M.; Campos, A. y Imaz, M. (1989). Estudio comparativo de la presencia de *Salmonella* en los coprocultivos y en las aguas residuales de Soria durante 1985. Rev. Esp. Microbiol. Clin. **4,** 643-646.
6. Castillo, J. y Gómez-Lus, R. (1986). Enteritis causadas por enterobacterias. Laboratorio. **82,** 301-323.
7. Echeita, M. A. and Usera, M. A. (1989). Prevalence of *Salmonella* serotypes isolated in Spain from human and non human sources (1983-1987). Microbiol. SEM. **5,** 95-103.
8. Ewing, W. H. y Martin, W. J. (1982). Enterobacteriaceas. En Manual de Microbiología Clínica, Lenette, E. H.; Balows, A.; Hausler, Y. J. y Truant, A. P. (eds.). Ed. Panamericana. Buenos Aires.
9. Ferrer, M. D. (1988). Evolución de la salmonelosis. Técnicas de Laboratorio. **146,** 460-477.
10. Harteman, P. et Foliget, J. M. (1985). Les bacteries pathogenes pour l'homme. In l'Epuration et le Traitment des Effluents. G. Martin (ed.). Technique et Documentation Lavoisier. Paris.
11. Lafarga, M. A. y Navarro, M. (1990). Nuestra experiencia en el aislamiento de *Salmonella enterica* en aguas residuales (1982-1989). Técnicas de Laboratorio. **158,** 378-382.
12. Le Minor, L. and Popoff, M. Y. (1987). Designation of *Salmonella enterica sp. no. nom. rev.*, as the type and only specie of the genus *Salmonella*. Int. J. Sys. Bacteriol., **37,** 465-468.
13. McFetters, B., Jezeski, J. J., Thompson, C. A. and Stuart, D. G. (1974). Comparative survival of indicator bacteria and enteric pathogens in well water. Appl. Microbiol. **27,** 823-829.
14. Mollaret, H. H., Alonso, J. M. et Bercovier, H. (1982). Aspects biologiques, diagnostiques et ecologiques des yersinioses. Med. Mal. Infect. **12,** 664-667.
15. Moriñigo, M. A., Borrego, J. J. and Romero, P. (1986). Comparative study of different methods for detection and enumeration of *Salmonella* spp. in natural waters. J. Appl. Bacteriol. **61,** 169-176.
16. Navarro, M., Castillo, J., Larraz, M. V. y Gómez Lus, R. (1985). Estudio de *Salmonella* en aguas residuales. Comparación con aislamientos clínicos. Res. X Congr. Nac. Microbiol. Valencia, p. 528.
17. O'Shanahan, L., Vega, S. y Campos, M. I. (1987). Aislamiento de *Salmonella* en agua de mar contaminada por vertidos domésticos. Res. XI Congr. Nac. Microbiol. Gijón, p. 714.
18. Perales, I. y Audicana, A. (1989). Métodos para el aislamiento de *Salmonella*. Alimentaria. Septiembre, 19-26.
19. Rhodes, P. and Quesnel, L. B. (1986). Comparison of Muller-Kauffman tetrathionate with Rappaport-Vassiliadis (RV) medium for the isolation of salmonellas from sewage sludge. J. Appl. Bacteriol. **60,** 161-167.
20. Rivera, M. J., Rivera, N., Castillo, J., Mazon, A., Lanas, A. and Gómez Lus, R. (1989). Molecular and epidemiological study of *Salmonella* clinical isolates. 16th Internat. Congr. Chemother. Jerusalem. Abstract. 144.

21. Romero, P. (1987). *Salmonellas* en aguas naturales. Res. XI Congr. Nac. Microbiol. Gijón, p. 247.
22. Therlfall, E. J. and Frost, J. A. (1990). The identification, typing and fingerprinting of *Salmonella:* laboratory aspects and epidemiological applications. J. Appl. Bacteriol. **68**, 5-16.
23. Van Leudsen, F. M., Van Schothorst, M. and Beckers, H. J. (1982). The Standard *Salmonella* Isolation Method. In: J. E. L. Corry, D. Roberts and F. A. Skinner (eds.). Isolation and Identification Methods for Food Poisoning Organisms. Technical Series **17**, pp. 35-49. London. Academic Press.

MICROBIOLOGIA SEM 7 (1991), 35-41

Numerical taxonomy of moderately halophilic Gram-positive cocci isolated from the Salar de Atacama (Chile)

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(Received July 9, 1990/January 16, 1991)

Summary

A taxonomic study has been carried out on 22 strains of moderately halophilic motile cocci isolated from the Salar de Atacama (Chile). The 112 phenotypic tests were analyzed by numerical taxonomy using S_{SM} coefficient and the unweighted pair group method of association (UPGMA). At the 67% similarity level, two phenons were obtained: phenon A included 11 strains and phenon B, 11 strains too, whereas the six reference strains did not cluster within these two phenons. These results suggest that moderately halophilic cocci with different phenotypic characteristics from previously described species can be isolated from the hypersaline habitat Salar de Atacama.

Key words: Taxonomy, halophilic bacteria, cocci.

Resumen

Se ha llevado a cabo un estudio taxonómico con 22 cepas de cocos halófilos moderados aislados del Salar de Atacama (Chile). Con los resultados de las 112 pruebas fenotípicas se realizó un análisis numérico, usando el coeficiente de semejanza de Sokal y Michener (S_{SM}) y la técnica de agrupación UPGMA, obteniéndose así 2 fenones a un nivel de semejanza del 67% (el fenón A incluye 11 cepas y 11 también el fenón B), mientras que las 6 cepas utilizadas como referencia no quedaron agrupadas dentro de estos fenones. Nuestros resultados sugieren que estas cepas aisladas del Salar de Atacama constituyen 2 grupos de cocos halófilos moderados de características fenotípicas diferentes de las especies descritas.

Introduction

Halophilic bacteria live in environments at high salinity conditions (4). According with their salt requirements for optimal growth, this group of microorganisms has been classified in different categories; among them, moderately halophilic bacteria are those which show their optimal growth in a range of salt concentrations of 3-15 % (w/v) NaCl (5).

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The taxonomic distribution of moderately halophilic bacteria is very heterogeneous, and so, this group includes bacterial species belonging to filogenetically very distant genera. The moderately halophilic Gram-positive cocci validly described up to date are: *Micrococcus halobius* (9), *Sporosarcina halophila*, a motile spore-forming coccus (1), *Marinococcus*, proposed by Hao *et al.* (3) as a new genus with two species *Marinococcus halophilus* and *Marinococcus albus*, to include the motile cocci previously designated as *Planococcus halophilus* (7), three strais of *Planococcus* sp. (20) and one unidentified motile coccus (3). Lately, Márquez *et al.* (6) described a new species, *Marinococcus hispanicus*. Recently, Ventosa *et al.* (22) have proposed a new genus and species of moderately halophilic Gram-positive coccus, *Salinicoccus roseus*.

The Salar de Atacama is a vaste salt deposit located in Northern Chile. The first studies on the microbial biota communities of the Salar have very recently been done, and dealt with the moderately halophilic Gram-negative rod shaped bacteria, which constitute the most abundant group within the heterotrophic organisms isolated from this habitat (8). The aim of this work was to study taxonomically a group of moderately halophilic Gram-positive cocci isolated from the Salar de Atacama, and to define their taxonomic position in accordance with the previously described species.

Materials and methods

Bacterial strains

The 22 moderately halophilic Gram-positive cocci selected for this study were isolated from saline water of Laguna de Tevenquiche located in the Salar de Atacama, region of Antofagasta, Chile. Sampling methods, as well as the isolation and further selection of the strains have been previously described (10, 21).

The following moderately halophilic Gram-positive cocci were used as reference strains: *Marinococcus albus* CCM 3517^T, *Marinococcus halophilus* CCM 2706^T, *Marinococcus hispanicus* ATCC 49259^T, *Micrococcus halobius* ATCC 21727^T, *Salinicoccus roseus* ATCC 49258^T and *Sporosarcina halophila* DSM 2266^T.

Maintenance medium

The strains were maintained on agar slants of MH medium (21) containing 10% (w/v) marine salts (13) and the following nutrients (%, w/v): Yeast extract (Difco), 1; Proteose-peptone no. 3 (Difco), 0.5 and glucose, 0.1. This medium was solidified with 2% (w/v) Bacto-agar (Difco). The pH was adjusted to 7.2 with 1N NaOH.

Phenotypic tests

The 22 isolates and the 6 reference strains were studied for 112 phenotypic characters, including morphological, physiological, biochemical, nutritional and antimicrobial susceptibility tests (see Table 1). All the tests were carried out as previously described (10, 11, 21).

Numerical analysis

A total of 89 taxonomic characters were coded in a binary form of the presence/absence type. Positive and negative results were coded as 1 and 0 respectively, and non-comparable or missing data were coded as 9. Strains similarities were estimated with the simple matching coefficient (S_{SM}) (15) and the cluster analysis was carried out by using the unweighted pair group method of associa-

TABLE 1
FREQUENCIES OF POSITIVE CHARACTERS FOUND IN THE TWO PHENONS OF MODERATELY HALOPHILIC GRAM-POSITIVE COCCI STUDIED. FREQUENCIES ARE EXPRESSED AS A PERCENTAGE OF THE TOTAL, SCORED EACH GROUP FOR THE GIVEN TEST

Characteristic	Phenon A (11 strains)	Phenon B (11 strains)	Characteristic	Phenon A (11 strains)	Phenon B (11 strains)
Oxidase*	100	0	D-Salicin	18	73
Pigmentation*	Orange	Yellowish	Starch	100	82
Salts: growth at % (w/v):			Sucrose*	27	91
0.5	54	45	D-Trehalose*	9	91
25	100	82	D-Xylose	9	27
30	100	63	Alcohols:		
pH growth at:			Adonitol	0	27
5	0	36	Dulcitol	9	36
Temperature: growth at (°C):			DL-Glycerol*	0	64
5	0	45	*meso*-Inositol	9	61
40*	91	0	D-Sorbitol*	9	73
Acid from glycerol	64	100	Carboxylic acids:		
Nitrate reduction to nitrite*	0	100	Caprilate	0	45
Arginine dehydrolase	9	0	Citrate	9	27
Phosphatase	45	18	Formate	18	27
ONPG*	91	0	Malonate	100	82
Hydrolysis of:			Oxalate	45	73
Gelatin*	100	0	Pyruvate	0	54
Casein*	91	0	Succinate*	0	64
Starch*	100	0	D-Tartrate*	0	64
Tween 20	0	9	Utilization of amino acids as sole source of carbon, nitrogen and energy:		
Utilization of organic compounds as sole source of carbon and energy:			L-Alanine*	27	100
Carbohydrates			L-Arginine	54	36
L-Arabinose	73	91	L-Asparagine	36	100
D-Cellobiose	0	36	L-Glutamine	45	100
D-Fructose	0	45	L-Histidine	9	9
D-Galactose*	0	64	L-Isoleucine	64	64
D-Glucose*	0	64	L-Lysine	73	54
Inulin	45	91	L-Ornithine	27	54
Lactose*	0	73	L-Serine	82	45
Maltose*	0	82	L-Threonine	27	45
Mannitol*	0	64	L-Tryptophan	27	27
L-Rhamnose*	18	91	L-Valine	82	27

* Characteristics with an asterisk are differential between the two phenons.

All strains were Gram-positive motile non spore-forming cocci and formed catalase. They grew between 3-20% (w/v) salts, at pH 6 to 10 and at 15, 25, 32 or 37.° C. They were strict aerobes. They produced acid from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, sucrose, and D-xylose. None formed indole, or reduced nitrite to gaseous compounds. All were phenylalanine deaminase, methyl-red, Voges-Proskauer, lysine and ornithine decarboxylase negative. They did not produce H_2S. None produced lecithinase, urease or DNAase, or hydrolyzed blood or Tween 80. They hydrolyzed aesculin. They did not use hypurate as sole source of carbon and energy, and cysteine as sole source of carbon, nitrogen and energy. They were sensible to ampicillin (100 μg), cephalothin (30 μg), cloramphenicol (30 μg), erythromycin (15 μg), penicillin G (10 IU), rifampin (5 μg) and tetracycline (30 μg).

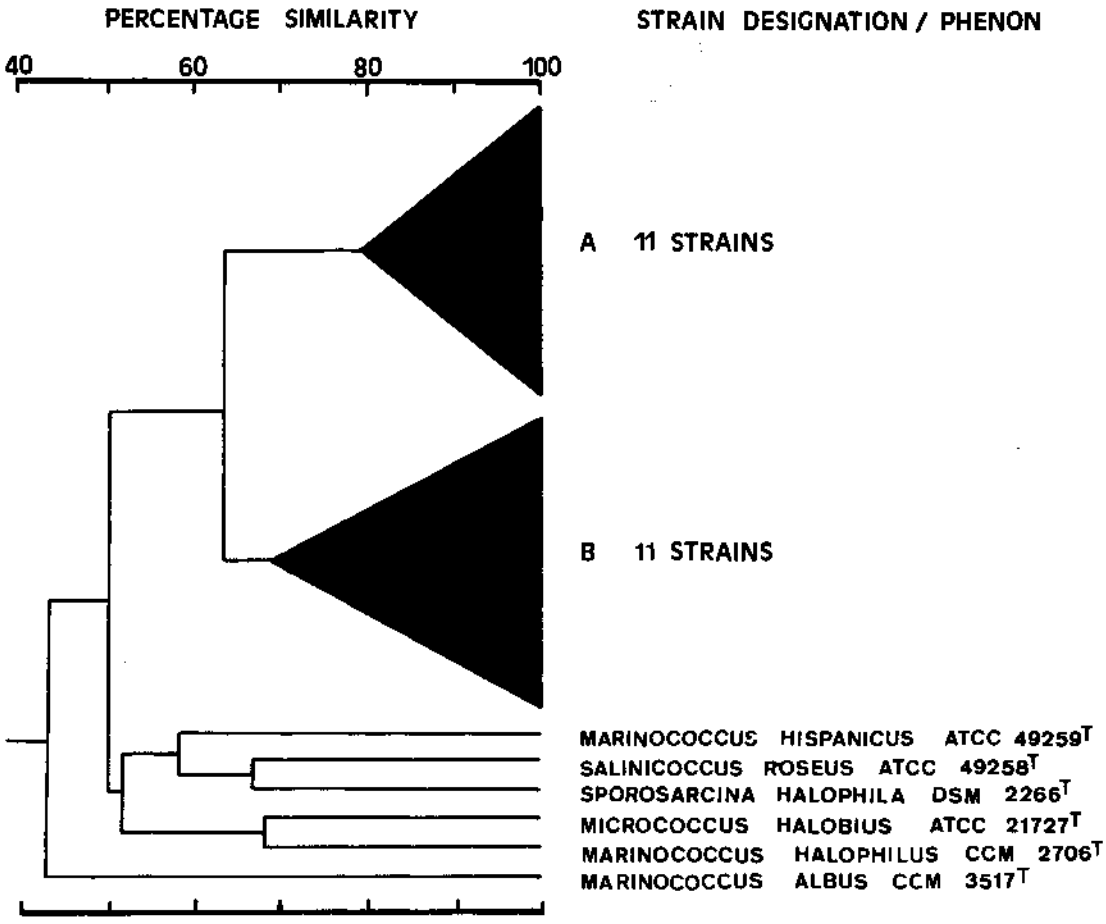

Fig. 1. Simplified dendrogram showing the clustering of 22 Gram-positive moderately halophilic cocci and six reference strains, based on the S_{SM} coefficient and UPGMA clustering method.

tion (UPGMA). The test error was calculated by examining 10% of the strains in duplicate (16). Cophenetic correlation was also studied (17). Computation was performed by using an Eclipse MV/10000 model computer with the MINT program (14).

Results and discussion

Recently, several studies have been carried out on the taxonomy of moderately halophilic bacteria isolated from different hypersaline environments. Thus, several new genera and species have been described (2, 6, 12, 18, 19, 22). However, the identification of some groups of moderately halophilic bacteria is still rather difficult, because they cannot be included into the previously described species on the basis of their phenotypic characteristics. In this work we describe 22 moderately halophilic motile Gram-positive cocci, isolated from the Salar de Atacama (Chile), comparing them with the previously described species.

The result of the numerical study of the 22 moderately halophilic cocci and the six reference strains grouped by means of the S_{SM} coefficient and UPGMA clustering method, is shown in a sim-

TABLE 2
DIFFERENTIAL CHARACTERISTICS OF THE TWO PHENA (A AND B) AND OTHER SPECIES OF MODERATELY HALOPHILIC GRAM-POSITIVE COCCI[a]

	Sporosarcina halophila	*Marinococcus halophilus*	*Marinococcus albus*	*Marinococcus hispanicus*	*Salinicoccus roseus*	*Micrococcus halobius*	*Phenon A*	*Phenon B*
Pigment	Orange	Yellow-orange	—	Red-orange	Pink-red	—	Orange	Yellow
Motility	+	+	+	—	+	—	+	+
Spores	+	—	—	—	—	—	—	—
Oxidase	+	—	+	+	+	+	+	—
Acid from:								
Fructose	—	—	—	+	—	ND	+	+
Galactose	—	—	—	+	—	+	+	+
Glycerol	—	+	—	+	—	+	D	+
Glucose	—	+	—	+	—	+	+	+
Mannitol	—	+	—	+	—	+	+	+
Xylose	—	+	—	—	—	+	+	+
Nitrate reduction	—	—	+	+	+	—	—	+
Urease	—	—	+	D[c]	—	—	—	—
Voges-Proskauer	—	—	—	—	—	+	—	—
Hydrolysis of:								
Aesculin	ND[b]	+	—	D	—	+	+	+
Casein	+	+	—	—	+	ND	+	—
Gelatin	+	+	—	+	+	—	+	—
Starch	+	—	—	D	+	—	+	—
DNAase	+	—	+	D	ND	ND	—	—
Haemolysis	ND	—	—	+	ND	ND	—	—

[a] Data from references 1, 3, 6, 9, 22 and from this study.
[b] ND: not determined.
[c] D: differs among strains.

plified dendrogram in which clusters are depicted as solid blocks (Fig. 1). The cophenetic value was 0.91 and the estimated test error was less than 2.9%. At a 67% similarity level the 22 isolates were grouped into two phenons, whereas none of the reference strains did cluster within them at that similarity level. Phenon A included 11 strains that clustered at 78% similarity level and phenon B contained 11 strains related at a similarity level of 67%. The phenotypic characteristics of the two phenons are listed in Table 1. The differential features are marked with as asterisk.

Our isolates constituted a quite homogeneous group with a number of similar characteristics. All the strains were moderately halophilic Gram-positive motile non spore-forming cocci, strict arerobes and catalase positive. They were moderate halophiles uncapable of growth on nutrient agar without additional salt. They grew between 3-20% (w/v) salts, at pH 6 to 10 and between 15-37° C, optimal growth occurring at 7.5-10% (w/v), pH 7 and 32° C. Phenon A included 11 strains that were oxidase positive and produced an orange diffusible pigment. They grew at 40° C and were unable to grow at 5° C or pH 5. They could not reduce nitrate to nitrite. ONPG test was positive. They hydrolyzed casein, gelatin and starch. Only starch and malonate were used as sole source of carbon and energy. They used L-serine and L-valine as sole source of carbon, nitrogen and energy. The most remarkable characteristic that differentiates the 11 strains of phenon B from phenon A is that all strains are oxidase negative and produce a yellowish pigment. Furthermore, they were unable to grow at 40° C; they reduced nitrate to nitrite; ONPG test was negative. They did not hydrolyze ca-

sein, gelatin or starch. L-arabinose, inulin, maltose, L-rhamnose, starch, sucrose, D-trehalose and malonate were used as sole source of carbon and energy; they could use L-alanine, L-asparagine and L-glutamine as sole source of carbon, nitrogen and energy.

On the basis of general resemblance, we determined that our isolates were more similar to *Marinococcus halophilus* than to any other described species of moderately halophilic Gram-positive cocci. *M. halophilus* was proposed to accommodate a group of 10 Gram-positive moderately halophilic cocci previously designated as *Planococcus halophilus* (7, 21), that were certainly very uniform in their phenotypic characteristics (3). However, the strains studied here are still different in many respects from *M. halophilus:* strains in phenon A were oxidase positive, produced acids from fructose and galactose and hydrolyzed starch; on the other hand, strains in phenon B differed from *M. halophilus* in the following features: acid production from fructose and galactose, nitrate reduction to nitrite, and aesculin, casein and gelatin hydrolysis (Table 2).

The moderately halophilic Gram-positive cocci isolated from the Salar de Atacama constitute a minor proportion within the heterotrophic moderately halophilic organisms present in this habitat (Prado *et al.,* unpublished data). Our results show that this group of moderately halophilic cocci have different phenotypic characteristics from previously described species. Further molecular studies would definitely help us to know the exact relationship between our strains and ohter groups of moderately halophilic bacteria isolated from hypersaline environments.

Acknowledgments

This investigation was partially supported by grants from the Ministerio de Educación y Ciencia (DGICYT PS88-0105) and from the Junta de Andalucía.

References

1. Claus, D., Fahmy, F., Rolf, H. J. and Tosunoglu, N. (1983). *Sporosarcina halophila* sp. nov., and obligate, slightly halophilic bacterium from salt marsh soils. Syst. Appl. Microbiol. **4,** 496-506.
2. Franzmann, P. D. V., Wehmeyer, E. and Stackebrandt, E. (1988). *Halomonadaceae* fam. nov., a new family of the class *Proteobacteria* to accommodate the genera *Halomonas* and *Deleya.* Syst. Appl. Microbiol. **11,** 16-19.
3. Hao, M. V., Kocur, M. and Komagata, K. (1984). *Marinococcus* gen. nov., a new genus for motile cocci with meso-diaminopimelic acid in the cell wall; and *Marinococcus albus* sp. nov. and *Marinococcus halophilus* (Novitsky and Kushner) comb. nov. J. Gen. Appl. Microbiol. **30,** 449-459.
4. Kushner, D. J. (1985). The Halobacteriaceae. In: C. R. Woese, R. S. Wolfe (eds.). The bacteria, vol VIII, pp. 171-214. Academic Press, Orlando.
5. Kushner, D. J. and Kamekura, M. (1988) Physiology of halophilic eubacteria. In: F. Rodríguez-Valera (ed.). Halophilic bacteria, Vol. **1,** pp. 109-141. CRC Press, Inc. Boca Ratón, Florida.
6. Márquez, M. C., Ventosa, A. and Ruiz-Berraquero, F. (1990). *Marinococcus hispanicus,* a new species of moderately halophilic Gram-positive cocci. Int. J. Syst. Bacteriol. **40,** 165-169.
7. Novitsky, T. J. and Kushner, D. J. (1976). *Planococcus halophilus* sp. nov., a facultatively halophilic coccus. Int. J. Syst. Bacteriol. **26,** 53-57.
8. Prado, B., del Moral, A., Quesada, E., Ríos, R., Monteoliva-Sánchez, M., Campos, V. and Ramos-Cormenzana, A. (1990). Numerical taxonomy of moderately halophilic Gram-negative rods isolated from the Salar de Atacama, Chile. Syst. Appl. Microbiol. (in press).
9. Onishi, H. and Kamekura, M. (1972). *Micrococcus halobius* sp. n. Int. J. Syst. Bacteriol. **22,** 233-236.
10. Quesada, E., Ventosa, A., Rodríguez-Valera, F. and Ramos-Cormenzana, A. (1983). Numerical taxonomy of moderately halophilic Gram-negative bacteria from hypersaline soils. J. Gen. Microbiol. **129,** 2649-2657.
11. Quesada, E., Ventosa, A., Ruiz-Berraquero, F. and Ramos-Cormenzana, A. (1984). *Deleya halophila,* a new species of moderately halophilic bacteria. Int. J. Syst. Bacteriol. **34,** 287-292.
12. Quesada, E., Valderrama, M. J., Béjar, V., Ventosa, A., Gutiérrez, M. C., Ruiz-Berraquero, F. and Ramos-Cormenzana, A. (1990). *Volcaniella eurihalina* gen. nov., sp. nov., a moderately halophilic nonmotile Gram-negative rod. Int. J. Syst. Bacteriol. **40,** 261-267.

13. Rodríguez-Valera, F., Ruiz-Berraquero, F. and Ramos-Cormenzana, A. (1981). Characteristics of the heterotrophic bacterial populations in hypersaline environments at different salt concentrations. Microb. Ecol. **11,** 235-243.
14. Rohlf, F. J. (1985). Numerical taxonomy system of multivariate statistical programs. State University of New York, Stony Brook, NY USA.
15. Sokal, R. R. and Michener, C. D. (1958). A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull. **38,** 1409-1438.
16. Sneath, P. H. A. and Johnson, R. (1972). The influence on numerical taxonomic similarities of errors in microbial test. J. Gen. Microbiol. **72,** 377-392.
17. Sneath, P. H. A. and Sokal, R. R. (1973). Numerical taxonomy. The principles and practice of numerical classification. Freeman WH and Co. San Francisco.
18. Ventosa, A., García, M. T., Kamekura, M., Onishi, H. and Ruiz-Berraquero, F. (1989). *Bacillus halophilus* sp. nov. a moderately halophilic *Bacillus* species. Syst. Appl. Microbiol. **12,** 162-166.
19. Ventosa, A., Gutiérrez, M. C., García, M. T. and Ruiz-Berraquero, F. (1989). Classification of «*Chromobacterium marismortui*» in a new genus *Chromohalobacter* gen. nov., as *Chromohalobacter marismortui.* Int. J. Syst. Bacteriol. **39,** 382-386.
20. Ventosa, A., Ramos-Cormenzana, A. and Kocur, M. (1983). Moderately halophilic Gram-positive cocci from hypersaline environments. Syst. Appl. Microbiol. **4,** 564-570.
21. Ventosa, A., Quesada, E., Rodríguez-Valera, F., Ruiz-Berraquero, F., Ramos-Cormenzana, A. (1982). Numerical taxonomy of moderately halophilic Gram-negative rods. J. Gen. Microbiol. **128,** 1959-1986.
22. Ventosa, A., Márquez, M. C., Ruiz-Berraquero, F. and Kocur, M. (1990). *Salinicoccus roseus* gen. nov., sp. nov., a new moderately halophilic Gram-positive coccus. Syst. Appl. Microbiol. **13,** 29-33.

MICROBIOLOGIA SEM 7 (1991), 42-48

On the role of trehalose in yeast cells subjected to hyperosmotic shock

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(Received December 11, 1990/February 2, 1991)

Summary

Plate counts on both high and low water activity (a_w) media were performed during the growth of *Candida utilis* in batch culture. The results revealed a marked discrepancy between the counts on the two media in the logarithmic phase. The discrepancy almost dissapeared in stationary phase revealing a higher resistance of these cells, as compared to growing cells, to the severe dehydration imposed by the hyperosmotic shock upon transfer to low a_w medium. Since the two types of cells differ in the level of endogenous trehalose the relation between plating discrepancy and trehalose content of the cells was investigated. Treatments that changed the intracellular trehalose concentration did not modify the plate counts on low a_w medium. It was therefore concluded that the amount of trehalose into the cells is not the only factor conferring resistance against the hyperosmotic shock. Glycerol content did not correlate with resistance to the water stress either. Congruent with the former conclusion, other yeast species *(Sporobolomyces salmonicolor, Schizosaccharomyces pombe)* showed no correlation between changes in the trehalose content and susceptibility or resistance to the osmotic stress in low a_w medium.

Key words: Hyperosmotic shock, trehalose, water stress, Candida utilis.

Resumen

Durante el crecimiento de *Candida utilis* en medio líquido se realizaron determinaciones de viables en medio sólido con alta y baja densidad de agua (a_w). Los resultados indicaron una notable discrepancia entre los recuentos de colonias en ambos medios durante la fase logarítmica. La discrepancia casi desapareció en fase estacionaria, revelando una mayor resistencia de estas células, en comparación con las exponenciales, a la deshidratación provocada por el choque hiperosmótico en medio de baja a_w. Como los dos tipos de células contienen diferentes niveles de trehalosa, se investigó la relación entre la discrepancia en el recuento de colonias y el contenido en trehalosa. Tratamientos que ocasionaron cambios en la concentración de trehalosa intracelular no modificaron la capacidad de formar colonias en medio de baja a_w. En consecuencia, la cantidad de trehalosa interna no parece ser el único factor que confiere resistencia frente al choque hiperosmótico. El contenido en glice-

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rol tampoco se correlaciona con la resistencia al estrés hídrico. En apoyo de la primera conclusión, otras especies que fueron analizadas *(Sporobolomyces salmonicolor, Schizosaccharomyces pombe)* no mostraron relación entre cambios en el contenido en trehalosa y la susceptibilidad o resistencia al estrés en medio de baja a_w.

Introduction

Yeast cells contain trehalose as an endogenous disaccharide whose physiological significance still remains unclear. In addition to be considered as a potential carbon source (12) recent observations have focussed on its possible role as a protectant under various stressing conditions (13). In baker's yeast the acquisition of thermotolerance and the survival after dehydration are examples of cell responses that occur in parallel to trehalose accumulation (5, 7). Water availability can be a limiting factor for growth in the culture media and the osmotic stress impossed by high solute concentrations in the media is often compensated by polyols storage (3). In this context, it has been propossed that under extreme conditions trehalose could help in maintaining the functional integrity of vital cell components by acting as a substitute for water throughout its interaction with the polar portion of some constituents such as membrane phospholipids (4).

Mackenzie *et al.* (9) described an apparent drop in viability by exponential cells of *Saccharomyces cerevisiae* when plated on media of low water activity (a_w). The effect of the hyperosmotic shock was called «water stress plating hypersensitivity» and, among fourteen species that were screened, it was only found during the exponential phase of growth in strains of *S. cerevisiae* and in *Candida kruzei.* Development of resistance by stationary phase cells was ascribed to accumulation of trehalose by these cells (10). In the present work we describe that a similar phenomenon occurs in the yeast *Candida utilis* and its relation to the trehalose content has been investigated. Contrary to earlier suggestions (10), the evidence linking trehalose accumulation and resistance to the hyperosmotic shock has been found to be merely circumstantial in this yeast.

Materials and methods

Microorganisms, culture conditions and counting

Candida utilis ATCC 60459, *Sporobolomyces salmonicolor* CECT 1274 and *Schizosaccharomyces pombe* L968 h^{90} were used in this work. Yeasts were grown in liquid Winge's medium till stationary phase (2). Aliquots of these cultures served as inocula to fresh media (initial O. D. 3 at 600 nm) that were incubated at 30° C; at timed intervals samples were withdrawn and the growth followed by total cell counting in a haemocytometer.

Appropriate dilutions of each sample were simultaneously plated in triplicate onto high a_w (WHA, Winge's mediun containing 2 % glucose, 0.3 % yeast extract and 2 % agar, w/v) and low a_w media (WLA, modified Winge's medium with 45 % glucose, 0.3 % yeast extract and 2 % agar w/v). After 36 h at 30° C counting was determined as colony-forming units. To express the different plating efficiency in orders of magnitude, «plating discrepancy» was defined for each culture sample as the log of plate counts on WHA medium minus the log of plate counts on WLA medium so that high values of plating discrepancy correspond to high sensitivity to the hyperosmotic shock.

Cell treatments and analytical procedures

To induce trehalose synthesis the procedure indicated by Hottiger *et al.* (7) was applied to *C. utilis* cells. Mid-exponential phase cultures were divided into two identical portions; one subculture was maintained at 30° C as control whereas the other was incubated at 40° C for 100 min. To decrease trehalose content in stationary phase cells the activation of trehalase was performed by adding 50mM glucose or 23 mM NaF as indicated earlier (2). Trehalose was extracted as previously described (2, 11). Glycerol was estimated according to Kates (8).

Results

Hyperosmotic shock in Candida utilis

Figure 1a shows comparative results on the ability of *C. utilis* to form colonies on solid media with high and low a_w during a partial growth cycle in liquid Winge's medium. On low a_w medium (WLA) a marked decrease in colony forming units was evident during the period of exponential growth. The cell population became resistant to the effects of the hyperosmotic shock in the low a_w medium as the culture reached the stationary phase. Because stationary phase cells were always employed as inocula in these experiments, the cultures were also partially resistant at the beginning of the growth cycle. Plating discrepancy (i. e., difference between the logs of plate counts on both solid media) reached a maximum value around 4 after 6-9 h of the inoculation of the stationary phase cells, which closely corresponds to the mid-exponential phase of growth. These results indicate that, on average, only 1 out of 10^4 exponentially growing cells is able to form at such stage a visible colony when the culture is plated on WLA medium containing high glucose concentration (low a_w). However, plating discrepancy for stationary phase cells never surpassed values above 1 when plated on the same two media.

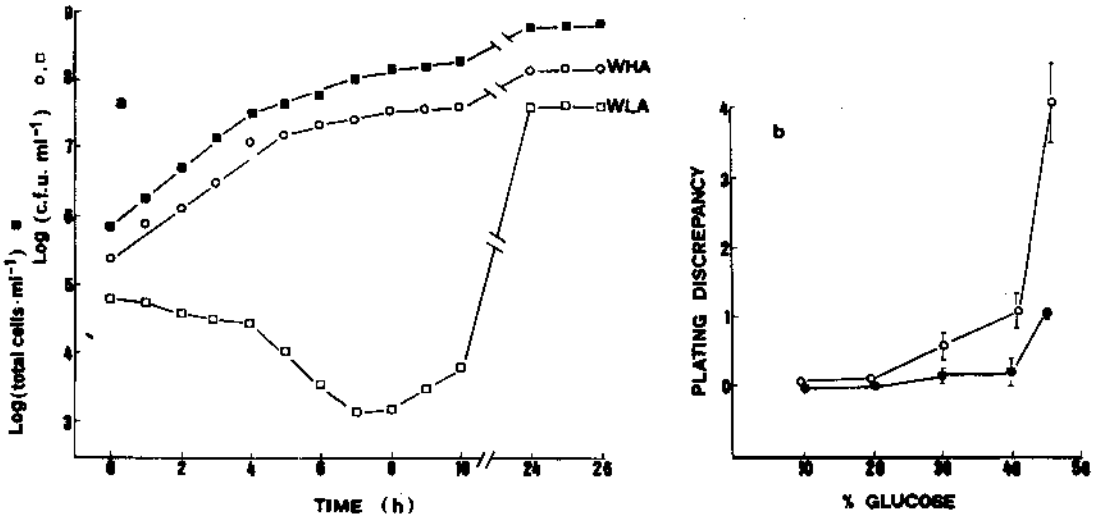

Fig. 1. Plating hypersensitivity in *Candida utilis* during growth. (a) Population counts: ■, total cells; ○, c.f.u. on WHA medium □, c.f.u. on WLA medium. (b) Plating discrepancy as a function of glucose concentration on solid media: ○, exponential phase cells; ●, stationary phase cells. Results are the mean of three experiments.

Physiological characteristics of the response

Figure 1b shows the relation between plating discrepancy and glucose concentration (as the major determinant of the inverse of the a_w value) for growing and resting cells. Below 20 % glucose (w/v) in the solid media there is not plating discrepancy but, as glucose concentration increases (and therefore a_w decreases), there is a pronounced plating discrepancy which is much more evident in the case of exponentially growing cells.

The ability shown by a restricted number of exponentially growing cells to divide and form colonies on media with low a_w suggested the possible existence in the culture of a cell subpopulation that was resistant to the hyperosmotic stress on genetic basis. To ascertain whether a selective process was taking place, colonies from plates containing WLA medium that had been seeded with exponential phase cells were isolated. The resulting cells were resuspended in sterile distilled water, the suspension serially diluted and aliquots plated again on both high and low a_w media to determine the initial colony forming units of this suspension on each media (Table 1). Another portion of the cell suspension served to inoculate fresh liquid Winge's medium and when the culture reached mid-exponential phase samples were also plated on the two solid media. Since the cultures arising from colonies derived from resistant cells showed again plating discrepancy (Table 1), the heterogeneity of the culture population growing at exponential phase, which is responsible for the results shown in Figure 1a, must rely on physiological rather than genetic grounds.

The effect of the hyperosmotic shock on the exponentially growing cells that are not able to form colonies on WLA medium is not lethal. Instead, the severe dehydration appears to induce a reversible state of latency. This conclusion was drawn from experiments (not shown) in which areas without colonies were cut out from plates containing WLA medium and reincubated in liquid normal Winge's medium. The subsequent cell growth that developed demonstrated that the cells unable to plate at low a_w were not dead but only dormant or supressed in their ability to divide.

Effect of changes in endogenous trehalose and glycerol content

Trehalose has been regarded as an intracellular protectan for yeast cells during some dehydration treatments (13). We therefore considered that this sugar could be also responsible for the differential behaviour of growing and resting cells in low a_w medium. This suggestion was further sustained by previous observations showing a higher trehalose content in stationary phase cells than in growing cells (2).

To show whether plating efficiency of growing cells increased at low a_w after induction of trehalose synthesis, exponential cultures were shifted al 40° C and the cells plated thereafter onto

TABLE 1
COLONY FORMING ABILITY ON WHA AND WLA MEDIA OF *CANDIDA UTILIS* CELLS SELECTED FROM WLA MEDIUM

Media	Inoculum[a] log (c.f.u. × ml^{-1})	Culture[b] log (c.f.u. × ml^{-1})
WHA	3.4	6.8
WLA	3.3	3.1

[a] Colony counts of the initial cell suspension from colonies selected on WLA medium:
[b] Colony counts of the mid-exponential phase culture originated from colonies selected on WLA medium.

WHA and WLA media. The results indicated that the increase in the level of intracellular trehalose after the moderate thermal shock was not reflected on a higher number of colony forming units at low a_w as compared to control cultures (Table 2). Conversely, we analyzed for changes in the response of stationary phase cells following mobilization of the endogenous trehalose by activation of the regulatory trehalase «in vivo». The addition of glucose or NaF to resting cells, which prompted a marked decrease in the intracellular level of trehalose, was not accompanied by a reduced plating efficiency in low a_w medium with respect to control cells (Table 2).

We also examined the possible implication in this response of glycerol, a compatible solute which functions in some cells as an osmoregulator and as an osmoprotector of enzyme activities under water-stressing conditions (14). No positive relationship was found between the intracellular concentration of glycerol and the resistance to the stress at low a_w. The glycerol content of mid-exponential phase cells (6.9×10^{-7} nmoles/cell) was in fact higher than that of stationary phase cells (1.8×10^{-7} nmoles/cell) suggesting that this metabolite is not among the factors conferring directly the ability to overcome the severe stress caused by the hyperosmotic shock.

Hyperosmotic shock in other yeasts

The plating at low and high a_w values was also analyzed in two additional yeasts and their trehalose content determined. The basidiomycetous yeast *S. salmonicolor* did not show significant plating discrepancy when cultured on solid media containing various glucose concentrations during the exponential or the stationary phase of growth (Fig. 2a). However, the endogenous trehalose in this yeast is about 2-fold higher in exponentially growing cells than in resting cells (Table 3), which clearly illustrates that differences in the trehalose content do not necessarily determine differences in the plating efficiency at low a_w.

Similar studies were carried out with the fission yeast *Shiz. pombe.* In this case, neither plating discrepancy at various a_w values nor significant changes in the trehalose content of the cells were found at different stages of the growth cycle (Fig. 2b; Table 3).

TABLE 2
PLATING EFFICIENCY OF *CANDIDA UTILIS* CELLS AFTER CHANGES IN THE TREHALOSE CONTENT

Exponentially growing cells[a]			
Treatment	Log (c.f.u. × ml^{-1})		nmoles trehalose per 10^{10} cells
	WHA	WHA	
None (control)	6.7	3.1	57
40° C, 100 min	6.6	3.2	212
Stationary phase cells[a]			
Treatment	Log (c.f.u. × ml^{-1})		nmoles trehalose per 10^{10} cells
	WHA	WLA	
None (control)	7.6	7.1	292
25 mM Naf, 100 min	7.4	7.0	15
50 mM glucose, 100 min	7.5	7.2	10

[a] Data from a single experiment for each type of cells are shown. Similar experiments were repeated twice with comparable cualitative results.

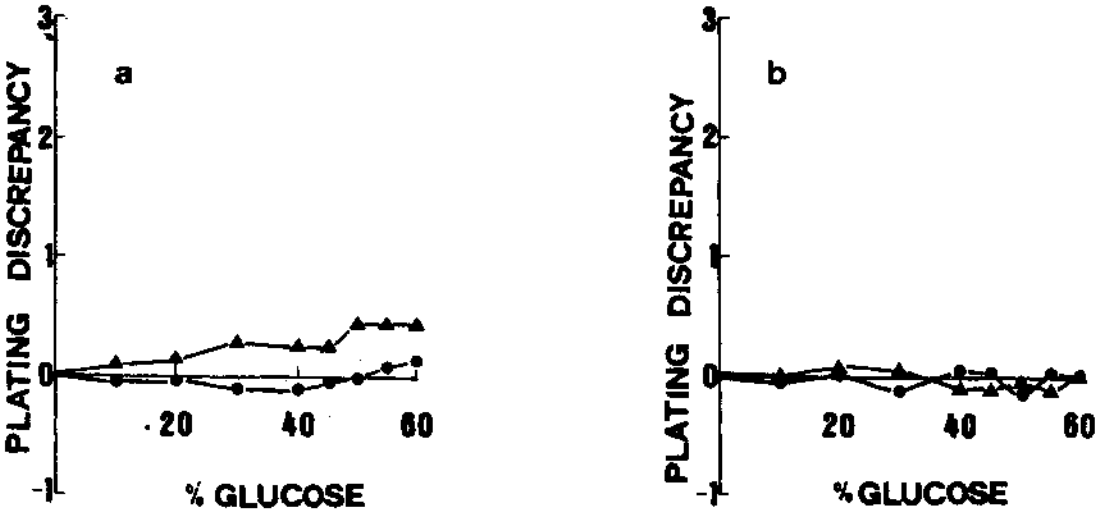

Fig. 2. Plating discrepancy in *Sporobolomyces salmonicolor* (a) and in *Schizosaccharomyces pombe* (b) as a function of the glucose concentration in solid media. ●, exponential phase cells; ▲, stationary phase cells.

Discussion

During growth of *C. utilis* the exponential cells appear to be critically sensitive to the water limitation stress in media of low a_w. The reduced ability to form colonies on media of low a_w is later recovered when the cells reach the stationary phase. Differences in the intracellular level of stored trehalose, however, can not account for the differential resistance to the hyperosmotic shock. Our results on the plating efficiency at low a_w of the cells that had been treated to induce synthesis or degradation of trehalose do not fit well with a suposed role of this sugar as a protectan in conditions of low water availability. Yeasts are generally more resistant to physical stresses when resting than when they are multiplying rapidly (14), but we consider unlikely that the higher trehalose content of stationary phase cells is the only determinant of their resistance of extreme solute stress. Glycerol also accumulates intracellularly in some fungi in response to increases in the concentration of solutes in the growth medium (1, 6, 14). Although we thought that differences in the initial glycerol content between growing and resting cells would offer an alternative explanation for the inequal ability of the two cell types to cope with the hyperosmotic stress, no relationship could again be established. Rather, the concentration of glycerol in *C. utilis* cells appears to follow an inverse trend

TABLE 3
INTRACELLULAR TREHALOSE IN *SPOROBOLOMYCES SALMONICOLOR* AND *SCHIZOSACCHAROMYCES POMBE*

Yeast	Growth phase	nmoles trehalose/mg dry weight
S. salmonicolor	Exponential	33.1 ± 2.0
	Stationary	14.0 ± 0.8
Schiz. pombe	Exponential	8.4 ± 0.4
	Stationary	10.8 ± 0.5

Results are the mean of two independent determinations.

to that expected in case of being involved in some protective role and the results are in agreement with the respiro-fermentative character of this yeast.

Mackenzie *et al.* (9) attributed the failure of exponential phase cells of *S. cerevisiae* to grow on a low a_w medium to death, not dormancy. In contrast, we have shown that the effect of the hyperosmotic shock in *C. utilis* consists solely in rendering the cells unable to divide under conditions of restricted water availability and that the same cells can revert to grow after transfer to high a_w medium.

The phenomenon of water stress plating hypersensitivity had been described previously in only two species (9). In *S. cerevisiae,* plating discrepancy was not evident for glucose concentrations lower than 40% in the low a_w media. In *C. utilis,* the existence of the phenomenon is already clear on low a_w media containing 30% glucose and it becomes more apparent as the glucose concentration increseases until 50%, limit at which no colonies are detected. This difference might reflect a minor xerotolerant nature of the yeast under study.

Our results do not allow to propose alternative candidates to trehalose or glycerol as the responsible agents for the relative tolerance of stationary phase cells to reinitiate growth under hyperosmotic conditions. The participation in this phenomenon of some stress-specific proteins being expressed at the stationary phase can not be dismissed.

References

1. Andre, L., Nilsson, A. and Adler, L. (1988). The role of glycerol in osmotolerance of the yeast *Debaryomyces hansenii.* J. Gen. Microbiol. **134,** 669-677.
2. Argüelles, J. C. and Gacto, M. (1985). Evidence for regulatory trehalase activity in *Candida utiis.* Can. J. Microbiol. **31,** 529-537.
3. Brown, A. D., Mackenzie, K. F. and Singh, K. K. (1986). Selected aspects of microbial osmoregulation. FEMS Microbiol. Rev. **39,** 31-36.
4. Crowe, J. M., Crowe, L. M. and Chapman, D. (1984). Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science **223,** 701-703.
5. Gadd, G. M., Chalmers, K. and Reed, R. H. (1987). The role of trehalose in dehydration resistance of *Saccharomyces cerevisiae.* FEMS Microbiol. Lett. **48,** 249-254.
6. Hocking. A. D. (1986). Effects of water activity and culture age on the glycerol accumulation patterns of five fungi. J. Gen. Microbiol. **132,** 269-275.
7. Hottinger, T., Boller, T. and Wiemken, A. (1987). Rapid changes of heat and desiccation tolerance correlated with changes in trehalose content in *Saccharomyces cerevisiae* cells subjected to temperature shifts. FEBS Lett. **220,** 113-115.
8. Kates, M. (1986). Analysis of glycerol. In: R. H. Burdon and P. H. van Knippenberg (eds.) Techniques in lipidology. pp. 140-141. Elsevier Science Pub. B. V. Amsterdam.
9. Mackenzie, K. F., Blomberg, A. and Brown, A. D. (1986). Water stress plating hypersensitivity of yeasts. J. Gen. Microbiol. **132,** 2053-2056.
10. Mackenzie, K. F., Singh, K. K. and Brown, A. D. (1988). Water stress plating hypersensitivity of yeasts: protective role of trehalose in *Saccharomyces cerevisiae.* J. Gen. Microbiol. **134,** 1661-1666.
11. Stewart, P. R. (1975). Analytical methods for yeasts. In: D. M. Prescott (ed.) Methods in Cell Biology **12,** pp. 117-147. Academic Press. New York.
12. Thevelein, J. M. (1984). Regulation of trehalose mobilization in fungi. Microbiol. Rev. **48,** 42-59.
13. Van Laere, A. (1989). Trehalose, reserve and/or stress metabolite? FEMS Microbiol. Rev. **121,** 1-9.
14. Yagi, T. (1988). Intracellular levels of glycerol necessary for initiation of growth under salt-stressed conditions in a salt-tolerant yeast, *Zygosaccharomyces rouxii.* FEMS Microbiol. Lett. **49,** 25-30.

MICROBIOLOGIA SEM 7 (1991), 49-52 **NOTAS**

Evaluación del Sistema Autoscan-4 en la identificación de cepas del género *Salmonella*

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(Recibido diciembre 12, 1990/enero 3, 1991)

Summary

A study was performed to compare the Autoscan-4 with conventional biochemical methods to identify isolates of the *Salmonella* genus. The Autoscan-4 yielded correct identification of the 99 % *Salmonella* isolates at the genus and species level, but failed to identify 74 % *Salmonella* isolates of the «Arizona» group, making necessary to perform subspecies biochemical reactions.

Key words: Salmonella, *identification.*

Resumen

Se ha realizado un estudio comparativo entre el método comercial Autoscan-4 y los métodos de identificación bioquímica convencionales para identificar cepas del género *Salmonella.* Los resultados obtenidos con Autoscan-4 coincidieron en el 99 % de los casos en el nivel del género y especie con los convencionales, pero hubo una discrepancia del 74 % en las cepas del grupo «Arizona». Los resultados obtenidos sugieren la necesidad de realizar en dicho grupo las reacciones bioquímicas de subespecie.

El sistema automatizado de lectura para identificación Autoscan-4 se utiliza en diferentes laboratorios de microbiología, en lugar de los métodos bioquímicos convencionales, siendo, por tanto, importante su comparación con los mismos para su correcta utilización.

La identificación de Autoscan-4 está basada en pruebas bioquímicas similares a otros sistemas comerciales existentes en el mercado, como son:

— Fermentación de los carbohidratos glucosa, sacarosa, sorbitol, rafinosa, ramnosa, arabinosa, inositol, adonitol y melibiosa.
— Hidrólisis de urea, esculina y ONPG.

(*) A quien debe dirigirse la correspondencia.

TABLA 1
DISTRIBUCION DE LAS 5.798 CEPAS DE *SALMONELLA* SEGUN EL RESULTADO OBTENIDO POR AUTOSCAN-4

Identificación de Autoscan	N.° de cepas	Grupos	% de fiabilidad	N.° de cepas
Salmonella Arizona	87	A	> 75	81
		B	50-55	6
S. typhi	115		> 75	
S. paratyphi A	5		> 75	
Salmonella sp	5.591		> 75	

— Utilización de citrato, malonato, tartrato y acetamida como única fuente de carbono.
— Producción de indol a partir de triptófano.
— Decarboxilación de lisina, arginina y ornitina.
— Presencia de triptófano deaminasa.
— Reacción de Voges-Proskauer.
— Producción de SH_2.
— Reducción de nitrato a nitrito.
— Tolerancia a la cetrimida.
— Resistencia a concentraciones específicas de penicilina, kanamicina, colistina, nitrofurantoína, cefalotina y tobramicina.

La interpretación de estas pruebas bioquímicas da lugar a la identificación de los siguientes grupos de *Salmonella: Salmonella spp, Salmonella typhi, Salmonella choleraesuis, Salmonella paratyphi A* y el grupo *Salmonella Arizona.*

Hemos comparado el sistema comercial Autoscan-4, comercializado por Baxter, S. A., en España, utilizando el panel Combo Negativo BP3 con los métodos convencionales utilizados en nuestro laboratorio (1). Las pruebas bioquímicas utilizadas son la fermentación de glucosa, lactosa y glicerol, la formación de gas a partir de glucosa, la producción de SH_2, utilización de citrato como única fuente de carbono, la hidrólisis de urea, la decarboxilación de lisina, arginina y ornitina, la producción de indol a partir de triptófano y la movilidad.

El estudio se realizó con las cepas de *Salmonella* recibidas en nuestro laboratorio para confirmación y serotipado en el año 1989 y primer semestre de 1990.

Las cepas recibidas se sembraron en placas de agar McC en aislamiento. Después de 18-24 horas a 37° C se seleccionaron varias colonias morfológicamente idénticas a partir de las cuales se inocularon los paneles comerciales siguiendo las instrucciones del fabricante y las pruebas bioquímicas de referencia elaboradas en nuestro laboratorio, poniéndose a incubar a 37° C tanto los paneles como las pruebas de referencia. Los paneles se leyeron a las 24 horas, después de añadir los reactivos correspondientes en el lector automático suministrado por Baxter, y las pruebas bioquímicas de referencia se leyeron a las 24 y 48 horas siguiendo las normas establecidas.

Todas las cepas se serotiparon de acuerdo con el método recomendado por el Instituto Pasteur (4). Las cepas identificadas por Autoscan-4 como «Sal/Arizona» se estudiaron para subespecie realizando las pruebas bioquímicas descritas por L. Le Minor (3).

Se estudiaron 5.798 cepas de *Salmonella* aisladas por distintos laboratorios de microbiología, tanto de hospitales como de salud pública y privados, de diferentes regiones de España.

Las 5.591 cepas identificadas por Autoscan-4 como *Salmonella sp* (Tabla 1), coincidieron en un 99 % con los resultados obtenidos por los métodos convencionales.

TABLA 2
DISTRIBUCION POR FUENTE DE AISLAMIENTO Y SUBESPECIE DE LAS CEPAS DE *SALMONELLA* IDENTIFICADAS COMO *SALMONELLA ARIZONA* POR AUSTOCAN-4

	FUENTE DE AISLAMIENTO										
	Humana		Alimento			Animal		Ambiente			
Subespecie	Heces	Sangre	Aves	Carne	Otros	Sangre caliente	Sangre fría	Agua corriente	Agua de río	Desconocido	Total
Grupo A*											
I										1	1
II	8	1	26	7	6	3			8	2	61
IIIa		1						1			2
IIIb				1		1	2		11	2	17
Grupo B**											
II	1		2			1					4
IIIb	1						1				2

* Grupo A: 81 cepas identificadas por Autoscan como *Salmonella Arizona* con un porcentaje de fiabilidad superior al 75%.

** Grupo B: 6 cepas identificadas por Autoscan como *Salnomella Arizona* con un porcentaje de fiabilidad entre el 50-55%.

Cinco cepas fueron identificadas como *S. paratyphi A* por Autoscan-4, de las cuales 4 fueron confirmadas bioquímica y serológicamente por los métodos convencionales (3 se aislaron de sangre y una de bilis) y una se identificó como *Salmonella abortusovis;* esta cepa se había aislado de placenta ovina.

El grupo de 115 cepas identificadas por Autoscan-4 como *S. typhi* supuso un 86% de coincidencia por ambos métodos (todas aquellas en las que coincidió la identificación fueron aisladas de muestras humanas). Las 16 cepas cuya identificación no coincidió fueron caracterizadas en nuestro laboratorio como *S. abortusovis,* dándose la circunstancia de que todas ellas se aislaron en muestras patológicas de ganado ovino.

Por último, las 87 cepas identificadas por Autoscan-4 como «Sal/Arizona» presentan importantes discrepancias (Tabla 2). Dichas cepas se pueden dividir en 2 subgrupos: subgrupo A (81 cepas), que comprende aquellas cepas cuyo margen de fiabilidad en la identificación según Autoscan-4 es igual o superior al 75 %, y subgrupo B, formado por las cepas con un grado de aceptabilidad entre el 50 y el 55 % según Autoscan-4 (6 cepas).

El grado de coincidencia entre ambos métodos en el subgrupo A fue del 26 %. Las 77 cepas que no coincidieron fueron identificadas por nuestro laboratorio como 76 de la subespecie II (el 82 % fueron *S. sofia* 1, 4, 12: b:-) y una de la subespecie I.

El subgrupo B estuvo formado por 4 cepas de la subespecie II y 2 cepas de la subespecie IIIb (anteriormente considerada como *Salmonella Arizona).*

Podemos concluir que la identificación a nivel de género realizada por el sistema Autoscan-4 es satisfactoria, tal como han encontrado otros autores (5), pero la identificación realizada por Autoscan-4 como *Salmonella Arizona* debe interpretarse con sumo cuidado, ya que solamente se pudieron identificar como subespecies IIIa o IIIb (anteriormente *S. Arizona)* el 26 % de las mismas.

Por esta razón sugerimos que cuando se obtenga por Autoscan-4 la identificación *Salmonella Arizona,* se realicen las pruebas bioquímicas necesarias para subespecie o en su defecto se envíe la cepa a un laboratorio de referencia, indicando que pertenecen al género *Salmonella.*

Las discrepancias observadas en la identificación de *S. paratyphi A* y *S. typhi* entre Autoscan-4 y el método de referencia, se pueden subsanar fácilmente atendiendo a la fuente de aislamiento de la cepa, ya que *S. paratyphi A* y *S. typhi* están adaptadas al ser humano, mientras que *S. abortusovis* está adaptada al ganado ovino.

Por último queremos agradecer a todos los laboratorios que nos envían cepas su colaboración, y a los técnicos de laboratorio Rosa Díez, Francisca Cerdán, Manuela de la Fuente y Rafaela Gutiérrez su inestimable trabajo en el laboratorio de referencia.

Bibliografía

1. 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.
2. Krieg, N. R. and Holt, J. G. (1984). Facultatively anaerobic Gram-negative rods. In: Tansill, B. (ed.). Bergey's Manual of Systematic Bacteriology, pp. 427-458. Williams and Wilkins. Baltimore.
3. Le Minor, L., Veron, M. and Popoff, M. Y. (1982). Proposition pour une nomenclarure des *Salmonella*. Ann. Inst. Pasteur (Microbiol.). **133 B,** 245-254.
4. Le Minor, L. and Popoff, M. Y. (1989). Antigenic formulae of the *Salmonella* serovars 5th revision. WHO Collaborating Centre for Reference and Research on *Salmonella*. Institut Pasteur. París.
5. Woolfrey, B. F., Lally, R. T. and Quall, C. O. (1983). Evaluation of the Autoscan-3 and Sceptor Systems for *Enterobacteriaceae* identification. J. Clin. Microbiol., **17,** 807-813.

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Comparison of quantitative analytical methods in evaluating acidophilic iron- and sulphur-oxidizing microorganisms liquid cultures

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(Received December 19, 1990/February 25, 1991)

Summary

Different quantitative methods to evaluate cell concentrations in acidophilic iron- and sulphur-oxidizing microorganisms liquid cultures have been compared with the «most probable number of viable germs» procedure. Plating and colony counting on recently developed solid media give good results when used with pure bacterial cultures, but plating efficiency decreases with natural mixed cultures.

Key words: Quantification, plating, acidophiles, metal-oxidizing.

Resumen

Diferentes métodos de análisis cuantitativo para la valoración de concentraciones celulares en cultivos líquidos de microorganismos oxidantes de hierro y azufre se han comparado con el procedimiento del «número más probable de gérmenes viables». El plaqueo y contaje de colonias sobre medios sólidos desarrollados recientemente produce buenos resultados cuando se usa con cultivos bacterianos puros, pero la eficacia de plaqueo disminuye en el caso de cultivos mixtos naturales.

Acidophilic microorganisms live at rather extreme conditions. Both, their environmental requirements and metabolism, lead them to build an acidic habitat which in turn affects the whole microflora growth (3, 9, 10, 11).

Mining industry has taken advantage of this very special environment, in those cases where acidophilic microorganisms show sulphur- or iron-oxidizing abilities. This kind of metabolic activities allows microbes to use reactions such as the sulphur to sulfate or the ferrous to ferric oxidations, as a source of energy (5, 10). As a result of these enzimatically catalized reactions, the pH of the medium decreases and in some cases the concentration of ferric ion gets very high levels, both factors

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TABLE 1
COMPARISON OF QUANTIFICATION RESULTS OBTAINED BY USING THE MOST PROBABLE (MPN) METHOD IN LIQUID MEDIUM AND PLATE CULTURE ON SOLID MEDIUM

Sample	MPN in ferrous (a) or sulphur (b) liquid cultures	Plating in ferrous (c) or thiosulphate (d) solid medium
Th. ferrooxidans ATCC23270	1×10^5 (a) ger/ml	6×10^6 (c) cfu/ml
Drainage mine water	4.5×10^6 (a) ger/ml	2×10^5 (c) cfu/ml
S-ozidizing T 3.2	1.5×10^8 (b) ger/ml	1.5×10^9 (d) cfu/ml
Drainage mine water	4.5×10^4 (b) ger/ml	1×10^3 (d) cfu/ml

contributing to create a very oxidant atmosphere which can lead to the solubilization of several metals (4, 7). Bioleaching is being used today for the metal recovery from low law minerals, and it is envisaged as one of the feasible methods to be used for metal extraction in the future (2, 10, 12).

On the other hand, some undesirable processes may also result of these microbial activities. Growth of some species of these kind of microorganisms on pyritic soils leads to sulfuric acid production and brings out the possibility of stream acidic pollution with its rather tragic effects in plant and animal populations (12).

Both, the applicability and control of these microorganism populations, requiere appropriate analytical methods to be available. Quantification procedures are especially important, due to the complexity of the populations to be studied. Culture plating and counting of grown colonies is the method most widely used for evaluating microbial cell concentration. However, the peculiar growing requirements of acidophilic oxidizing microorganisms, namely pH and also temperature in the case of thermophiles, have made very difficult the development of solid media suitable to get a good plating efficiency (6, 8, 13). Today, the method known as the determination of the most probable number (MPN) of viable germs, based on the dilution of a liquid culture and the detection of oxidant activity, is the most often used for this kind of analysis (12).

Nevertheless, microbial isolation and purification from colonies grown on solid media is still the best way to prepare pure strains, and has been repeatedly tried during last years with these acidophilic oxidizing microorganisms (6, 8, 13). These attempts have led to the improvement of plating efficiency. This paper shows the comparative results of different plating methods and MPN when used with an acidophilic microbial population.

Thiobacillus ferrooxidans ATCC 23270 was used as a pure acidophilic iron-oxidizing typical standard microorganism. A sulphur-oxidizing, recently isolated bacteria, T 3.2, has been chosen to monitor sulphur-oxidizing activity and bacterial growth. As a mixed and more natural population, water from the drainage stream of an uranium mine has been used.

Quantification assays of liquid cultures were made by the «most probable number (MPN) of viable germs» determination method (12).

Quantification assays on solid medium were made on agarose plates (5 g/l) with ferrous (13.9 g/l $FeSO_4 \cdot 7H_2O$) or thiosulphate (5 g/l $Na_2 S_2 O_3$) in saline MS solution (0.2 g/l $(NH_4)_2 SO_4$, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l KCl, 0.1 g/l $K_2 HPO_4 \cdot 3H_2O$, pH 2.0). Bromophenol blue (3 mg/ml) was added to the thiosulphate medium in order to improve colonies visualization (1).

TABLE 2
RESULTS OF GROWING EXPERIMENTS ON DIFFERENT TYPES OF PLATES

Sample	Ferrous plates			Thiosulphate plates		
	Standard	Pre-dried	Overlaid	Standard	Pre-dried	Overlaid
Th. ferrooxidans ATCC 23270	2×10^7 cfu/ml	2×10^7 cfu/ml	2×10^7 cfu/ml			
Drainage mine water	1.2×10^5 cfu/ml	spots	3×10^3 cfu/ml	No growth	No growth	No growth
S-oxidizing T 3.2				1.5×10^8 cfu/ml	2×10^8 cfu/ml	2×10^7 cfu/ml

Drying of plates was carried out by keeping them open into a 37° C oven for 30 min. Plates overlay was made by mixing 2 ml of molted solid medium at 40° C with 0.1 ml of bacterial culture and pouring it on a standard agarose plate.

Bacterial cultures quantification results obtained by the most probable number (MPN) method and expressed as number of viable germs per ml are showed in Table 1. In these experiments, *Thiobacillus ferrooxidans* ATCC 23270 and S-oxidizing T 3.2 pure cultures were iron- and sulphur-oxidizing controls, respectively. Mine water was used as a natural culture, and analized both in ferrous- and in sulphur-containing media. Also in Table 1, these numbers are compared with those obtained by counting colonies grown on ferrous or thiosulphate standard solid medium, expressed as colony forming units (cfu's) per ml. Even lacking a deep statistical analysis of the data shown, some points can be discussed.

As can be seen, cell quantification by plating and colony counting yields results rather similar or even slightly higher than those obtained by the MPN method when evaluating pure bacterial cultures. However, when both procedures are used on a natural, mixed microbial population like that occurring in the mine water, the situation is the opposite, MPN giving the highest results.

These data are explained on the basis of two different factors. One is the presence in the mixed population of other microorganisms which could affect the growth of iron- and/or sulphur-oxidizing bacteria on the solid medium used for these assays. The second one is the fact that pure strains have gone through several purification steps on solid medium and are probably adapted to this kind of growth.

Trying to find better conditions for counting colonies on solid medium, some modifications in preparing plates were checked, namely drying and overlaying. Pre-dryed plates were used trying to get a better absorption of the cellular suspension. On the other hand, overlaying might make cell implantation on the solid substrate easier and more homogeneous. Both alternative ways were analyzed and their results are showed in Table 2, in comparison with those obtained with standard plates. Data shown are an average of three experiments. No statistical study of these results was made.

Thiobacillus ferrooxidans shows identical plating efficiency on standard, pre-dried and overlaid plates. S-oxidizing T 3.2 gives slightly lower results on overlaid plates. Mixed population in mine water behaves, however, in a very different way; cell growth and colony formation on pre-dried plates apparently proceed with some difficulty, giving rise to large and heterogeneous spots which prevent any counting of colonies. This same microbial population, when overlaid on solid medium, shows a very homogeneous colony distribution on the plates, although yields rather lower numbers in comparison with those from standard plating.

So, plating can be recommended for analysing pure (adapted) bacterial cultures, while it would

not be the method of choice when working with natural mixed populations. Plating alternatives checked here seem not to offer advatanges when compared to MPN method in evaluating cell concentrations in natural liquid cultures. At the moment, this last procedure is, in our opinion, the most reliable one. Nevertheless, time consumption is an important drawback of MPN, and more attempts in developing faster quantification methods will probably be made.

References

1. Bryant, R. D., McGroarty, K. M., Costerton, J. W. and Laishley, E. J. (1983). Isolation and characterization of a new acidophilic *Thiobacillus* species (T. albertis). Can. J. Microbiol. **29,** 1159-1170.
2. Gentina, J. C. and Acevedo, F. (1985). Microbial ore leaching in developing countries. Trends in Biotechnology **3,** 86-89.
3. Huber, H. and Stetter, K. O. (1990). *Thiobacillus cuprinus* sp. nov., a novel facultatively organotrophic metal-mobilizing bacterium. App. Env. Microbiol. **56,** 315-322.
4. Ingledew W. J. (1982). *Thiobacillus ferrooxidans,* the bioenergetics of an acidophilic chemolithotroph. Biochim. Biophys. Acta **683,** 89-117.
5. Kelly, D. P. (1988). Evolution of the understanding of the microbiology and biochemistry of the mineral leaching habitat. In: P. R. Norris and D. P. Kely (eds.) Biohidrometallurgy, Proc. Intern. Symp. Warwick. pp 3-14. Science and Technology Letters. Kew, U. K.
6. Lindström, E. B. and Shelin, H. M. (1989). High efficiency of plating of the thermophilic sulphur-dependet archaebacterium *Sulfolobus acidocaldarius* App. Env. Microbiol. **55,** 3030-3021.
7. Lizama, H. M. and Suzuky, I. (1989). Rate equations and kinetic parameters of the reactions involved in pyrite oxidation by *Thiobacilus ferrooxidans.* App. Env. Microbiol. **55,** 2918-2923.
8. Manning, H. L. (1975). New medium for isolating iron-oxidizing and heterotrophic acidophilic bacteria from acid mine drainage. App. Microbiol. **30,** 1010-1016.
9. Miroshnichenko, N. L., Bonch-Osmolovskaya, E. A., Neuner, A., Kostrikina, N. A., Chernych, N. A. and Alekseev, V. A. (1989). Thermococcus stetteri sp. nov., a new extremely thermophilic marine sulphur metabolizing archaebacterium. System. App. Microbiol. **12,** 257-262.
10. Norris, P. R. (1990). Acidophilic bacteria and their activity in mineral sulphide oxidation. In: H. L. Ehrlich and C. L. Brier ley (eds.) Microbial Mineral Recovery. pp. 3-54. McGraw Hill Pub. Co. New York.
11. Pledger, R. J. and Baross, J. A. (1989). Characterization of an extremely thermophilic archaebacterium isolated from a black smoker polychaete (Paravinella sp.) at the Juan de Fuca ridge. System. App. Microbiol. **12,** 249-256.
12. Silver, M. (1987). Distribution of iron-oxidizin bacteria in the nordic uranium tailings deposit, Elliot Lake, Ontario, Canadá. App. Env. Microbiol. **52,** 846-852.
13. Tuovinen, O. H. and Kelly, D. P. (1974). Studies on the growth of *Thiobacillus ferrooxidans.* Arch. Microbiol. **98,** 351-364.