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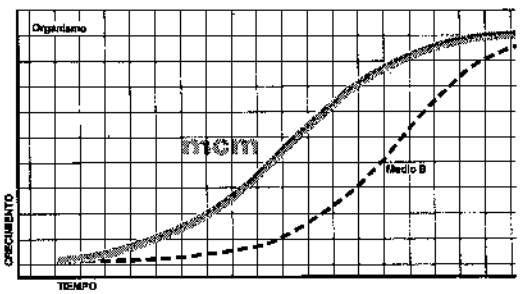

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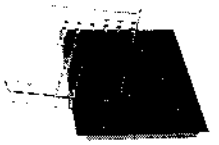

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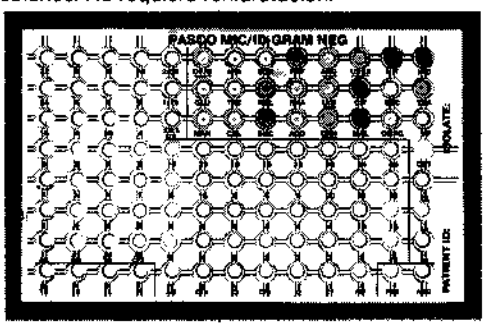

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INDICE

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MICROBIOLOGIA SEM 5 (1989), 5-12 **MINIREVIEW**

DNA replication of plasmids from Gram-positive bacteria in *Bacillus subtilis*. Plasmid pUB110 as a model system

Juan C. Alonso

Max-Planck-Institut für Molekulare Genetik. Ihnestrasse 73, D-1000 Berlin 33. F. R. Germany
(Received March 27, 1989)

Summary

The small high copy plasmids from gram-positive bacteria like pUB110 replicate via an asymmetric rolling circle mechanism. On the based of several criteria those plasmids could be subdivided in four different families. By analysing pUB110 replication in *B. subtilis* as a model system we have obtained information on the way by which initiation, elongation and termination in replication is accomplished in *B. subtilis*.

Key words: Bacillus subtilis, *DNA replication, plasmids.*

Resumen

En bacterias Gram-positivas los plásmidos pequeños de alto número de copias, como pUB110, replican vía un círculo rotatorio asimétrico. Basándonos en varios criterios, hemos subdividido a dichos plásmidos en cuatro familias diferentes. Utilizando pUB110 como sistema modelo para estudiar replicación de plásmidos en *B. subtilis*, hemos obtenido información sobre los procesos de iniciación, elongación y terminación de replicación del DNA plasmídico en *B. subtilis*.

The replication cycle of the small high copy plasmids from gram-positive bacteria can be divided into two stages: leading and lagging strand synthesis. In the first stage the replication of the leading strand is accomplished, requiring besides host functions the plasmid encoded initiator protein (Rep). In the second stage the synthesis of the complementary lagging strand is either initiated as soon as an initiation signal is exposed in the single-stranded form (plasmid pUB110) or after the leading strand synthesis terminates (pC194). Mechanistically this mode of plasmid replication (sigma replication) resembles the DNA replication of single-stranded *E. coli* phages [e.g. ΦX174, see (6) for a review]. Unlike ΦX174, initiation of pUB110 or pT181 plasmid replication is indirectly controlled and the replication initiation protein (Rep) which is trans-active is used only once (rate limiting factor). This mode of plasmid replication clearly differs from that described for plasmids from gram-negative bacteria. There, theta type replication is the common mode, and leading and lagging strand synthesis might be primed in close vicinity and both strands remain covalently closed. The elongation step could be uni- or bidirectional and finally the relaxed concatemeric dimer is resolved into monomeric rings [see (36) for a review]. Furthermore, this mode of replication differs from sigma or rolling circle replication commonly found in double-stranded DNA bacteriophages, because in those phages leading and lagging strand DNA synthesis are coupled.

TABLE 1
FAMILY GROUPING OF THE GRAM-POSITIVE PLASMIDS, AND THEIR BEHAVIOUR IN *B. SUBTILIS*

Family	Plasmid	Presence of SS(c) DNA	Marker[f]	Host	References
pT181[a]	pT181	yes	Tc^r	*Sau*	32
	pS194	yes	Sm^r	*Sau*	32
	pC221	yes	Cm^r	*Sau*	32
	pUB112	yes	Cm^r	*Sau*	32
	pC223	yes	Cm^r	*Sau*	32
	pNS1	nd	Tc^r	*Sau*	32
	pTZ12	nd	Cm^r	*Cxe*	5
pIM13[b]	pIM13	yes	Em^r	*Bsu*	31
	pE5	nd	Em^r	*Sau*	31
	pE12	nd	Em^r	*Sau*	31
	pSN2	nd	cryptic	*Sau*	31
	pNE131	nd	Em^r	*Sep*	31
pLS1[c]	pLS1	yes	Tc^r	*Sag*	20
	pE194	yes	Em^r	*Sau*	18
	pADB201	nd	cryptic	*Mmy*	7
pUB110[d]	pUB110	no	Nm^r, Pm^r	*Sau*	21
	pC194	yes	Cm^r	*Sau*	18
	pAMα1	nd	Tc^r	*Sfa*	30
	pRBH1	no	Nm^r	*Bacilli*[e]	26
	pTHT15	nd	Tc^r	*Bacilli*[e]	17
	pFTB14	nd	cryptic	*Bam*	27
	pBAA1	no	cryptic	*Bsu*	11
	pSN1981	nd	Tc^r	*Bsu*	34
	pBC16	no	Tc^r	*Bce*	8
	pCB101	nd	cryptic	*Cbu*	25

The initiation replication protein Rep with a molecular weight mass of ~37 kDa[a], ~17 kDa[b] ~24 kDa[c] and ~39 kDa[d] defines the families. The SS(c) DNA threshold was arbitrarily fixed as 10 % of total intracellular plasmid DNA as SS(c) DNA. The species they were isolated from are shown in their conventional three letter code and[e] thermophilic Bacilli; nd, not done. [f] Cm^r = chloramphenicol; Em^r = erythromycin; Nm^r = kanamycin; Pm^r = phleomycin and Tc^r = tetrocycline resistance.

The majority of the small multicopy plasmids of gram-positive bacteria can be classified into at least four families. Plasmids from the same family share, to a certain extent, the same genome organization, a high degree of DNA sequence homology in their replication regions and use the same basic copy control mechanism. Each of those plasmids codes for a positive trans-acting product (Rep) that is negatively regulated by an antisense RNA (*cop* or *inc)* and is transcribed in the same direction in which leading strand replication proceeds.

The first family of plasmids were isolated either from *Staphylococcus aureus* or *Corynebacterium xerosis*. Plasmid pT181 is the prototype of this family (see Table 1). The second family of plasmids were either isolated from *Staphylococcus* or *Bacillus subtilis*, with pIM13 as prototype. The third family of plasmids were isolated either from *S. aureus, Mycoplasma mycoides* or *Streptococcus agalactiae*, pLS1 is its prototype. The plasmids placed into these three families naturally accumulate high amounts of circular single-stranded [SS(c)] DNA (33, our unpublished results) which lead to a cell physiological stress in *B. subtilis* (41).

In this review we will be concerned with the fourth family of small multicopy plasmids which show a wider host distribution. They were either isolated from *Staphylococcus aureus, Streptococcus faecalis, Clostridium butyricum, Bacillus amyloliquefaciens, Bacillus cereus, Bacillus subtilis,* or thermophilic Bacilli. Plasmids belonging to this family encode a Rep protein of about 39 kDa and they accumulate very little SS(c) DNA [except for pC194 (1, 33)]. pUB110 is the best characterized representative of this family of plasmids.

Plasmid pUB110 leading strand synthesis is normally closely associated with lagging strand synthesis, but they can be easily dissociated from each other by *in vitro* manipulation. Therefore, from studying pUB110 plasmid or pUB110 derivatives with decoupled leading and lagging strand DNA synthesis, we can obtain information on the way by which initiation, elongation and termination of the replication process of pUB110 is accomplished in *B. subtilis*. We choose *B. subtilis* as the species to perform this studies. Hence, a large body of information on well characterized plasmids in *Staphylococcus aureus* and *Streptococcus pneumoniae* will not be discussed.

Concerning pUB110 replication I shall first discuss the effectors involved. Recently the determinants for the negative (IncA) and the positive (RepU) trans-acting products of pUB110 have been mapped. These define the minimal region that is necessary and sufficient for autonomous plasmid replication [coordinates 3118-4316 (23), Fig. 1 A] in Rec^+ cells (22). RepU which has a molecular mass of 39 kDa was shown to bind to the *oriU* DNA region, and to trigger initiation of leading strand synthesis (22).

In analogy to *E. coli* single stranded phages fl or ΦX174 also in pUB110 the use of an internal initiator codon within the RepU open reading frame leading to a polypeptide that is 92 amino acids smaller than RepU, termed RepB, was proposed (26). Although a striking analogy can be drawn between RepB, protein X of phage fl (12) and protein A* of ΦX174 we failed to detect RepB protein synthesis in an heterologous host as *E. coli* (22). Furthermore, this internal open reading frame is absent in highly related plasmids as pFTB14 or pBAA1 (see above).

The RepU-dependent replication origin, termed *ori*U (Fig. 1 B), has been localized within a 24 bp segment at coordinates 4293-4316 that lies upstream of the RepU coding region (Fig. 1 A). Immediately downstream of *ori*U a «termination enhancer» region that is not absolutely required, but rather serves to potenciate termination of leading strand synthesis has been reported [(2); Fig. 1 B]. However, the pC194 RepH recognition site (non-covalent binding) seems to be located downstream the nicking site (our unpublished results).

The major lagging strand origin, *ori*L, has been identified withing a 140 nucleotide segment between coordinates 1380 to 1520 [(41), Fig. 1 C]. The *ori*L DNA sequence is a cis-activated and orientation-dependent determinant required for normal plasmid replication (40, 41). Furthermore, it was shown that *in vivo* the *ori*L region does not constitute an origin of replication of its own. pUB110 derivatives in which the *rep*U open reading frame was truncated or *ori*U was deleted *in vitro* were unable to form colonies at non-permissive temperature when fused to a thermosensitive replicon (22). Plasmid pC194 although belonging to the same family accumulates SS(c) DNA (see Table 1). However, insertion into pC194 of the *ori*L region of pUB110 in the proper orientation for priming eliminates the accumulation of SS(c) DNA (41) pointing to the independence of the effectors concerned.

With the signal structures and effectors defined, replication proceeds as follows (see Fig. 2): Leading strand synthesis of plasmid pUB110 can be divided into three steps: i) Initiation: the rate limiting RepU protein binds at the leading strand origin (*ori*U) upstream nicking site (non-covalent binding) [(22), see Fig. 1 B]. Within this region sequence deviations can occur among highly related plasmids (2), which indicate that not all 24 nucleotides are essential for RepU-*ori*U interaction. Similar results were reported for plasmid pC221 (38). No high energy cofactor is required but the substrate must be negatively supercoiled (2, 3). It has been reported that RepD protein of plasmid pC221 makes a single strand DNA cleavage at *ori* (leading strand origin) and remains attached to the

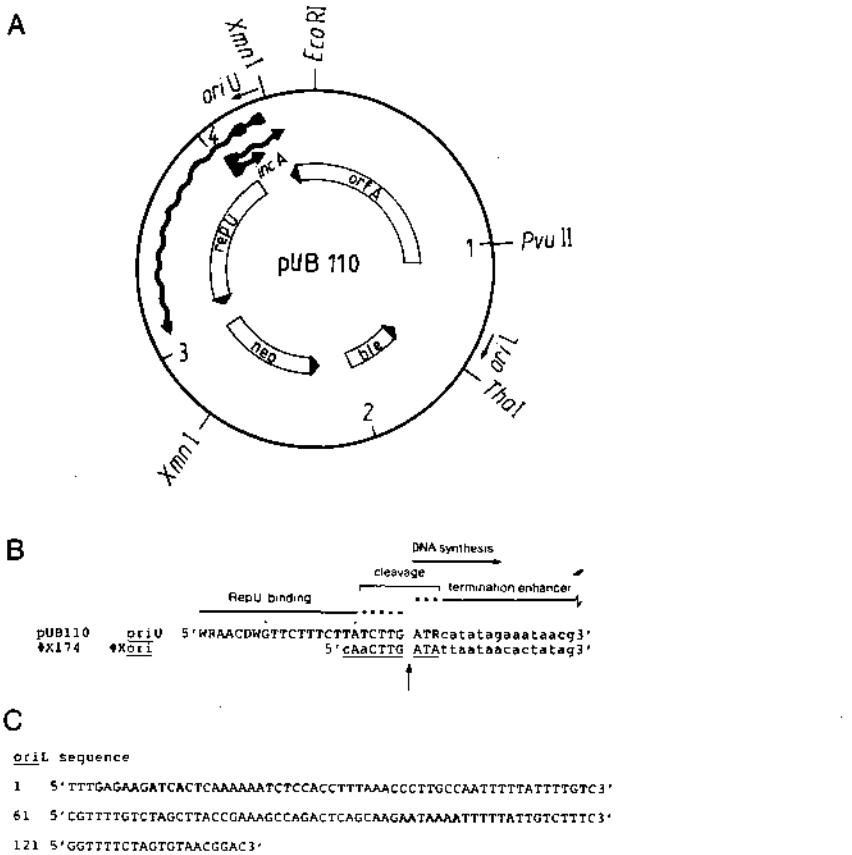

Fig. 1 A. Physical map of pUB110. The wavy lines represent RNA transcripts of *rep*U and *inc*A (copy number repressor) and the boxes in the wavy lines denote the putative promoters. Internal arrows indicate the open reading frames. The leading (*ori*U) and lagging (*ori*L) strand origins and the direction of DNA synthesis are indicated. Fig. 1 B. The DNA sequence of the leading strand origin is aligned with the ΦX174 origin. In the case of pUB110 upper case letters indicate the *ori*U DNA sequence and lower case letters indicate sequence outside the minimal origin for initiation, but for termination (termination enhancer). RepU binding and cleavage regions are indicated. The dots over the DNA sequence indicate nucleotides 4310 and 4300, respectively. The ΦX174 minimal sequence for protein A *in vitro* cleavage is underlined. The ΦX174 and postulated pUB110 nicking sites are indicated by a vertical arrow. R = purine, W = adenine or thymidine and D = no cytosine (UIB). Fig. 1 C. The DNA sequence of the 140 base pair *ori*L region (coordinates 1380 to 1520). The displaced strand corresponds to the non-coding strand of the RepU effector.

5' terminus (by a tyrosine residue) during strand displacement. This seems to hold true also for plasmid pUB110, but there RepU produces the nick, and the non-covalent binding to *ori*U is upstream the nicking site [see (2), our unpublished results]. The RepU protein may provide a «functional gap» which allows a 3' to 5' DNA helicase (putative *B. subtilis* Rep-like helicase) to bind and initiate unwinding of DNA. ii) Elongation: the 3' hydroxyl (primer) end at the nicking site is then extended by DNA Polymerase III (2, 37). As soon *ori*L becomes single-stranded, lagging strand synthesis initiates. However for simplicity we describe lagging strand synthesis separately. iii) Termination: After one full round of replication, RepU terminates the strand displacement by cleaving the regenerated *ori*U to produce mainly unit length single-stranded circular [SS(c)] DNA similar to the ΦX174 bacteriophage (19). However, plasmids bearing deletions of a DNA sequence immediately downstream the

nicking site (see Fig. 1 B) fail to recognize *ori*U as a termination signal at a high frecuency. This permits the replication to continue around the DNA circle leading to the accumulation of oligomeric plasmid products (2). Therefore, a termination enhancer and perhaps a host-encoded product are needed for precise termination. When certain host component(s) become rate limiting the DNA sequence requirement for cleavage by RepU protein is «more relaxed». Additional sites (resembling the *ori*U termination domain, 5 to 6 nucleotides in length) can be cleaved (22) and termination takes place (2). Hence, we hypothesized that termination of leading strand synthesis requires an active RepU protein bound to the 5' terminus, a complete *ori*U sequence, the «terminator enhancer» and a host component(s).

Lagging strand synthesis can be divided into five steps («prepriming», priming, elongation, primer removal and gap filling): i) «prepriming», at *ori*L, takes place 2.7 kb from the RepU target site (41). At present it is not clear what signal(s) in the DNA template is responsible for such event to occur. We have failed by computer assistance to predict putative stable secondary structures which may serve as such signal. Recently, however, it has been suggested that a palindromic sequence (*pal*A) is required for priming lagging strand synthesis of plasmids belonging to the pT181 and pLS1 family (see Table 1) as well as pC194 (10, 14). Such *pal*A structure was not found in plasmid pUB110 (14). ii) Priming: it is thought that the primosome may move in a direction opposite to that in which the DNA chain is elongated to generate multi-primers (3, 19). Since, a multi-primer seems to be formed, we hypothesize that neither RNA polymerase (as required for filamentous phages) nor

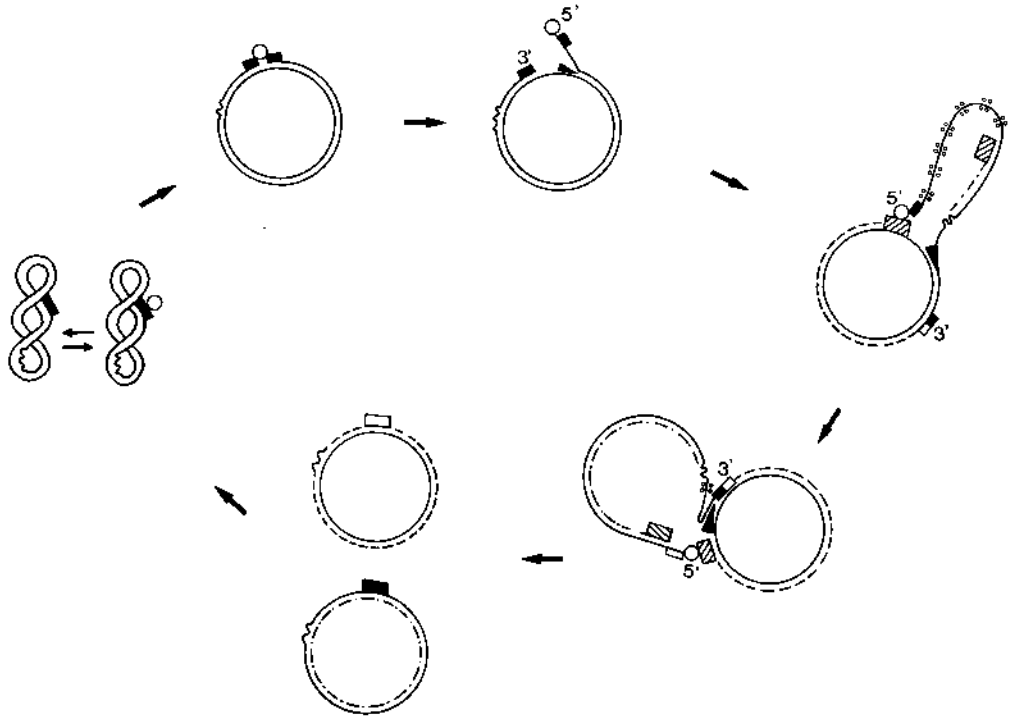

Fig. 2. Model of pUB110 plasmid replication. The RepU protein binds to the pUB110 supercoiled DNA origin, generating a specific nick and a «functional gap». DNA helicase and DNA pol III account for asymmetric replication. RepU terminates DNA synthesis after a full round of replication. Broken lines represent DNA pol III-dependent newly synthesized DNA initiated at the RepU-dependent 3' -end, dotted broken lines correspond to the DNA pol III-dependent chain elongation at primed 3' -end Primase-dependent lagging strand origin. ○ Plasmid-encoded RepU protein. ▨ DNA polymerase III. ▶ DNA helicase. ∞ SSB. ■ and □ leading strand origin and ~ lagging strand origin.

DNA primase alone (required by G4-like phages) are responsible for lagging strand priming at *ori*L [see (6) for a review]. iii) Elongation: of the primer is then performed by DNA polymerase III. iv) Primer removal: DNA polymerase I may account for removal of the RNA primers, and v) Ring closure: DNA ligase performes the closure reaction and DNA gyrase introduces superhelical turns (2).

In *ori*L$^-$ pUB110 derivatives, a second priming mechanism was identified. There, the DnaB protein may direct DNA primase and perhaps other components to a normally not used, uncharacterized lagging strand origin, to synthesize the primer (3). Alternatively, a RecE-mediated conversion from SS(c) to dsDNA occurs. No mutants in the postulated pre-primosome complex and DNA ligase are available so far.

Although belonging to the pUB110 family, plasmid pC194 differs in the mechanism by which leading and lagging strand syntheses start. Both, pC194 and pUB110 plasmids share a high degree of homology at their replication origin and their initiation replication proteins [RepU and RepH respectively (22)]. However, RepU which binds to a DNA sequence 3' -end of the nicking site (Fig. 1 B) could use for early events in such DNA recognition a putative helix-turn-helix DNA binding motif; whereas RepH seems to recognize a DNA sequence 5' -end of the nicking site and might utilize a different mechanism for sequence recognition because such a DNA binding motif remained undetected.

Unlike pUB110, lagging strand synthesis in pC194 is thought to initiate at or within an asymmetric DNA palindrome, *pal*A (10, 14). Such DNA synthesis could initiate after a full displacement of the leading strand. This is consistent with the fact that a high accumulation of SS(c) DNA with unique polarity is observed during pC194 replication in *B. subtilis* (33).

However, in the absence of *pal*A, as reported for *ori*L$^-$ derivatives of pUB110 (40) pC194 is not stably maintained, has a reduced copy number and cannot be transformed in *rec*E4 mutants under certain condition (low temperature and rich media). This suggests that the *pal*A signal is recognized in *B. subtilis* but priming at *pal*A may occur only after full displacement of the leading strand.

Evolutionary considerations

It is surprising that the low dG + dC content of all plasmids listed in Table I (32 % to 34 %) does with the exception of *S. aureus* not follow the genomic dG + dC content of the species from which the above plasmids were isolated (24 %, *M. mycoides)*, (42 %, *B. subtilis)* or (57 %, *C. xerosis)* (15). This bias in base composition is not compatible with the neutral theory of molecular evolution (28). It is conceivable that selective forces favouring high dA + dT content were operative during plasmid evolution. A high dA + dT content would e.g. reduce the free energy required for strand separation during plasmid replication. Indeed, this assumption is compatible with the observation that by increasing *in vitro* the dG + dC content aberrant replication forms can be detected [(40), our unpublished results].

Intergeneric and interspecies, transfer of genetic material has been documented (42) and either involves plasmid mobilization between closely related genera, such as *Escherichia, Shigella* or *Salmonella* or a wider interspecies mobilization. The promiscuity of interspecies transfer could be accomplished mainly by two routes: (a) plasmids may be highly autonomous in their replication machinery as in the case of plasmid RSF1010. This plasmid codes for its own initiation replication protein, DNA primase and DnaB-like (5' → 3' helicase) proteins (35) and requires from the host only highly conserved functions like a processive DNA polymerase, SSB, DNA topoisomerases, etc., or (b) plasmids as those from gram-positive bacteria which use a very simple replication mechanism. In plasmid replication as discussed above leading strand synthesis requires the plasmid encoded initiator-terminator protein and relies on highly conserved host functions like a processive DNA polymerase, SSB, DNA topoisomerases and any 3' → 5' helicase. Lagging strand synthesis is accomplished only by the usage of highly conserved functions (41). In that different avenues may be followed as reported for the *E. coli* single-stranded phages as M13, G4 or ΦX174 [see (6) for a review].

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Utilización de nitrato por bacteroides y citosol de nódulos formados por *Rhizobium leguminosarum*

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Summary

Nitrite production by nodules and roots of pea plants (*Pisum sativum* L., cultivar Alaska) inoculated with *Rhizobium leguminosarum* strain 3855 has been studied. Nitrate reductase (NR) activity and nitrite reductase (NiR) activity of the bacteroidal and citosolic fractions of the nodules were also determined, as well as the nitrite content of the nodules cytosol. Nitrite production by nodules and roots from plants treated with 5 mM KNO_3 was higher than that of nodules and roots from plants not treated with nitrate, and regardless of the nitrate treatment, nitrite production increased with the incubation period. The presence of nitrate, propanol or both compounds in the incubation mixtures significantly increased the nitrite production by nodules and roots.

Nitrite reductase activity was detected in fresh by isolated bacteroids of *R. leguminosarum* strain 3855, although the presence of nitrate reductase activity could not be detected both in bacteroids of nodules isolated from plants treated or not with 5 mM KNO_3. After isolation, when bacteroids were incubated in a mixture with nitrate, nitrate reductase activity developed after incubation for 12 h. Consequently, there was an increase in nitrite reductase activity, which resulted in the disappearance of the nitrite previously accumulated in the incubation medium. Nitrate utilization by bacteroids was not detected until 5 h from the beginning of the incubation period. Since the presence of chloramphenicol or rifampicin in the incubation medium prevented the development of the nitrate reductase activity, such activity was induced in bacteroids.

Nitrite content and nitrate reductase and nitrite reductase activities of the cytosol from nodules of pea plants treated or not with 5 mM KNO_3 varied with the buffer used for nodules homogeneization. However, no nitrite was found when nodules were homogeneized with ethanol, what indicates that nitrite accumulation in the cytosol occurs during the homogeneization process of the nodules.

Key words: Rhizobium leguminosarum. Pisum sativum, *bacteroid, cytosol, nitrate and nitrite reductase.*

Resumen

Se ha estudiado la producción de nitrito por los nódulos y las raíces de plantas de guisante (*Pisum sativum* L. variedad Alaska) inoculadas con la raza 3855 de *Rhizobium leguminosarum*. Tam-

(*) A quien debe dirigirse la correspondencia.

bién se han determinado las actividades nitrato reductasa (NR) y nitrito reductasa (NiR) de las fracciones bacteroidal y citosólica de los nódulos, así como el contenido en nitrito del citosol de los mismos. La producción de nitrito por los nódulos y las raíces de plantas tratadas con KNO_3 5 mM fue mayor que la de los nódulos y raíces de plantas no tratadas con nitrato, e independientemente del tratamiento, la producción de nitrito aumentó con el tiempo de incubación. La adición de nitrato, propanol o de ambos compuestos produjo incrementos estadísticamente significativos en la producción de nitrito por los nódulos y las raíces.

En bacteroides recién aislados de *R. leguminosarum* 3855 se detectó actividad nitrito reductasa, aunque no se pudo demostrar la presencia de actividad nitrato reductasa, tanto en bacteroides aislados de nódulos de plantas no tratadas como tratadas con KNO_3. Cuando después de su aislamiento los bacteroides se incubaron en una mezcla con nitrato se observó la aparición de actividad nitrato reductasa después de 12 h de incubación. Consecuentemente, hubo un aumento en la actividad nitrito reductasa que resultó en la desaparición del nitrito acumulado en el medio de incubación. No se detectó consumo de nitrato por los bacteroides hasta 5 h después del inicio del período de incubación. La presencia de rifampicina o cloranfenicol en el medio de incubación previno el desarrollo de la actividad nitrato reductasa, por lo que puede considerarse que tal actividad fue inducida en los bacteroides.

El contenido en nitrito y las actividades nitrato reductasa y nitrito reductasa del citosol de los nódulos de plantas de guisante, tratadas o no con KNO_3 5 mM, varió con el tampón utilizado para homogeneizar los nódulos. Sin embargo, no se detectó nitrito cuando los nódulos se homogeneizaron con etanol, lo que parece indicar que el nitrito sólo se acumula en el citosol durante el proceso de homogeneización de los nódulos.

Introducción

Los nódulos de las leguminosas de los géneros *Glycine, Vigna* y *Medicago* expresan actividad nitrato reductasa (NR) tanto en el citosol como en los bacteroides, mientras que los nódulos de los géneros *Phaseolus* y *Pisum* sólo presentan tal actividad en la fracción citosólica (3). Ambas enzimas son diferentes ya que la NR del citosol, como las de otros tejidos vegetales, utiliza NADH como donador de electrones y su peso molecular aproximado es de 500 kDa (9, 15, 27). La NR bacteroidal tiene un peso molecular estimado de 70 kDa y aunque los bacteroides intactos pueden utilizar succinato como fuente de energía (17), los extractos de NR sólo aceptan electrones de derivados del viológeno, siendo el metil viológeno el más efectivo (15).

Basándose en el empleo $^{15}NO_3^-$, algunos autores han estimado que la expresión de actividad NR en los nódulos representa del 1,5-3 % del contenido en nitrógeno reducido total de la planta (29). También se ha calculado que los bacteroides contribuyen con más del 90 % al total de la actividad NR de los nódulos (4, 14, 26).

La mayoría de las especies de los géneros *Rhizobium* y *Bradyrhizobium* y sus bacteroides, también contienen NR de tipo desasimilatorio (respiratorio), lo que capacita a estas células para utilizar el nitrato como aceptor final de electrones. Este mecanismo permitiría a los bacteroides sobrevivir durante períodos de anoxia de las plantas o en condiciones de microaerofilia (19, 23).

Se ha sugerido, sin embargo, que el nitrato no tiene acceso a la zona infectada por los bacteroides, quedando su localización restringida a la región externa del córtex de los nódulos (25) y que, por tanto, el nitrato no es metabolizado por los bacteroides (11).

Este trabajo se inició con el objeto de estudiar la actividad NR en los nódulos formados en la simbiosis *R. leguminosarum-Pisum sativum*, así como examinar la utilización de nitrato por el citosol y los bacteroides de *R. leguminosarum*. En los bacteroides de esta especie no se ha demostrado la existencia de actividad NR.

Materiales y métodos

Plantas y microorganismos

Se ha utilizado guisante (*Pisum sativum* L.), var. Alaska en simbiosis con la raza 3855 de *Rhizobium leguminosarum*. Los procesos de esterilización e inoculación de las semillas, así como la forma de cultivo y las condiciones de crecimiento de las plantas, se han publicado previamente (5). Las plantas crecieron dependiendo exclusivamente de la fijación de N_2 o se trataron con KNO_3 5 mM cinco días antes de la toma de las muestras.

Determinación de las actividades enzimáticas

La actividad NR de los nódulos y raíces se estudió mediante ensayos *in vivo* siguiendo una técnica de infiltración al vacío (16). Para ello, los nódulos enteros y las raíces homogéneamente troceadas (2-3 mm de longitud) se sumergieron en 4 ml de tampón fosfato potásico (pH 7,5) adicionado de EDTA-Na_2 1 mM. Este medio de incubación contenía o carecía de KNO_3 50 mM y propanol (1 %, v/v). El material infiltrado se incubó a 30° C durante 120 min.

Previa a la determinación de la actividad NR en bacteroides y citosol, ambas fracciones se obtuvieron homogeneizando 2 g de nódulos en 10 ml de los siguientes medios de extracción: tampón Tris/fosfato potásico 200 mM/50 mM, pH 7,5; cisteína 5 mM; FAD 10 μM; EDTA-Na_2 5 mM; albúmina 1 % (p/v), sacarosa 300 mM y polivinilpolipirrolidona (PVP) 2,5 % (p/v) (11); tampón maleico/KOH 100 mM, pH 6,8; sacarosa 100 mM, 2-mercaptoetanol 2 % (v/v); etilenglicol 15 % (v/v) y PVP (1/3 del peso fresco de nódulos); tampón fosfato potásico 50 mM, pH 7,5; EDTA-Na_2 2 mM; ditiotreitol (DTT) 2 mM; albúmina 1,5 % (p/v) y PVP (1/3 del peso fresco de nódulos). Los nódulos también se homogeneizaron en presencia de etanol (96 %, v/v).

Después de homogeneizar los nódulos, la mezcla resultante se filtró a través de cuatro capas de gasa y se centrifugó a 250 × g durante 5 min para eliminar la PVP y los residuos vegetales más gruesos. El sobrenadante se recentrifugó a 10.000 × g durante 10 min para sedimentar los bacteroides. El sobrenadante se volvió a centrifugar a 20.000 × g durante 20 min, utilizándose el nuevo sobrenadante como fracción citosólica en la que se determinaron las actividades NR y NiR, así como su contenido en nitrito.

Los bacteroides sedimentados se resuspendieron y lavaron 2 veces con tampón MOPS/KOH, pH 7,5, 50 mM, y se recogieron finalmente en 5 ml del mismo tampón.

Los procesos de homogeneización y filtrado se llevaron a cabo en condiciones anaeróbicas, utilizándose una campana de metacrilato, diseñada de forma que permite la entrada y salida de gases, así como la manipulación del material que se introduce en ella. Se utilizó argon para conseguir la exclusión de oxígeno. La centrifugación de los bacteroides se realizó en tubos previamente gaseados con argon y provistos de cierre hermético para evitar entrada de oxígeno. Igualmente, los tampones se gasearon con argon antes de su empleo.

Los bacteroides aislados se incubaron en un medio que contenía: 0,65 ml de tampón MOPS/KOH, pH 7,5, 50 mM; 0,05 ml de KNO_3, 10 mM; 0,1 ml de una mezcla de glucosa (50 mM) y succinato sódico (50 mM), y 0,2 ml de bacteroides (0,1-0,3 mg de proteína, aproximadamente). En algunos casos, a la mezcla de incubación se adicionó rifampicina (20 μg/ml) o cloranfenicol (100 μg/ml).

Los bacteroides se incubaron anaeróbicamente (atmósfera de argon) a 28° C durante 25 horas. Se tomaron alícuotas de 2 ml a intervalos de tiempo regulares y se centrifugaron a 8.000 × g durante 10 min. En el sobrenadante se determinó el contenido en nitrato y nitrito. Los bacteroides se lavaron 2 veces con tampón MOPS/KOH, pH 7,5, 50 mM y se resuspendieron finalmente en 1 ml de tampón MOPS/KOH, pH 7,5, 125 mM, adicionado de EDTA-Na_2 0,25 mM.

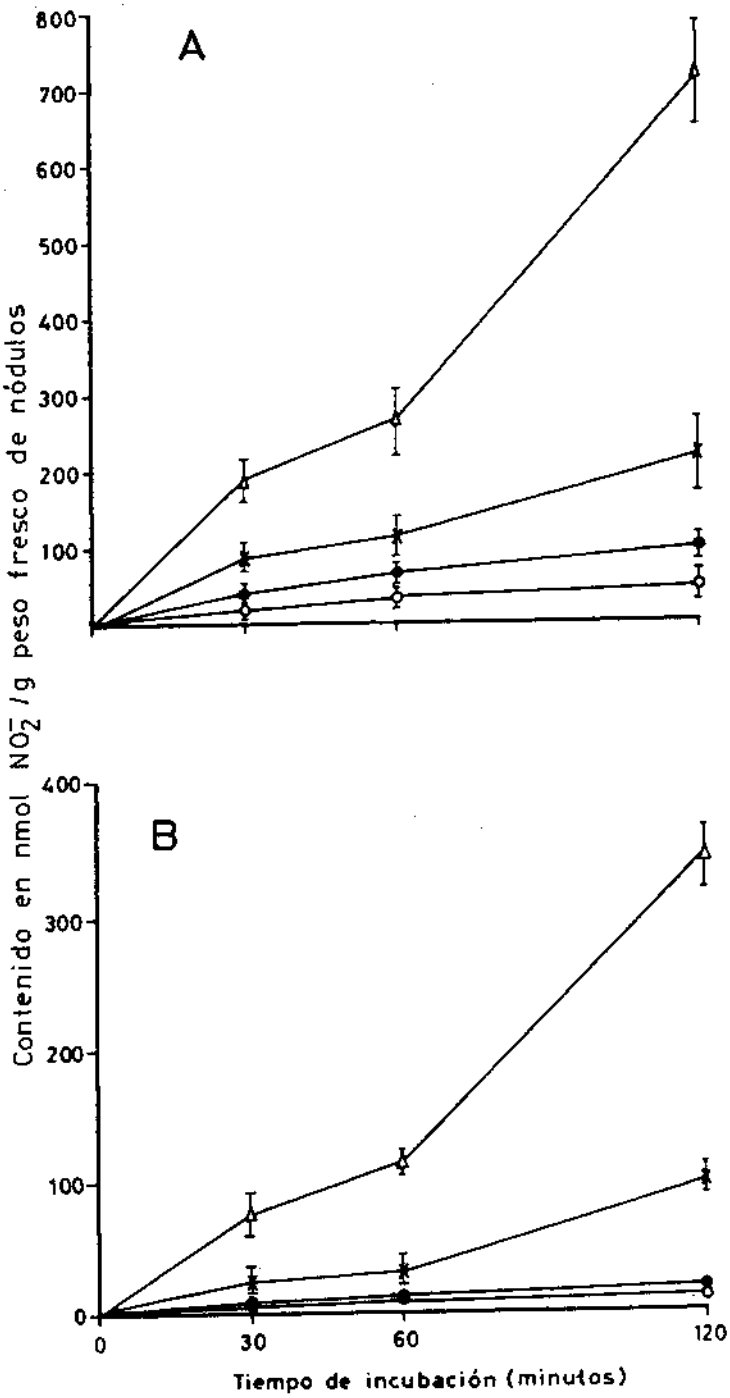

Fig. 1. Actividad nitrato reductasa (NR) *in vivo* de los nódulos formados por *Rhizobium leguminosarum* 3855 en simbiosis con *Pisum sativum* L. variedad Alaska. Las plantas fueron (A) o no (B) tratadas con KNO_3 5 mM. Las mezclas de incubación para la determinación de actividad NR contenían o carecían de KNO_3 (50 mM) y propanol (1 %, v/v). Ensayos en tampón sin nitrato y sin propanol –○–○–; sin nitrato y con propanol –●–●–; con nitrato y sin propanol –×–×; con nitrato y con propanol –△–△.

La actividad NR y NiR *in situ* de los bacteroides se determinó en las mezclas de reacción descritas por Guerrero *et al.* (12), adicionadas de 50 μg/ml de mezcla de alquil y trimetil de bromuro de amonio para permeabilizar las células. La actividad NR de la fracción citosólica se determinó mediante la técnica descrita por Hageman y Hucklesby (13). La actividad NiR del citosol se ensayó siguiendo las recomendaciones de Vega *et al.* (30). El contenido en nitrito de citosol se determinó en alícuotas de 0,5 ml, después de la adición al extracto de 25 μl de acetato de zinc 1 M y centrifugación a 10.000 × g durante 10 min.

El contenido en nitrato se determinó mediante la técnica de Cawse (7), y el de nitrito se valoró mediante la reacción de diazotación descrita por Snell y Snell (24). El contenido en proteína se estimó por el procedimiento de Markwell (18) utilizando albúmina como proteína de referencia.

Resultados

En la Fig. 1 se presentan los valores correspondientes a la producción de nitrito por los nódulos de plantas de guisante tratadas (Fig. 1 A) y no tratadas (Fig. 1 B) con KNO_3 5 mM después de 30, 60 y 120 min de incubación en presencia y en ausencia de nitrato, propanol o de ambos compuestos.

Los resultados obtenidos, basados en la media de los valores correspondientes al nitrito producido por los nódulos aislados de las plantas tratadas y de las no tratadas con nitrato después de 30 min de incubación, indican que la inclusión de propanol en las mezclas de incubación (ensayos $-NO_3^-$ + $-CH_2OH$) incrementó dos veces el contenido en nitrito, respecto al nitrito presente en las mezclas que carecían de nitrato y de propanol (ensayos $-NO_3^-$ – $-CH_2$ OH) (Figs. 1 A y 1 B). La adición de nitrato a las mezclas de incubación (ensayos + NO_3^- – –CHOH) incrementó 5 veces el contenido en nitrito, mientras que la presencia simultánea de ambos compuestos (ensayos + NO_3^- + $-CH_2OH$) multiplicó por 20 la producción de nitrito por los nódulos.

De manera similar, la producción de nitrito por las raíces (Fig. 2) se incrementó cuatro veces cuando se adicionó propanol (ensayos $-NO_3^-$ + $-CH_2OH$) a las mezclas de incubación, en relación con el nitrito contenido en las mezclas no adicionadas ni de nitrato ni de propanol (ensayos $-NO_3^-$ – $-CH_2OH$). Sin embargo, la inclusión de nitrato (ensayos $+NO_3^-$ – $-CH_2OH$) en las mezclas de incubación aumentó 10 veces el contenido en nitrito, y la adición simultánea de nitrato y propanol (ensayos $+NO_3^-$ + $-CH_2OH$) produjo que el contenido en nitrito fuera 17 veces mayor (Figs. 2 A y 2 B).

En bacteroides recién aislados de nódulos de plantas de guisante no tratadas con nitrato se detectó actividad NiR y no se encontró actividad NR (Fig. 3 A). Cuando los bacteroides se incubaron en anaerobiosis (atmósfera de argon) se pudo observar la presencia de actividad NR después de 12 h de incubación y que tal actividad incrementó hasta las 25 h (Fig. 3 A). La actividad NiR aumentó desde el inicio del período de incubación, lo que resultó en una desaparición progresiva del nitrito acumulado en el medio (Fig. 3 A). La concentración de nitrato se mantuvo constante durante las primeras horas, observándose la desaparición del mismo después de 5 h de incubación. Pasado este tiempo, la utilización del nitrato trajo consigo una mayor acumulación de nitrito (Fig. 3 A). La adición de rifampicina o cloranfenicol a las mezclas de incubación de los bacteroides resultó en una disminución progresiva de la actividad NiR, en la ausencia de acumulación de nitrito y en la no utilización del nitrato presente en el medio (Fig. 3 B). Igualmente, no se pudo observar la aparición de actividad NR a lo largo del período de incubación (Fig. 3 B).

Idénticos resultados a los indicados en la Fig. 3 A se obtuvieron cuando los bacteroides se aislaron de nódulos de plantas tratadas con KNO_3 2,5, 5 y 10 mM (los datos no se presentan). En ningún caso se detectó actividad NR en bacteroides recién aislados y los valores de actividad NiR fueron parecidos a los indicados en la Fig. 3 A.

No se observó incremento en la densidad óptica de las mezclas de incubación a lo largo del período de incubación.

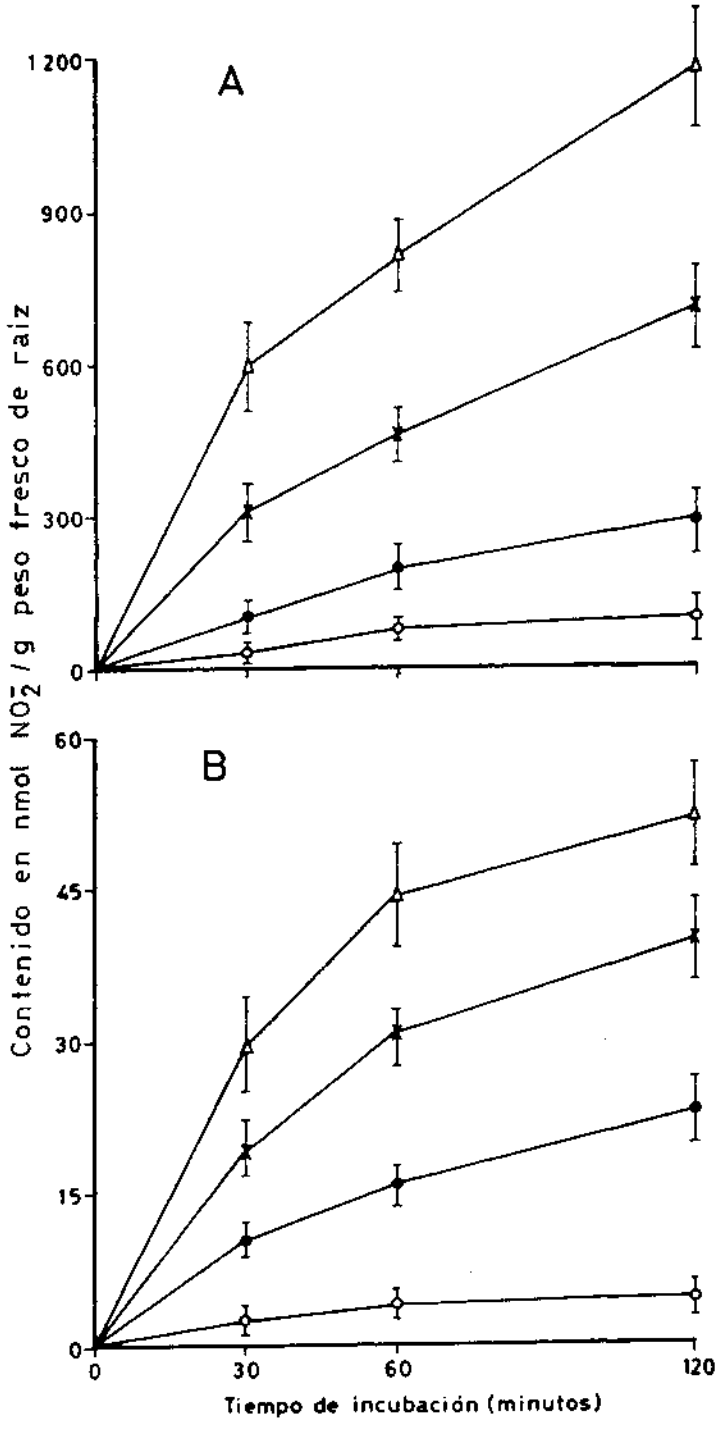

Fig. 2. Actividad nitrato reductasa (NR) *in vivo* de las raíces de plantas de *Pisum sativum* L. variedad Alaska, inoculadas con *Rhizobium leguminosarum* 3855. Las plantas fueron (A) o no (B) tratadas con KNO_3 5 mM. Las mezclas de incubación para la determinación de actividad NR contenían o carecían de KNO_3 (50 mM) y propanol (1 %, v/v). Ensayos sin nitrato y sin propanol –O–O–; sin nitrato y con propanol –●–●–; con nitrato y sin propanol –x–x–; con nitrato y con propanol –△–△–.

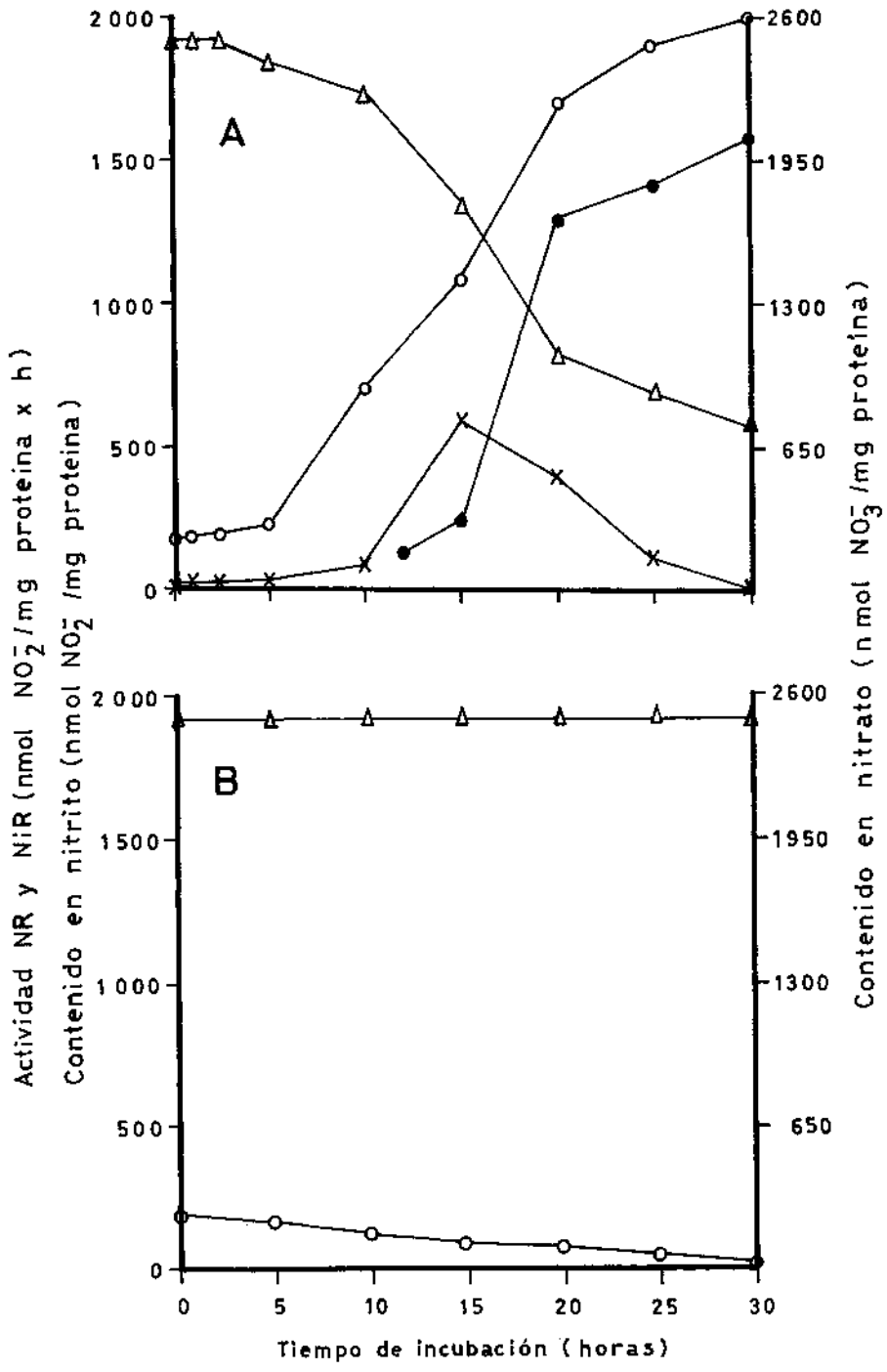

Fig. 3. Actividad nitrato reductasa (NR) y nitrito reductasa (NiR) *in situ*, acumulación de nitrito y consumo de nitrato con los bacteroides de *Rhizobium leguminosarum* 3855 incubados anaeróbicamente en un medio con nitrato, en ausencia (A) y en presencia (B) de cloranfenicol. NR, –●–●–; NiR, –○–○–; nitrito, –×–×–; nitrato, –△–△–.

En la Tabla 1 se presentan los datos de actividad NR y NiR del citosol de nódulos de plantas de guisante no tratadas o tratadas con KNO_3 5 mM. Los valores de ambas actividades variaron significativamente según el tampón utilizado para la homogeneización de los nódulos e incluso no se detectó actividad NR cuando se empleó el tampón maleico/KOH (Tabla 1). Independientemente del medio de extracción utilizado para homogeneizar los nódulos, las actividades NR y NiR del citosol de nódulos de plantas tratadas con nitrato fue 1,8 y 1,2 veces mayores, respectivamente, que las actividades NR y NiR del citosol de nódulos de plantas no tratadas con nitrato (Tabla 1). Igualmente, la utilización de diversos tampones para la homogeneización de los nódulos produjo cambios significativos en los valores del contenido en nitrito del citosol (Tabla 1). El contenido en nitrito del citosol de los nódulos de plantas tratadas con nitrato fue, aproximadamente, 2,3 veces mayor que el del citosol de los nódulos de plantas no tratadas (Tabla 1). No se observó nitrito en el citosol cuando se utilizó tampón maleico/KOH o etanol para homogeneizar los nódulos (Tabla 1).

Discusión

Aunque en tejidos vegetales la enzima NR suele ser constitutiva, también se puede inducir en presencia de su substrato (6). En nódulos y raíces de plantas de guisante existe actividad NR constitutiva, ya que tal actividad se pudo detectar cuando las plantas crecieron en ausencia de nitrógeno combinado y los ensayos de actividad se llevaron a cabo en mezclas que no incluían nitrato (Figs. 1 B y 2 B). Por otra parte, hubo inducción de la actividad NR puesto que se produjo un incremento de tal actividad cuando las plantas se cultivaron en presencia de nitrato (Figs. 1 A y 2 A).

La actividad NR que se determina mediante ensayos *in vivo* + NO_3^- indican la capacidad de un tejido para reducir nitrato cuando el substrato no es limitante y se considera que representan la actividad NR medida *in vitro* (1). En los ensayos *in vivo* – NO_3^-, tanto el nitrato como el poder reductor son los propios de los tejidos, por lo que estos ensayos se relacionan directamente con la actividad NR medida *in situ* (1). Por otra parte, los ensayos de actividad *in vivo* que incluyen propanol en las

TABLA 1
EFECTO DEL NITRATO Y DEL MEDIO DE EXTRACCION SOBRE EL CONTENIDO EN NITRITO Y LAS ACTIVIDADES NITRATO REDUCTASA (NR) Y NITRITO REDUCTASA (NiR) DEL CITOSOL DE LOS NODULOS FORMADOS POR *R. LEGUMINOSARUM* 3855 EN SIMBIOSIS CON *P. SATIVUM*, VAR. ALASKA

Medio de extracción	NR		NiR		Nitrito	
	Tratamiento con KNO_3 (mM)					
	0	5	0	5	0	5
Tris (200 mM)/fosfato potásico (50 mM) pH 7,5	16 ± 3	28 ± 3	1.537 ± 57	1.927 ± 72	55 ± 7	158 ± 18
Maleico/KOH (100 mM) pH 6,8	0,0	0,0	3.945 ± 162	4.910 ± 143	0,0	0,0
Fosfato potásico (50 mM) pH 7,5	40 ± 5	77 ± 9	2.148 ± 88	2.674 ± 12	146 ± 16	268 ± 14
Etanol (96 %)	0,0	0,0	0,0	0,0	0,0	0,0

Los valores de actividad NR y NiR se expresan en nmol de NO_2^-/g peso fresco de nódulo × h y los de contenido en nitrito en nmol de NO_2^-/g peso fresco de nódulos. Los valores representan la media ± SE de tres repeticiones.

mezclas proporcionan una sobreestimación de la actividad NR, ya que la alcohol deshidrogenasa puede utilizar el propanol como substrato para reducir NAD^+ (2).

Por tanto, se puede establecer que la actividad NR de los nódulos y raíces de plantas de guisante (determinada mediante ensayos *in vivo* + NO_3^-) fue más sensible a la presencia de nitrato que la del citosol de los nódulos (determinada mediante ensayos *in vitro*). Efectivamente, la actividad NR de los nódulos y raíces de plantas tratadas con nitrato fue 4 y 16 veces mayor, respectivamente, que la de los nódulos y raíces de plantas no tratadas con nitrato (Figs. 1 A y 2 A), mientras que la actividad NR del citosol sólo incrementó 1,8 veces (Tabla 1).

La existencia de actividad NR constitutiva en nódulos fue demostrada por Evans en 1954 (10). Las evidencias experimentales actualmente disponibles indican que la mayoría de los bacteroides de *Rhizobium* y *Bradyrhizobium* pueden contener dos tipos de NR, asociadas con la asimilación y desasimilación o respiración del nitrato, respectivamente (3, 6). En este sentido, se ha establecido que los bacteroides de *R. leguminosarum* no expresan actividad NR constitutiva (8, 17, 28).

Los resultados obtenidos en este estudio indican que es posible inducir actividad NR y NiR en bacteroides de *R. leguminosarum* 3855 incubados anaeróbicamente en un medio con nitrato (Fig. 3 A). Los bacteroides, por tanto, poseen el equipo enzimático necesario para la síntesis de ambas proteínas. Una inducción similar de actividad NR y NiR se ha descrito en bacteroides de *B. japonicum* (11). Puesto que la adición de rifampicina o de cloranfenicol a la mezcla donde se incubaron los bacteroides impidió la inducción de tales actividades (Fig. 3 B), fue necesaria la síntesis de *novo* de las dos enzimas y no la reutilización de otras preexistentes en los bacteroides. Al contrario que la actividad NR, se observó la existencia de actividad NiR constitutiva en los bacteroides (Fig. 3 A). Estos resultados coinciden con los previamente publicados por otros autores en bacteroides de *R. leguminosarum* (28).

Cuando los bacteroides se aislaron de nódulos de plantas no tratadas o tratadas con KNO_3 2,5, 5 ó 10 mM, en ningún caso se pudo detectar la presencia de actividad NR, lo que sugiere que el nitrato no es metabolizado por los bacteroides puesto que no hubo inducción de la actividad NR. No hay que descartar, sin embargo, la posibilidad de que otras concentraciones más elevadas de nitrato o tratamientos más prolongados puedan permitir que el nitrato alcance la zona de los nódulos infectada por los bacteroides. Estos resultados prestan apoyo a la hipótesis de Pate y Atkins (21), que indica que el nitrato no penetra en el interior de los bacteroides. Hunter (14), basándose en ensayos *in vivo* de actividad NR, ha sugerido también que el nitrato no alcanza la zona infectada de los nódulos. Otra evidencia a favor de la hipótesis que supone que el nitrato no penetra en el interior de los nódulos ha sido proporcionada por Sprent *et al.* (25). Estos autores han separado, mediante micromanipulación de los nódulos, el córtex de la región infectada y han determinado la actividad NR y el contenido en nitrato de ambas fracciones. De los resultados obtenidos por ellos se puede concluir que la presencia de nitrato queda restringida a los tejidos corticales de los nódulos.

Nuestros resultados, sin embargo, contrastan con los publicados por otros autores que indican que la actividad NR de los nódulos contribuye de forma sustancial a la asimilación de nitrato (20, 22, 29).

De los datos que se presentan en la Tabla 1 se puede concluir que tanto las actividades NR y NiR como el contenido en nitrito del citosol variaron ampliamente cuando los nódulos, procedentes de plantas tratadas o no con nitrato, se homogeneizaron con distintos medios acuosos de extracción. Puede observarse, además, una relación entre la existencia de actividad NR y el contenido en nitrito del citosol. De hecho, cuando la homogeneización de los nódulos se efectuó con tampón maleico/KOH, no se pudo detectar nitrito en el citosol, lo que podría deberse a la ausencia de actividad NR en este tampón (Tabla 1). De nuestros resultados, sin embargo, no se puede concluir la(s) causa(s) por las que no se detectó actividad NR en el tampón maleico/KOH. Que la no expresión de actividad NR trajo aparejada la no acumulación de nitrito en el citosol se pudo comprobar cuando los nódulos se homogeneizaron con etanol, que inactiva la enzima. Estos hechos sugieren que el nitrito

no se acumula en el citosol cuando los nódulos se encuentran en condiciones fisiológicas, y que este fenómeno sólo ocurre durante el proceso de homogeneización de los nódulos al permitirse la puesta en contacto de la enzima con su substrato específico. Es posible, por tanto, que la acumulación de nitrito en el citosol sea un artefacto originado durante el proceso de su extracción.

Agradecimientos

Los autores expresan su gratitud a Ricardo García Vélez y a José Antonio Miquel por su inestimable colaboración durante el desarrollo de estos experimentos. Este trabajo ha sido subvencionado por el Proyecto BT 87-0003 de la CAICYT.

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Description of four species of the genus *Vannella* isolated from freshwater

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Summary

Four species of the genus *Vannella* have been isolated and identified from samples of different freshwater habitats. The present work is an attempt to bring up to date the descriptions of *V. simplex, V. platypodia, V. mira* and *V. miroides.*

Key words: Limax amoebae, freshwater amoebae, Vannella.

Resumen

Cuatro especies del género *Vannella* han sido aisladas e identificadas de muestras procedentes de diferentes biotopos de agua dulce. El presente trabajo tiene por objeto aportar datos sobre las descripciones de *V. simplex, V. platypodia, V. mira* y *V. miroides.*

Introduction

Limax amoebae are protozoa belonging to the Subpylum Sarcodina; the clinical importance of such protozoa is increasing since some of them cause important diseases (10); members within this group of amoebae can also transmit pathogenic bacteria from water and cause intestinal diarrhoea (7, 8, 9, 18). Systematic studies on amoebae of the genus *Vannella* are scarce. *V. mira* and *V. platypodia* have been the most frequently species found on swimming pools (2, 3, 4, 17). These species were isolated by Michel and Schneider (12) from a hospital physiotherapeutic swimming pool; Michel and Just (11) identified three amoebae of the genus *Vannella* in the refrigeration and mouthwash water of 49 units of dental treatment (11), with the following frequency: *V. mira* (19 % of the samples), *V. platypodia* (7,1 %) and *V. simplex* (7,1 %). In our country, two strains of *V. mira* have been isolated and identified from covered and uncovered swimming pools (5); *V. simplex* and *V. platypodia* were isolated from bottled water (5, 6).

In the present work a fauna study was carried out in the provinces of Sevilla and Córdoba (Spain) with the object of determining the amoebic fauna in different freshwater habitats. Four species of the genus *Vannella* have been found besides other species belonging to other genera. This fau-

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na study is a first step towards the final objetive of evaluating the risk that the freshwater amoebic fauna can present for human health, because of the existence of pathogenic limax amoebae as well as other limax amoebae with doubtful or unknown pathogenicity. This latter case refers especially to the species of the genus *Vannella*, whose presence has been demonstrated in freshwater habitats, but whose possible pathogenic capacity will be evaluated in further studies.

Materials and methods

The water samples were collected from different freshwater habitats: covered swimming pool (strain 8SE87), pond (strain 9SE87), well (strain 10SE87), bottled water (strain 11SE87) and river (strain 12SE87). In each case, the volume of water was of 1000 ml. Methods of cultivation, measurement and flagellation tests were those described by Page (13). The isolation, culture and maintenance were performed on Petri dishes containing non-nutrient agar with NaCl, and incubated at 28° C (strain 10SE87 was also grown at 37° C). For the maintenance of these strains, reseed was carried out every 3-4 days, because their incapacity to encyst. The flagellar stage was absent in all the strains.

TABLE 1
DATA OF BIOMETRIC PARAMETERS REPORTED FOR THE TROPHIC FORMS OF DIFFERENT SPECIES OF THE GENUS *VANNELLA* (1, 5, 14, 16, 19)

Parameters*	*V. platypodia*	*V. simplex*	*V. mira*	*V. miroides*
Locomotive forms:				
Lenght	10-23	35-80	23-55	25-35
Breadth	—	—	—	20-30
Length/Breadth	Undefined tendency	< 1	< 1	Undefined tendency
Diameter of nucleus	3.4-4.8	7.7-10.8	4.8-8.3	4-4.5
Diameter of nucleolus	2.1-3.4	—	2.1-4.1	1.75
Rate of locomotion (micrometers/minute)	21-34	—	—	—
Diameter of the contractile vacuole	3.4-4.1	7-8	4.7-7.6	5-6
Period of pulsation (second)	40-115	Slow	55-115	60-180
Diameter of the food vacuole	3.4-4.8	—	4.8-8.3	2-3
Usual external form	Spatulate	Triangular-ovoid	Fan-triangular	Fan
Hyaline veil aspect	Smooth	Almost smooth	Smooth-ragged	Somewhat wavy
Number of nuclei	1	1-2	1	1
Floating form:				
Persistence	Brief	—	Large	Large
Number of pseudopods	2-9	5-8	10	6-8
Form of the pseudopods	Straigh-curved	Fine	Spiral	Fine
Length of the pseudopods	—	—	32-117	50-60
Diameter of central mass	6-13	—	12-24	8-9
Aspect	Assymetric	Asymmetric	Asymmetric	Symmetric
Culture:				
Temperature (° C)				
Fecal pellets	No	No	Yes	No

* All the measurements are expressed in micrometers.

The identification included *in vivo* studies on resting forms and locomotive trophozoites: the latter in both floating and locomotive forms. Giemsa and Feulgen techniques were used for the nuclear studies (13).

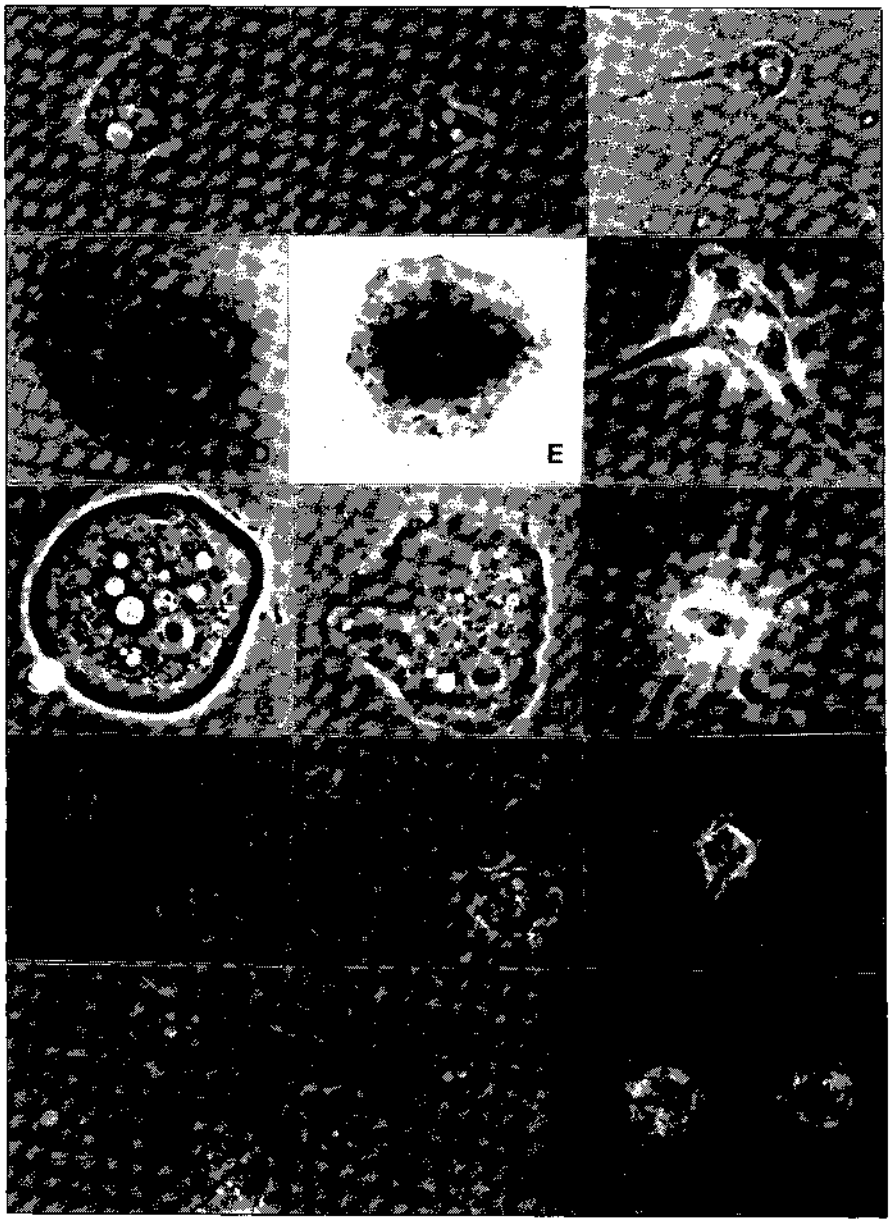

Fig. 1. *V. platypodia:* locomotive (A, B) and floating (C) forms; *V. simplex:* locomotive (D) and binucleate (E) forms (Giemsa) and floating form (F); *V. mira* (strain 10SE87): resting form (G), locomotive (H) and floating (I) forms; *V. mira* (strain 11SE87): locomotive form (J) and fecal pellets (K) in the cultures, floating form (L); *V. miroides:* locomotive (M) and floating (N) forms (A, B, C, E, F, G, H, I, X 1000; D, K, L, M, N, X 400; J X 200).

Results

Strain 8SE87

Amoebae appeared in different forms during locomotion; sometimes, the anterior ectoplasmic edge appeared as a broad arc, while the posterior edge was slightly rounded (Fig. 1 A). The most frequent form observed in the trophozoites in locomotion was spatulate with a much broader anterior ectoplasmic region and a granular posterior endoplasmic region which became progressively narrower (Fig. 1 B). The amoebae had a non eruptive movement through a steady and more or less slow ectoplasmic flow. The rate of locomotion was 30-58 μm per minute with a median of 45 μm per minute. The lenght of the amoebae in locomotion was 12-23 μm and the breadth was of 6-18 μm, demonstrating a long rather than broad strain. A 30 per cent of the specimens were twice as long as they were broad and in 100 per cent of these the greatest diameter was that of the length (Table 2). The single nucleus was vesiculate, and was located between the ectoplasm and the endoplasm (Fig. 1 A). This nucleus showed a central nucleolus with a clear nucleoplasm and a well defined nuclear membrane. The diameter of the nucleus was 3-4 μm and that of the nucleolus was 2 to 3 μm. One or two contractile vacuoles were observed with a diameter of 3-6 μm; and a period of 82-268 seconds (Table 2).

The floating form appeared after the amoebae were hung in a fluid medium. This phase was very brief. This floating form showed a granular central mass with a diameter of 7-12 μm; 2 to 8 conical pseudopods were present with a length of 7-21 μm. This radiate phase demonstrated and assymetric aspect because of the different lengths of the pseudopods. The degeneration of this floating form was produced by the coiling of the pseudopods like a corkscrew, and these then finally disappeared (Fig. 1 C).

Strain 9SE87

Amoebae in active locomotion are constantly changing shape: spatulate, triangular (Fig. 1 D). Granular endoplasm and clear ectoplasm extended along the anterior end. The hyaline edge was smooth, although with occasional nicks. The sizes of 100 trophozoites measured in locomotion were 21-47 μm in length and 14-45 μm in breadth. Fifty eight per cent of the trophozoites in locomotion showed the ratio length/breadth above 1, while 26 per cent showed this ratio under 1, so the amoebae tended to present forms which were longer than they were broad; nevertheless, this was not a constant character (Table 2). Binucleated forms have been especially observed in Giemsa stained preparations (Fig. 1 E), but the majority of trophozoites showed only one single nucleus with a diameter of 5-7 μm. The diameter of nucleolus was 4-5 μm. A single contractile vacuole was present with a diameter of 5-7 μm; the period was of 34-103 seconds. Food vacuoles appeared in variable numbers and diameters, and always in an inverse ratio (Table 2).

The floating form was observed after the amoebae were suspended with a fluid medium, but the time of permanence was not very long because these forms transformed quickly into a locomotive state (Fig. 1 F). This floating form showed a rounded central mass with a diameter of 14-31 μm, and long, sharp pseudopods with broad bases; these pseudopods, in number of 5 to 8, had oscilating movements; the lengths of these were of 25-45 μm (Table 2).

Strain 10SE87

Resting amoebae appeared in large rounded forms (20-50 μm), with a granular endoplasm, sometimes surrounded by a clear and hyaline ectoplasm. The nucleus was patent. Many vacuoles were observed in the endoplasm (Fig. 1 G).

TABLE 2
BIOMETRIC PARAMETERS OF THE TROPHOZOITE OF THE STRAINS STUDIED IN THE PRESENT WORK

Parameters*	Strain 8SE87	Strain 9SE87	Strain 10SE87	Strain 11SE87	Strain 12SE87
Locomotive forms:					
Length	12-23	21-47	24-47	17-54	12-35
Breadth	6-18	14-45	24-47	21-68	21-47
Length/breadth	> 1	> 1	< 1	< 1	< 1
Diameter of nucleus	3-4	5-7	5-8	6-9	2.7-5.5
Diameter of nucleolus	2-3	4-5	3-5	3-5	1.6-2.2
Rate of locomotion (micrometers/minute)	30-58	45-68	7.2-13.8	—	20.5-38.8
Diameter of the contractile vacuole	3-6	5-7	6-8	6-9	6-7
Period of pulsation (second)	82-268	34-103	45-121	—	89-146
Diameter of the food vacuole	—	—	—	4-9	4-6
Usual external form	Spatulate	Spatulated-triangular	Fan-triangular	Fan triangular	Fan
Hyaline veil aspect	Smooth	Smooth-ragged	Almost smooth-slightly ragged	Almost smooth-slight ragged	Somewhat wavy
Number of nuclei	1	1-2	1	1	1
Floating form:					
Persistence	Brief	Brief	Large	Brief	Large
Number of pseudopods	2-8	5-8	6-10	4-7	5-7
Form of the pseudopods	Straight-curved	Fine	Straight-curved	—	Curved-straight
Length of the pseudopods	7-21	25-45	20-40	15-35	30-64
Diameter of central mass	7-12	14-31	12-25	14-20	12-26
Aspect	Asymmetric	Asymmetric	Asymmetric	Asymmetric	Symmetric
Culture:					
Temperature (°C)	28°	28°	28-37°	28°	28°
Fecal pellets	No	No	Yes	Yes	No

* All the measurements are expressed in micrometers.

Amoebae in locomotion showed typical fan and triangular forms with a clear difference between the granular endoplasmic region and the hyaline ectoplasmic zone. Sometimes, the ectoplasmic edge appeared slightly ragged and wavy (Fig. 1 H). In about 50 per cent of the individuals the breadth was greater than the length; the rest of the amoebae showed either the length slightly greater than the breadth or forms longer than wider. The slow and constant movement was produced by a flow of the ectoplasmic region; the rate of locomotion was of 7.2-13.8 μm per minute. The direction changed by a lateral expansion of the ectoplasmic zone. The single vesiculate nucleus had a diameter of 5-8 μm, and the nucleolus 3-5 μm, the latter located in a central position surrounded by a clear nucleoplasm and a nuclear membrane which was not always patent. The pulsatil plasm showed a single vacuole formed by the successive coalescence of several smaller vacuoles. The maximum diameter was 6-8 μm;

the mean period was 70 seconds (45-121 seconds). The length of this amoebae in locomotion was 24-47 μm while the breadth was 24-47 μm (Table 2).

The floating form showed a more-or-less rounded central mass, wider at its base, and sharp pseudopods. These, frequently, were straight, curved, sometimes spiral or tightly coiled, during retraction (Fig. 1 I). The central mass was 12-25 μm in diameter, and the number of pseudopods of 6-10 with a length of 20-40 μm, giving this floating form an assymetric aspect. This form was retained for a short period (Table 2). Fecal pellets of different forms and size were present both in culture and in hanging drop slides.

Strain 11SE87

The amoebae in repose showed a rounded form with a granular endoplasm surrounded by a narrow zone of ectoplasm. The transformation of this form to locomotive amoebae was through the development of the ectoplasmic zone, with the amoebae in fan, triangular forms and other forms. In this strain, approximately 76 per cent of the motile trophozoites were wider than long. The anterior edge of the hyaline zone sometimes appeared ragged (Fig. 1 J). The lenghts of 100 trophozoites in locomotion were of 17-45 μm and the breadth 21-68 μm. Only one single vesiculate nucleus was observed inside the endoplasm, with a diameter of 6-9 μm; the patent nucleolus showed a diameter of 3-5 μm. The diameter of the single vacuole was of 6-9 μm. A variable number of food vacuoles were observed with a diameter of 6 μm.

The floating forms were present during a short time; the diameter of the central mass was 14-20 μm, presenting a variable number (4-7) of conical pseudopods with a length of 15-35 μm (Table 2); showing an assymetric aspect (Fig. 1 L). Focal pellets were present in culture dishes (Fig. 1 K).

Strain 12SE87

The active locomotion form showed a broader rather than longer fan aspect with a granular mass or endoplasm, usually located in median posterior positions, and a clear ectoplasmic region with a slightly rough outline; this sometimes extended ahead of and along both sides of the endoplasmic zone (Fig. 1 M). A 90 per cent of the motile trophozoites showed the breadth as the greatest diameter and 6 per cent had a ratio length/breadth of 1. The movement was smooth with a constant trayectory during a short time; the rate of locomotion was 20.5-38.3 μm per minute. The length of the locomotive trophozoites was 12-35 μm and the breadth 21-47 μm. The nucleus (2.7-5.5 μm) had a clear membrane and a nucleolus with a diameter of 1.6-2.2 μm. The vacuolar complex was composed of pulsatile and food vacuoles; one or two contractile vacuoles were observed with a diameter of 6-7 μm, the period was of 89-146 seconds. The largest food vacuoles presented a diameter of 4-6 μm observed in a variable number, including a maximum of 9. The phagocytosis was very active (Table 2).

The floating forms demonstrated a rounded central mass with a median diameter of 13.5 μm (12-26 μm). Radiate and sharp pseudopods were observed with similar lengths (30-64 μm) (Fig. 1 N); the median number of these was 6 (5-7) (Table 2). These floating forms were observed during a long period of time.

Nuclear studies

All the strains studied presented a typical mesomitosis before the binary division; the description of this has been carried out on *V. platypodia;* similar figures were observed in the other species

studied. In order to study the mitotic division Feulgen technique was carried out. The interphase nucleus showed the chromosomic material near the nuclear membrane (Feulgen +) presenting a hyaline central zone corresponding with the nucleolus (Feulgen –) (Fig. 2 A). In prophase, the nucleolus disappeared and some fibres arranged as shown in Figure 2 B and Figure 2 C were observed. The mitotic stages corresponding to the metaphase are those of Figures 2 D and 2 E. In anaphase the chromosomic material was deeply stained near the nuclear membrane and arranged in the poles of the cell; the nuclear membrane disappeared progressively (Figs. 2 F, 2 G and 2 H). In telophase (Fig. 2 I) two nuclei were observed in the poles of the cell and subsequently a constriction of the cell took place giving rise to the cytokinesis (Fig. 2 J). The division is a typical mesomitosis with later disappearance of the nuclear membrane.

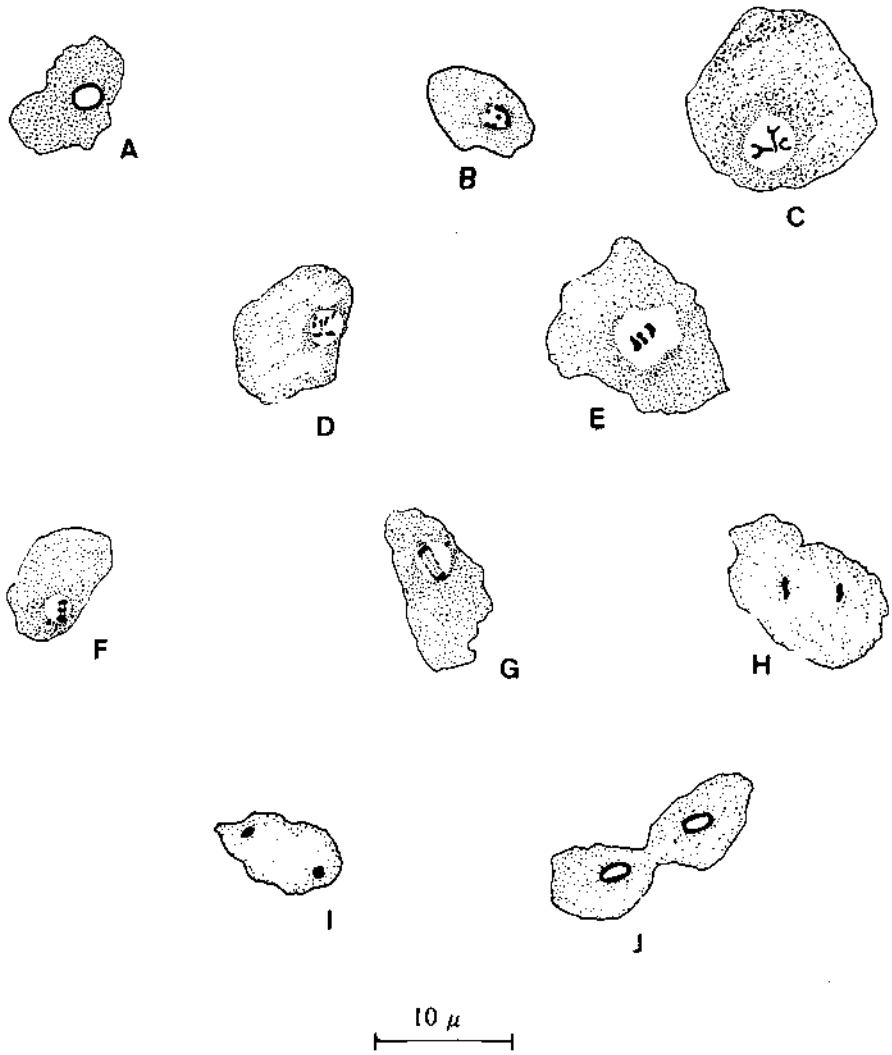

Fig. 2. Division stages of *V. platypodia:* Interphase nucleus (A); Prophase (B, C); Metaphase (D, E); Anaphase (F, G, H); Telophase (I); Cytokinesis (J).

Discussion

According to the characteristics cited by Bovee, 1965 (1), all the isolated and identified strains were included in the family *Thecamoebidae* Schaeffer, 1926, genus *Vannella* Bovee, 1965. Four freshwater species belonging to the genus *Vannella,* have been described by Page (1976) (16): *Vannella platypodia* Glaser, 1912, *V. simplex* Wolfarth-Botterman, 1960, *V. mira* Schaeffer, 1926 and *V. miroides* Bovee, 1965. The morphological characteristics of these four species reported by different authors (1, 5, 14, 16, 19) have been compiled in Table 1 in order to make a comparative study as well as to provide new parameters for the specific differentation.

Strain 8SE87 was identified as *Vannella platypodia* according to the characteristics reported for this species (14). Nevertheless, this author observed that 60 % of the motile trophozoites of our strain (8SE87) were longer than wider, as this author has pointed out (14), the length and width of the forms of *V. platypodia* varied continuously.

Strain 9SE87 has been identified as *V. simplex* Wolfarth-Botterman, 1960 (19) according to the size of the motile trophozoites, the presente of two nuclei in some trophozoites and the aspects of the pseudopods of the floating form. Nevertheless, there are some differences between our material and the material studied by other authors; so, in *V. simplex* the majority of the specimens were wider than longer while in strain 9SE87 the majority were longer than wider; and the diameter of the nucleus is larger in *V. simplex* than in our strain.

Strain 10SE87 was identified as *V. mira* (1, 14, 16) as it coincided in the following aspects: size of the locomotive trophozoite, nucleus and nucleolus, diameter of the contractile vacuole, period of pulsation, uninucleate amoebae, number of pseudopods in the floating form and persistence of this form, and the presence of the fecal pellets. Strain 10SE87 was smaller than *V. mira,* but this difference was also observed by Page in 1968 (14) in his studies of the Alabama and Wisconsin strains of *V. mira.*

All the morphological characteristics observed in the 11SE87 strain were those of *V. mira* Schaeffer, 1926, the presence of the fecal pellets being the specifying phenomenon. Thus, strains 10SE87 and 11SE87 corresponded to *V. mira* and only one difference was observed between both strains: 11SE87 presented larger trophozoites than 10SE87.

Strain 12SE87 was similar to *V. miroides* (1, 16) in the following characteristics: the size of the locomotive trophozoite, the diameter of the nucleus and nucleolus, the diameter of the contractile and food vacuoles, the morphology of the trophozoite, the aspects of the hyaline ectoplasm, the number of nuclei, the persistence of the floating form, the number of pseudopods of the floating form and the symmetric aspects of the floating form.

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Host range and particle morphology of some bacteriophages affecting pathovars of *Xanthomonas campestris*

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Summary

Seven bacteriophages active against different pathovars of *Xanthomonas campestris* were isolated from naturally infected plant material. All showed polyhedral heads and could be separated into two morphological groups according to their tail structures. Phages active against *X. campestris* pv. *cucurbitae* (XCU-P1 and XCU-P3) and *X. campestris* pv. *holcicola* (XHOL-P1) were described for the first time. Ninety nine bacterial strains belonging to 5 genera *(Xanthomonas, Pseudomonas, Agrobacterium, Clavibacter* and *Erwinia)* were tested for sensitivity to the different phage suspensions. More than 60 % of the *Xanthomonas* cultures were susceptible to one or more phages. The other genera did not react with any of the phages. The 7 phages were specific at a generic level but showed a low degree of specificity at the pathovar level.

Key words: Xanthomonas campestris *pathovars*, X. campestris *p.v.* cucurbitae, X. campestris *p.v.* holcicola, *bacteriophages.*

Resumen

Se aislaron siete bacteriófagos a partir de plantas infectadas que se mostraron activos frente a distintos patovares de *Xanthomonas campestris*. Todos los fagos presentaron cabezas poliédricas y se separaron en dos grupos de acuerdo con la estructura de sus colas. Se describen por primera vez bacteriófagos activos frente a *X. campestris* pv. *cucurbitae* (XCU-P1 y XCU-P2) y *X. campestris* pv. *holcicola* (XHOL-P1). Se emplearon 99 líneas bacterianas pertenecientes a cinco géneros *(Xanthomonas, Pseudomonas, Agrobacterium, Clavibacter* y *Erwinia)* con el objeto de determinar su sensibilidad frente a diferentes suspensiones de los fagos. Más del 60 % de las cepas de *Xanthomonas* resultó susceptible a uno o más fagos. El resto de los géneros no reaccionó con ninguno de los fagos. Los siete bacteriófagos resultaron específicos a nivel genérico pero presentaron un bajo nivel de especificidad a nivel de patovar.

Introduction

Many plant pathogenic bacteria are difficult to distinguish from closely related pathogens, especially within the genera *Pseudomonas* and *Xanthomonas* where conventional culture and physiological tests are often inadequate for the identification of many species (8), now classified as pathovars

(10, 33). Techniques involving serology and bacteriophages are useful for rapid identification of pathogens for diagnostic purposes or for the recognition of strains of epidemiological significance. Several studies have demonstrated that phages active against *X. campestris* pathovars have a high degree of specificity, such as *X. campestris* pv. *campestris* (21); *X. campestris* pv. *malvacearum* (6); *X. campestris* pv. *pruni* (7, 11) and other pathovars (17-20, 25). However, other *Xanthomonas* bactriophages present a broad spectrum of activity (9, 13, 28).

The purpose of the present study was the isolation, from infected plant material, of phages active against different pathovars of the *X. campestris* group and the development of a useful technique for the obtention of high titer stocks. Phage sensitivity tests were made in order to find out whether the phages isolated were pathovar specific or capable of lysing different pathovars.

Materials and methods

Bacteria and bacteriophages

The bacteriophages and bacterial propagating strains isolated from plant material are listed in Table 1. The bacterial cultures were received from Dr. M. Moffet (DPI Plant Pathology Branch, Queensland, Australia); Dr. A. C. Hayward; Mr. A. Shelley and the UQM Culture Collection (Department of Microbiology, University of Queensland, Australia). Other cultures were isolated by the author.

The following list gives the cultures, the hosts, the number of strains tested of each organism and the code letters used: *X. campestris* pv. *alfalfae* (from lucerne, 01-alf); *X. campestris* pv. *begoniae* (from begonia, 03-be); *X. campestris* pv. *campestris* (from cabbage 04-ca, 05-ca, 06-ca and 62-ca, from cauliflower 07-ca, from broccoli 08-ca and from rape 63-ca); *X. campestris* pv. *corylina* (from hazelnut 09-co); *X. campestris* pv. *cucurbitae* (from pumpkin 10-cu, 11-cu, 13-cu, 54-cu, 65-cu, 66-cu, 92-cu and from zucchini 12-cu); *X. campestris* pv. *dieffembachiae* (from dieffembachia 14-di and from *Anthurium* sp. 45-di); X. campestris pv. *eucalypti* (from eucalyptus 15-eu); *X. campestris* pv. *glycines* (from soybean 16-gly, 17-gly, 69-gly); *X. campestris* pv. *holcicola* (from maize 18-hol and from sorghum 19-hol, 67-hol, 68-hol); *X. campestris* pv. *juglandis* (from walnut 20-ju); *X. campestris* pv. *malvacearum* (from cotton 21-ma, 22-ma, 23-ma, 24-ma, 25-ma, 26-ma, 27-ma, 48-ma); *X. campestris* pv. *mangiferaindicae* (from mango 28-man); *X. campestris* pv. *phaseoli* (from bean 29-pha, navy bean 30-pha, 70-pha, from mung bean 31-pha and from French bean 71-pha); *X. campestris* pv. *pelargonii* (from pelargonium 32-pe); *X. campestris* pv. *poinsetticola* (from poinsettia 33-po); *X. campestris* pv. *pruni* (from peach 34-pr and from plum 35-pr, 36-pr, 37-pr, 72-pr, 73-pr); *X. campestris* pv. *translucens* (from Japanese millet 74-tr); *X. campestris* pv. *vesicatoria* (from pepper 38-ve, 40-ve, 75-ve, 77-ve, from tomato 39-ve, 42-ve and from cape goosberry 41-ve); *X. campestris* pv. *vitians* (from lettuce 43-vi, 44-vi); *X. campestris* pv. *zinniae* (from zinnia 02-zi); *X. albilineans* from sugar cane 47-alb); *Agrobacterium tumefaciens* (60-At); *Clavibacter michiganense* subsp. *michiganense* (from tomato 78-Cm); *Erwinia chrysanthemi* (from chrysanthemun 61-Ec); *Pseudomonas andropogonis* (from sorghum 55-Pa); *P. aeruginosa* (80-Pae); *P. maltophilia* (from soil 50-malt, 51-malt, 52-malt, 76-malt); *P. solanacearum* Biovar IV (from ginger 97-Pso, 100-Pso, 103-Pso, from blackberry nightshade 101-Pseo, 102-Pso); *P. solanacearum* B. III (from potato 54-Pso, from blackberry nightshade 96-Pso, from ginger 97-Pso, from eggplant 98-Pso, from tomato 99-Pso); *P. solanacearum* B. II (from potato 93-Pso, 95-Pso, from ginger 94-Pso); *P. syringae* pv. *syringae* (from apricot 58PS); *P. syringae* pv. *pisi* (from pea 57-PSp); *P. syringae* pv. *phaseolicola* (from lucerne 58-Psph); *P. syringae* pv. *tabaci* (from tobacco 56-PSt); *P. rubrisubalbicans* (from sugar cane 81-PR); *P. fluorescens* (from milk, 83-PF, 84-PF, 85-PF, 86-PF, 87-PF) and *Pseudomonas* sp. (from milk, 88-P, 89-P, 90-P, 91-P).

TABLE 1
HOST RANGE OF BACTERIOPHAGES AND INDICATOR STRAINS

Bacteriophages	Propagating strain	Source material
XcaP1 (UQM 69 BV).	*Xanthomonas campestris* pv. *campestris* 06-ca (UQM 2705).	*Brassica oleracea* var. *capitata* (cabbage). From leaves.
XcuP1 (UQM 71 BV).	*X. campestris* pv. *cucurbitae* 12-cu (UQM 2700).	*Cucurbita maxima* var. *melopepo* (zucchini). From leaves.
XcuP3 (UQM 72 BV).	*X. campestris* pv. *cucurbitae* 13-cu (UQM 2701).	*C. moschata* (pumpkin). From fruits.
XholP1 (UQM 67 BV).	*X. campestris* pv. *holcicola* 19-hol (UQM 2696).	*Sorghum* sp. (sorghum). From leaves.
XmaP1 (UQM 73 BV).	*X. campestris* pv. *malvacearum* 26-ma (UQM 2702).	*Gossypium hirsutum* (cotton). From leaves vein-lesions.
XprP1 (UQM 66 BV).	*X. campestris* pv. *pruni* 37-pr (UQM 2695).	*Prunus salicina* cv. Elephant's heart (plum). From stem cankers.
XveP1 (UQM 68 BV).	*X. campestris* pv. *vesicatoria* 41-ve (UQM 2697).	*Physalis peruviana* (cape goosberry). From leaves.

Media

The following media were used: sucrose peptone agar —SPA— (15) for bacterial isolation. Nutrient yeast glycerol agar —NYGA— (4), nutrient glycerol yeast broth —NYGB— (4) and phosphate buffered tryptone water —PBT— (22) for phage isolation and purification. Phosphate-buffered saline + 1 % gelatine (0.01 M phosphate, PH 7.6) (PBSG) (12) for dilutions.

A. Bacteriophage isolation

A phage active against *X. campestris* pv. *pruni* (XPr-P1) was isolated from plum stem cankers using the technique described by Crosse and Hingorani (5).

The remaining phages were isolated from diseased plant material showing typical lesions by the following procedure: single lesions were cut and placed in tubes with 10 ml of sterile distilled water and then homogenized in a vortex-mixer. The tubes were incubated at 28° C for 48 h. The liquid was then centrifuged at 1,000 rpm for 30 min. The supernatant was transferred to a sterile 25 ml screwcapped bottle and shaken vigorously with 0.1 ml chloroform. After the chloroform had settled, the supernatant was tested for presence of phages by the surface plating method (3, 4) using the corresponding indicator strains (Table 1). Plates were examined for plaque formation after 24 h of incubation at 28° C.

B. Phage purification

Single plaques were cut from the isolation plates, suspended in 5 ml of NGYB (one tube per plaque) and incubated at 28° C for 48 h (4).

The tubes were centrifuged at 1,000 rpm for 30 min and drops of the supernatant were immediatly streaked on dried NGYA plates lawn with the respective propagating strain (each suspension was prepared by suspending a loopful of bacterial growth from a 48 h culture on SPA in sterile distilled water). The plates were incubated at 28° C for 24 h. Each phage was purified by 4 successive single-plaque isolation.

C. Obtention of high titer stock solution (HT)

The phage suspension (0.1 ml of the supernatant of the last purification) was fully streaked onto plates with 20 ml NGYA to which 1 ml suspension of indicator strain was added before setting and incubated at 28° C for 24 h.

Plates showing nearly confluent lysis were washed with 5 ml of PBT and left for 8 h at room temperature. The liquid was centrifuged twice, first at 3,000 rpm for 30 min, and then at 18,000 rpm for 60 min. The resulting pellet as washed and resuspended in PBSG.

High titer stocks were stored in sterile screw-capped bottles and chloroform was added in a proportion of 1:9. Stocks were maintained at 4° C. Titers of 10^{10} PFU ml^{-1} were obtained.

D. Electron microscopy

High titer solutions were examined under EM (Hitachi M 800). A 1 % phosphotungstic acid (PH 6.5) was employed as a negative contrast stain (Hayward, Pers. comm).

E. Routine test dilutions

Values of plaque forming units per ml (PFU ml^{-1}) at high titer (HT) and routine test dilution (RTD) were calculated for each phage by means of the agar layer method (1, 3).

The RTD is defined as the highest dilution just giving confluent lysis of the propagating host (21).

F. Phage susceptibility tests

Phage susceptibility tests were determined at HT, RTD and RTD × 100 by the spot method of Jindal and Patel (16) but using 5 μl of each phage suspension. In the case of positive reaction, the lytic spots were recorded as clear, semi-turbid and turbid.

Only results at RTD and RTD × 100 were considered as positive because with concentrated phage suspensions, the lytic reaction of spot tests may occasionally even be simulated by antibiotic effect, lethal adsorption or bacteriocin action, as reported by Stolp and Starr (27).

Results and discussion

Phage plaque morphology

All phages formed clear plaques with defined margins. Plaque characteristics are shown in Table 2. Plaque sizes were in relation with phage morphology. Phages with long flexuous tails formed large plaques and phages with contractile tails formed small plaques. The only exception was XVE-P1.

TABLE 2
DESCRIPTION OF BACTERIOPHAGE ISOLATES

Isolate	Source	Plaque morphology (size: diameter)	Virion morphology		
			Particle size (in nm) head	tail	Classification
XcaP1	Cabbage	Small, 1-1.5 mm clear	50	100 × 26	*Myoviridae*
XcuP1	Zucchini	Large, 3 mm clear	50	150 × 10	*Styloviridae*
XcuP3	Pumpkin	Large, 3 mm clear	50	150 × 10	*Styloviridae*
XholP1	Sorghum	Large, 2-3 mm clear	50-55	150 × 10	*Styloviridae*
XmaP1	Cotton	Large, 3-4 mm clear	45	150 × 10	*Styloviridae*
XprP1	Plum	Small, 1 mm clear	55	140 × 15	*Myoviridae*
XveP1	Cape gossberry	Small, 1.5-2 mm clear	45	200	*Styloviridae*

According to Matthews (24).

Particle morphology

All phages showed polyhedral heads of 50 nm average diameter and may be separated into 2 groups according to their tail structures (Table 2). These groups fall in 2 families of viruses as proposed by Matthews (24).

Group I: Phages XCA-P1 (Fig. 1) and XPR-P1 were similar with contractile tails separated from the head by a neck region. Family *Myoviridae* (24).

Group II: Phages XCU-P1; XCU-P3; XHOL-P1 (Fig. 2); XMA-P1 and XVE-P1 showed long flexuous non-contractile tails. Matthews (24) proposed the name *Styloviridae* for the family. Particle sizes are listed in Table 2. The results here were similar to those reported by Bergamin Filho *et al.* (2); Liew and Alvarez (22) and Watanabe *et al.* (32) for *X. campestris* pv. *campestris* phages.

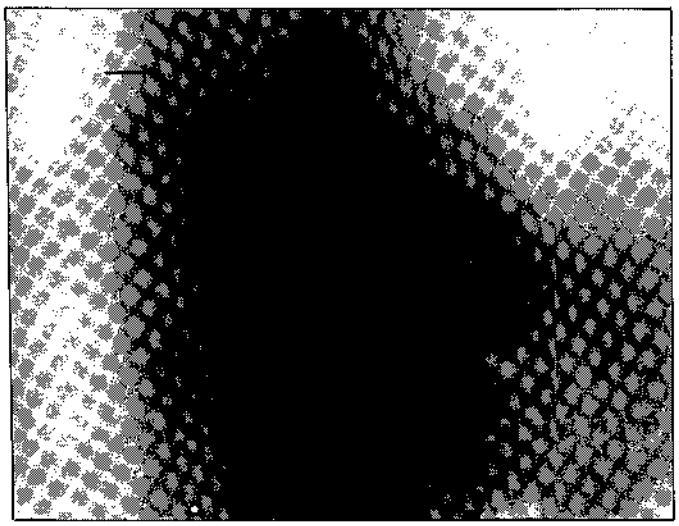

Fig. 1. Bacteriophages active against *X. campestris* pv. *campestris*, isolated from cabbage (XCA-P1). Stain: 1 % PTA (phosphotungstic acid PH 6.5). Scale: bar 50 nm.

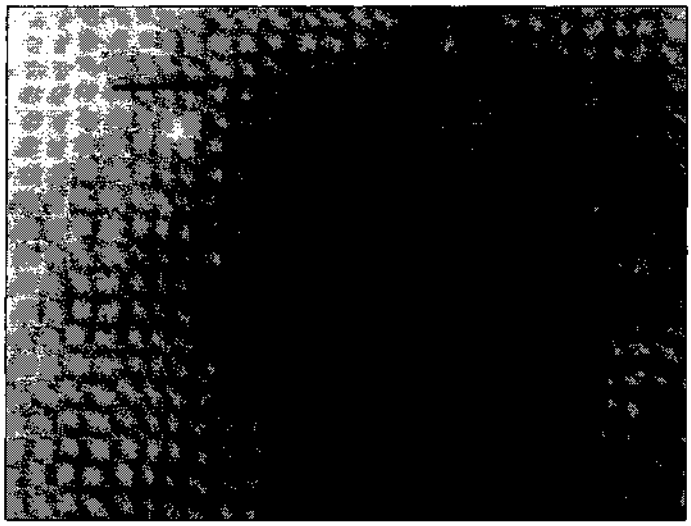

Fig. 2. Bacteriophages active against *X. campestris* pv. *holcicola*, isolated from sorghum (XHOL-P1). Stain: 1 % PTA (phospotungstic acid PH 6.5). Scale: bar 50 nm.

Regarding phages active against *X. campestris* pv. *cucurbitae* this is believed to be the first description. There were no morphological differences between the phages isolated from pumpkin (XCU-P1) and zucchini (XCU-P3) (Table 2) and the lysis patterns (Table 3) were similar. The only reference is a «polyphage» isolated from soil and plant debris by Sutton et al. (28) that reacted *in vitro* with several *Xanthomonas* spp., including *X. campestris* pv. *cucurbitae*.

XHOL-P1 has polyhedral heads of 50-55 nm in diameter and long non-contractile tails of 150 x 10 nm (Fig. 2), this is the first report for a phage active against *X. campestris* pv. *holcicola* which showed a high degree of specificity (Table 3). The morphology of XMA-P1 was the same as reported by Das *et al.* (6) and the characteristics of XPR-P1 were similar to those described by Ghei *et al.* (13). All the phages were chloroform-resistant.

Phage susceptibility tests

Patterns of phage sensitivity —only positive reactions— are listed in Table 3. More than 60 % of *Xanthomonas* strains tested were susceptible to one or more phages. Pathovars *begoniae, corylina, eucalypti, glycines, juglandis, mangiferaindicae, phaseoli, pelargonii, poinsetiicola, translucens, vitians, zinniae*, one strain of *X. campestris* pv. *dieffembachiae*, 4 strains of *X. campestris* pv. *vesicatoria* (from pepper) and *X. albilineans* were negative against the 7 phages tested. The other genera *(Pseudomonas, Agrobacterium, Clavibacter* and *Erwinia)* did not react with any of the bacteriophages. The results reported herein confirm the similarity among the *Xanthomonas* at a generic level, in contrast with the pattern within pathovars of *X. campestris* which are heterogeneus.

The activity of any particular phage was not restricted to isolates of one particular pathovar. Some bacteriophages, such as XCU-P1 and XCU-P3 reacted with a large number of *Xanthomonas*, while others, as XHOL-P1 and XCA-P1 only gave positive reactions with a limited number of strains. The specificity of *X. campestris* pv. *campestris*-phages was previously reported by Liew and Alvarez (22). XMA-P1 reacted with *X. campestris* pv. *malvacearum* strains and some isolates of *X. campestris* pv. *cucurbitae* which means a satisfactory level of specificity.

TABLE 3

BACTERIOPHAGE REACTION PATTERNS

Strain	XCA-P1	XCU-P1	XCU-P3	XHOL-P1	XMA-P1	XPR-P1	XVE-P1
X. campestris pv.:							
alfalfae (01-alf)	–	∅	–	–	–	–	∅
campestris (04-ca)	●	∅	–	–	–	–	–
campestris (05-ca)	●	–	–	–	–	–	–
campestris (06-ca)*	●	○	–	–	–	–	–
campestris (07-ca)	●	–	–	–	–	–	–
campestris (08-ca)	●	–	–	–	–	–	–
campestris (62-ca)	∅	–	–	–	–	–	∅
campestris (63-ca)	∅	∅	–	–	–	–	∅
cucurbitae (10-cu)	–	∅	∅	–	–	–	–
cucurbitae (11-cu)	–	●	●	●	●	●	–
cucurbitae (12-cu)*	–	●	●	●	●	●	–
cucurbitae (13-cu)*	–	●	●	●	●	●	–
cucurbitae (64-cu)	–	○	○	–	–	–	–
cucurbitae (65-cu)	–	●	●	∅	∅	–	–
cucurbitae (66-cu)	–	●	●	●	●	●	–
cucurbitae (92-cu)	–	○	○	–	–	–	–
dieffembachiae (45-di)	–	●	–	–	–	●	–
holcicola (18-hol)	–	∅	●	●	–	●	–
holcicola (19-hol)*	–	∅	●	●	–	●	–
holcicola (67-hol)	–	∅	●	●	–	●	–
holcicola (68-hol)	–	●	●	●	–	●	–
malvacearum (21-ma)	∅	∅	∅	–	●	–	–
malvacearum (22-ma)	∅	–	–	–	●	–	–
malvacearum (23-ma)	–	∅	–	–	●	–	–
malvacearum (24-ma)	–	–	–	–	●	–	–
malvacearum (25-ma)	–	∅	–	–	●	–	–
malvacearum (26-ma)*	∅	∅	∅	–	●	–	–
malvacearum (27-ma)	∅	∅	∅	–	●	–	–
malvacearum (48-ma)	–	∅	∅	–	●	–	–
pruni (34-pr)	–	–	–	∅	–	●	–
pruni (35-pr)	●	–	–	∅	–	●	●
pruni (36-pr)	●	∅	∅	∅	∅	●	●
pruni (37-pr)*	∅	–	–	∅	–	●	–
pruni (72-pr)	–	–	–	∅	–	●	–
pruni (73-pr)	●	–	–	∅	–	●	●
vesicatoria (39-ve)	–	∅	–	–	–	–	∅
vesicatoria (41-ve)*	–	–	–	–	–	–	●
vesicatoria (42-ve)	–	∅	–	–	–	–	●

(*) Indicator stain.

Reactions:
● Clear (positive).
∅ Semiturbid (positive).
○ Turbid (positive).
– Negative.

Du Plessis *et al.* (7), Eisentark and Bernstein (11) and Thronberry *et al.* (30) reported a high degree of specificity in *X. campestris* pv. *pruni*-phages. In contrast, here, XPR-P1 showed a low degree of specificity at the pathovar level because it not only reacted with all the *X. campestris* pv. *pruni* isolates but also with several strains of *X. campestris* pv. *cucurbitae, X. campestris* pv. *dieffembachiae* and *X. campestris* pv. *holcicola.* There is no conflict between the results reported in this paper and those of Lovrekovich and Klement (23) and Klement (19). Isolates of *X. campestris* pv. *vesicatoria* from tomato, pepper and cape gooseberry are not uniform and could be distinguished by the use of bacteriophages. In contrast, Dye *et al.* (9) concluded that pepper strains can not be distinguished from tomato strains by means of phages.

Four cultures of *P. maltophilia* showed clearing when they were exposed to HT shock solutions of some bacteriophages. Such reactions, positive only when tested at HT, were not considered indicative of sensitivity in the present work because they were negative at RTD and RTD × 100 and such behaviour may be due to antibiotic effects, lethal adsorption or bacteriocin action. Nevertheless, this is an interesting datum in relation to the similarities between *P. maltophilia* and the *Xanthomonas* group, which were pointed out by Swings *et al.* (29) who proposed the transference of that species to the genus *Xanthomonas* as *X. maltophilia.*

The results clearly show that *Xanthomonas*-phages are useful and reliable at a generic level, but not at the pathovar level since one phage isolated from one pathovar is capable of lysing different pathovars. Nevertheless, at the pathovar level, certain strains could be distinguishable by parameters such as their relationships between phage sensitivity and host specificity.

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Note: The bacteriophages and bacterial propagating strains isolated have been deposited in the University of Queensland Department of Microbiology Culture Collection (UQM) and are listed in Table 1.

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Production of Riboflavin (Vitamin B_2) by Hydrocarbon-utilizing Yeasts

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Summary

Candida guilliermondii Wickerham was found to be superior to the other yeasts tested for growth yields and riboflavin production when cultivated on different carbohydrate and hydrocarbon-containing media. Among the refined petroleum fractions and cheap hydrocarbons tested, solar was selected as a carbon source best suited for the fermentation process. The highest growth yield and riboflavin output (10.64 mg/100 ml) by *C. guilliermondii* Wickerham were achieved by placing aliquots of 20 ml medium (pH 6.0) in 100 ml Erlenmeyer flasks and incubating the inoculated medium at 30° C for 14 days in the dark.

Key words: Riboflavin, Hydrocarbons, Candida guilliermondii.

Resumen

Se ha encontrado que *Candida guilliermondii* Wickerham crece mejor y produce más riboflavina que otras levaduras estudiadas cuando se cultiva en diferentes medios que contienen carbohidratos o hidrocarburos. Entre las fracciones de petróleo refinado y de hidrocarburos baratos probados, «solar» fue seleccionado como la mejor fuente de carbono para el proceso de fermentación. El mejor crecimiento y la mayor producción de riboflavina (10,64 mg/100 ml) por *C. guillermondii* Wickerham se consiguió colocando alícuotas de 20 ml de medio (pH 6,0) en matraces Erlenmeyer de 100 ml e incubando el medio inoculado a 30° C durante 14 días en la oscuridad.

Introduction

Although different carbohydrates have been used as a carbon source for the microbial production of riboflavin (3, 5, 15), studies concerned with the formation of the vitamin from the fermentation of hydrocarbons have been increased drastically since the first report of Sato *et al.* (13). Thus, different hydrocarbons have been found of use for the microbial production of vitamin B_2 (1, 9, 12, 18, Sabry *et al.* 1989, in preparation).

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The systematic exploitation of hydrocarbons for riboflavin production has not been previously investigated in Egypt, although they have been tested for the production of single cell protein, amino acids and lipids (4, 19 and Olama, Z., 1985. Ph. D. Thesis, Alexandria University). We were therefore, prompted to explore the potentiality of different microorganisms to produce riboflavin from hydrocarbons. In a previous article, Sabry *et al.* (under press) described a successful fermentation method for the vitamin B_2 production by certain mold fungi. In the present communication the work has been extended to evaluate the capacity of several yeasts to utilize a local crude oil and some of its fractions for the production of riboflavin.

Materials and methods

Microorganisms

The identities as well as the sources of the different yeasts used are presented in Table 1. Stock cultures were maintained on glucose-peptone slopes.

Composition of culture media (g/L.):

Medium I: glucose, 50; peptone, 5; yeast extract, 3; beef extract, 2.

Medium II: glucose, 10; peptone, 5; KH_2PO_4, 1.5; $MgSO_4.7H_2O$, 0.5; corn steep liquor, 10; yeast extract, 2.

Medium III: crude beet molasses, 60; KH_2PO_4, 1; $MgSO_4.7H_2O$, 1; $(NH_4)_2HPO_4$, 5.

Medium IV: glucose, 40; asparagine, 2; urea, 2; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5; thiamine, 0.5.

Medium V: glucose, 30; corn oil, 10; peptone, 5; yeast extract, 2.

TABLE 1
PRODUCTION OF RIBOFLAVIN BY THE TESTED YEASTS GROWN IN DIFFERENT TYPES OF MEDIA (AS SHOWN BY THE TLC ANALYSES)

Yeasts	Source	I	II	III	IV	V
Candida guilliermondii Wickerham	DSM (Deutsche sammlung von Mikroorganismen) Gottingen, Germany.	+	+	+	+	+
C. guilliermondii strain 1	DSM.	+	+	+	+	+
C. tropicalis Y-21	NRRL (Northern Regional Research Laboratory, Poria, 111, USA).	–	–	–	–	–
C. utilis Y-30	NRRL.	–	–	–	–	–
C. utilis Y-900	NRRL.	–	–	–	–	–
Geotrichum tropicum	MIRCEN (Microbiological Resource Centre Ain Shams University).	–	–	–	–	–
Hansenula polymorpha Y-7	NRRL.	–	–	–	–	–
Pichia polymorpha	MIRCEN.	+	+	+	+	+
Rhodotorula rubra Y-18	NRRL.	–	–	–	–	–
Saccharomyces cerevisiae 1	MIRCEN.	–	–	–	–	–
S. cerevisiae 2	MIRCEN.	–	–	–	–	–
S. cerevisiae SB	MIRCEN.	–	–	–	–	–
S. cerevisiae Y-4332	MIRCEN.	–	–	–	–	–
S. cerevisiae Y-2034	MIRCEN.	–	–	–	–	–
S. cerevisiae Y-1347	NRRL.	–	–	–	–	–
S. cerevisiae Y-2235	NRRL.	–	–	–	–	–

Hydrocarbons

The hydrocarbons, which were used as the only carbon source throughout the present work, were kindly provided by the refinery plant at Alexandria of Misr Petrol Company. These were mainly composed of El-Alameen crude oil and some of its fractions; the boiling point ranges of these are: C_6-fraction (60-80° C); C_9-fraction (148-150° C); C_{11}-fraction (195-196° C); C_{12}-fraction (214-216° C); C_{13}-fraction (235-236° C); C_{14}-fraction (236-240° C); C_{15}-fraction (258-262° C); C_{16}-fraction (283-286° C); solar (248-379° C); kerosene (153-239° C) and gasoline (44-150° C); crude oil and mixture 1.

Cultivation

For inoculum cultivation in Erlenmeyer flasks of 250 ml, a suspension of the contents of two slants after mixing each with 10 ml sterile distilled water was shaked with 50 ml of the fermentation medium for 48 hours to initiate growth. All fermentation media received a 5 % aliquot (v/v) of this inoculum.

The organisms were allowed to grow in 20 ml portions of the medium placed in 100 ml Erlenmeyer flasks. Initial pH's of the medium were adjusted using 1N HCl or 1N NaOH.

All nutrient solutions were sterilized by autoclaving. The hydrocarbon substrate was added to the cooled autoclaved, medium at 5 % (v/v). The sterilized media were inoculated, and incubated in the dark under static condition at 30 ± 2° C for the requisite time. The content of each flask was then centrifuged and the necessary analyses were made.

Analyses

Extraction and estimation of riboflavin

The cultures obtained at the end of the incubation period were heated for 30 min at 75° C in order to liberate the vitamin bound to the cells into the culture medium (8). The cells were then removed from the cultures by centrifugation.

Identification of riboflavin was carried out by thin layer chromatography (TLC) using a solvent system of n-butanol-acetic acid-water (4:1:5 v/v) (18). Spots giving a lemon yellow fluorescence were identified as riboflavin as compared with an authentic sample. Quantitative estimation of riboflavin was carried out spectrophotometrically at $\lambda = 450$ nm (6, 7).

Determination of growth yield

After centrifugation the yeast grown, was washed and dried at 70° C to constant weight.

Results and discussion

Screening experiments

Among the tested organisms, *Candida guilliermondii* Wickerham, *C. guilliermondii* (Strain 1) and *Pichia polymorpha* showed the ability to produce riboflavin (Table 1). The flavinogenesis of *C. guilliermondii* and *Pichia* sp. by carbohydrate fermentation has previously been reported (11, 15).

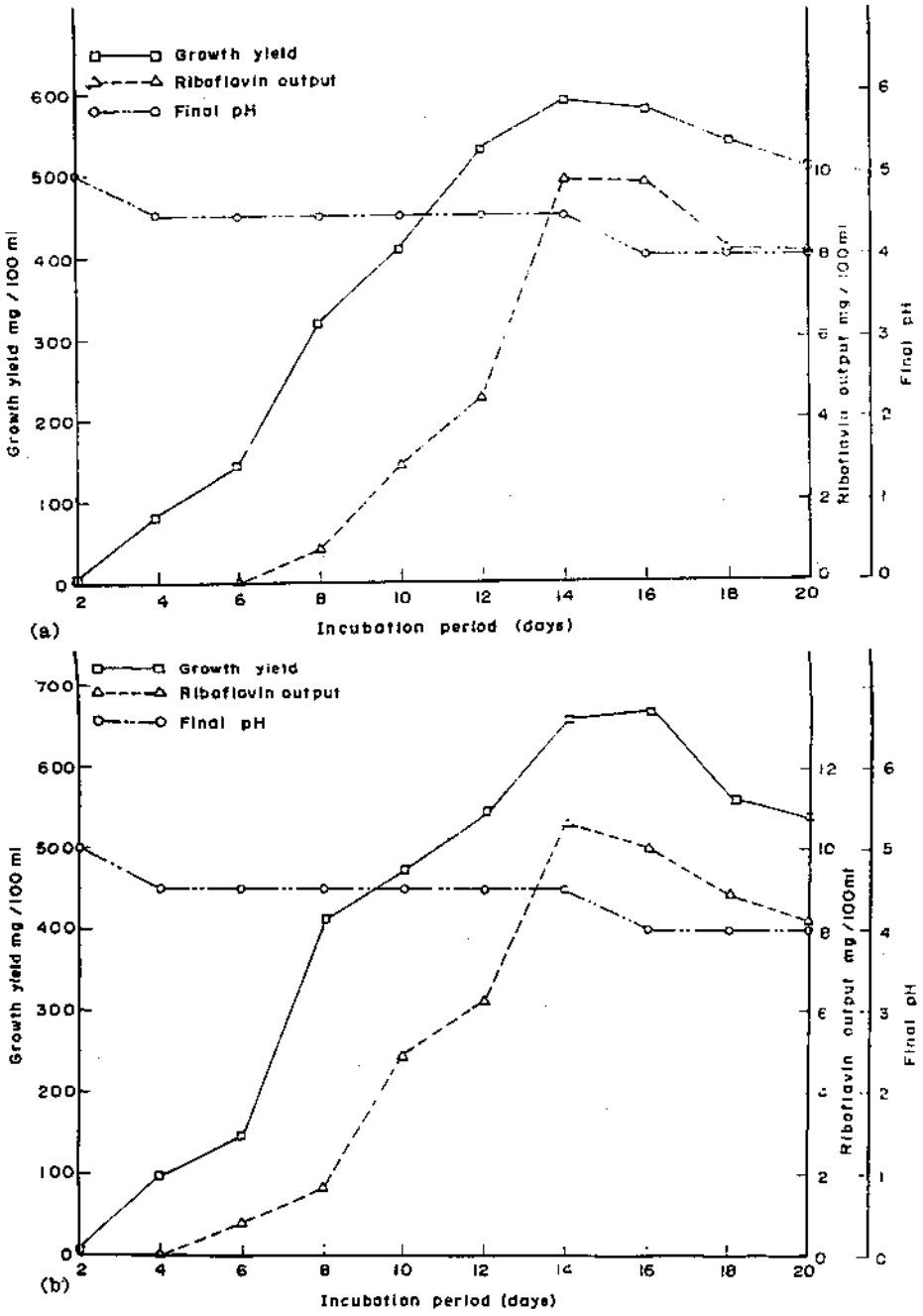

Fig. 1. Growth yield as dry weight and riboflavin output of *Candida guilliermondii* Wickerham during different periods of incubation under static (a) and shaked conditions (b).

TABLE 2
YEAST GROWTH AS DRY WEIGHT AND RIBOFLAVIN YIELD (BOTH IN MG/100 ML) OF THE SELECTED YEASTS CULTIVATED ON GLUCOSE-CONTAINING MEDIA

Organism	I		II		III		IV		V	
	Dry weight	Ribo-flavin	Dry weight	Ribo-flavin	Dry weight	Ribo-flavin	Dry weight	Ribo-flavin	Dry weight	Ribo-flavin
Candida guilliermondii Wickerham	1742	8.07	501	10.93	401	4.29	893	5.56	592	10.09
Candida guilliermondii Strain 1	993	7.47	493	9.98	420	4.08	901	4.63	621	9.89
Pichia polymorpha	980	4.62	490	6.23	420	2.50	930	2.46	640	5.02

Incubation period 14 days.
Initial pH, 6.0.

As clearly shown in Table 2, the *Candida* tested showed relatively higher growth yields and riboflavin productivity compared to *Pichia polymorpha*. These results are in agreement with those of Demain (3) who reported that *C. guilliermondii* is an over producer of vitamin B_2.

The nature and chemical composition of the media tested exerted a remarkable effect on the yeast growth as well as on riboflavin output. Evidently, the production of the vitamin seemed not to be consistently related to the growth yields of the yeasts. These observations have been previously noted for several yeasts (Osman, H. G. and Shaheen, F. A. 1966, the 1st Arab Chemical Congress, Cairo, Shaheen, F. 1981. Ph. D. thesis, Cairo University). The corn steep-liquor and corn oil containing media supported a good vitamin yield. The stimulatory effect of corn-steep liquor and corn oil on the flavinogenesis of yeasts has been reported previously (Ragab, A., 1968. M. Sc. thesis, Ain Shams University) (16).

Appropriatness of different hydrocarbons

The two *Candida* strains selected were tested for their potentiality to grow and produce riboflavin on different hydrocarbon fractions as well as some cheap hydrocarbons. Both strains failed to grow on hexane (C_6) as a sole carbon source, but were able to efficiently utilize the refined petroleum fraction from C_9 to C_{16} as well as mixture 1 (Table 3). The appropriatness of n-alkanes as carbon sources for riboflavin production has been reported (2, 8, 10).

The (C_{13}-C_{16}) n-alkanes supported the highest values of growth yield and riboflavin production. C_{15} was the best among the other tested refined fractions.

Among the cheap hydrocarbons tested, solar seemed to be the best carbon source. This might be due to the wide boiling range of the solar distillate fraction (containing n-paraffins with short and long chains). Crude oil was less effective, while kerosene and gasoline were inhibitory to vitamin production.

Candida guilliermondii Wickerham was selected as the experimental organism for subsequent studies. Solar, being a cheap carbon source, and supporting relatively higher riboflavin yield (9.86 mg/100 ml) compared to glucose (8 mg/100 ml), was also selected for further experimentations.

Some factors affecting riboflavin production

1. *Temperature.* The growth and riboflavin biosynthesis by *C. guilliermondii* Wickerham were markedly affected by the incubation temperature. The maximal growth value and riboflavin output (590 and 9.84 mg/100 ml medium) were achieved at 30° C. Growth did not continue at higher temperatures, while lower temperature seem to be inhibitory. Similar results have been reported (8, 10, 17).

2. *Aeration.* In hydrocarbon fermentation, aeration is a significant factor for microbial growth as well as emulsification of the substrate (15). Under our experimental conditions, 20 ml of the culture medium in 100 ml Erlenmeyer flask allowed optimal cell yield and riboflavin production. Maximum production of the vitamin with 75 ml of medium in a 500 ml flask was reported (9).

3. *pH relations.* It is evident from Table 4, that cell mass yield and riboflavin biosynthesis by the tested yeast responded differently to the pH value of the fermentation medium. Initial adjustment of the basal medium to pH values below 4 seems to be inhibitory for growth and vitamin production. However, the fermentation parameters increased in a linear and regular fashion between pH 4 and 6. Thus maximal growth and riboflavin output were recorded at pH 6.0. The suitability of the slightly acidic medium for vitamin B_2 production was also reported (10).

To overcome the observed shift in the initial pH of the medium, aliquots of the fermentation medium were adjusted with acetate or phosphate buffer to different pH values. The acetate-buffered medium completely inhibited vitamin formation, while the maximal yield of riboflavin achieved

TABLE 3
UTILIZATION OF DIFFERENT HYDROCARBONS AS A SOLE CARBON SOURCE BY THE TESTED RIBOFLAVIN-PRODUCING YEASTS

Hydrocarbons 5 % (v/v)	*Candida guilliermondii* Wickerham		*C. guilliermondii* Strain 1	
	Growth yield as dry weight	Riboflavin yield	Growth yield as dry weight	Riboflavin yield
	mg/100 ml medium		mg/100 ml medium	
C_6	0.00	0.00	0.00	0.00
C_9	90	0.62	90	0.60
C_{11}	328	4.09	324	3.20
C_{12}	423	4.63	396	4.02
C_{13}	372	5.32	313	6.20
C_{14}	393	6.14	401	4.81
C_{15}	491	8.23	382	7.68
C_{16}	525	7.41	456	6.82
Mixture 1 (C_9-C_{14})	313	5.38	310	5.39
Crude oil	622	2.87	621	2.08
Solar	590	9.86	489	9.79
Kerosone	472	0.00	423	0.00
Gasoline	189	0.00	540	0.00

The basal medium has the following composition (g/l).
$(NH_4)_2 SO_4$, 3.75; $NH_4H_2PO_4$, 3.75; KH_2PO_4, 2.5; K_2HPO_4, 2.5; $MgSO_4.7H_2O$, 1; pH, 6.0.
Incubation period 14 days.

TABLE 4
GROWTH AS DRY WEIGHT AND RIBOFLAVIN YIELD (BOTH AS MG/100 ML) OF *CANDIDA GUILLIERMONDII* WICKERHAM GROWN IN THE BASAL MEDIUM INITIALLY ADJUSTED TO DIFFERENT pH VALUES

pH value		Dry weight	Riboflavin
Initial	Final		
2	2.0	98	1.05
3	2.5	166	1.58
4	3.5	277	3.15
5	4.5	348	7.02
6	4.5	590	9.84
7	6.5	409	4.30
8	7.0	247	2.17
9	7.5	136	1.08

with the phosphate-buffered medium adjusted to pH 5.5 was 8.8 mg/100 ml. This value is lower than that obtained with medium initially adjusted to pH 6 (9.84 mg/100 ml).

4. *Fermentation time course.* The changes in the growth and riboflavin yields of *C. guilliermondii* Wickerham during the fermentation period were recorded for different time intervals. Figure 1 shows that under static culture conditions, significant growth was observed after 6 days of incubation and reached maximum levels at the end of the 14th day, after which it started declining. On the other hand, riboflavin was only traced after 6 and 8 days in shaken and static cultures respectively, and increased concurrently up to the 16th day. However a slight increase of the yeast yields was recorded upon using the shaken culture technique.

Solar is a promising substrate for the production of riboflavin from *Candida guilliermondii* Wickerham. Highest vitamin yield (10.64 mg/100 ml) was obtained when the organism was grown in 20 ml portion of the medium in a 250 ml Erlenmeyer flask and incubated in the dark at 30° C shaked for 14 days. Some other culture conditions affecting the flavinogenesis of *C. guilliermondii* Wickerham was recently published by the same authors (14).

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MICROBIOLOGIA SEM 5 (1989), 53-55 **NOTES**

Actual identity of six micrococcal strains selected as potential starter for dry fermented sausages production

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Summary

The DNA guanine + citosine contents of six strains previously selected as potential starters for their use for the dry fermented sausages production have been determined. Five strains were characterized as *Micrococcus spp.* and the remainder as *incertae sedis.*

Key words: Micrococci, *dry sausages.*

Resumen

Se ha determinado el contenido de guanina + citosina del DNA de seis cepas de micrococáceas seleccionadas previamente como posibles integrantes de un cultivo iniciador útil para la fabricación de embutidos madurados. Cinco cepas se caracterizaron como *Micrococcus* spp. y la restante se consideró como *incertae sedis.*

In a previous work, sixty two strains of micrococci isolated from Spanish dry fermented sausages had been studied in order to select the most appropriate ones for their use as potential starters for the sausage production (5). In that work the micrococci were only tentatively characterized according to the routine lysostaphin test developed by Schleifer and Kloos (4). To achieve a definite separation of micrococcal from staphylococcal strains, it would be necessary to use a more discriminatory test. The most conclusive method to achieve this goal is the determination of guanine + citosine (G+C) content but this test is not applicable for routine purposes. The present work was carried out to confirm the identity of the six strains finally selected as potential starters in a previous investigation (5).

The organisms used were six strains (3, 10A, 10C, 11, 12 and 30) of presumptive micrococci previously isolated from Spanish dry fermented sausages (5). *Staphylococcus aureus* ATCC 6538-P was used as control. The isolation and purification of DNA were carried out as reported by Marmur (2). The determination of the (G+C) content of the DNA was carried out according to Ulitzur (6). The sensitivity to the lysostaphin was studied by the turbidimetric, semiquantitative method developed by Zygmunt *et al.* (7).

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Table 1 shows the G+C content of the DNAs of the six strains of micrococci and that of *S. aureus* ATCC 6538-P and the lytic action of lysostaphin on these organisms. It has been reported (1) that the percentage of reduction in turbidity after 20 min of incubation at 37° C is the most appropriate time to establish the degree of sensitivity to lysostaphin. According to this criterion and using *S. aureus* as reference, five of the six strains (3, 10A, 10C, 11 and 30) would be considered as micrococci, whereas the strain *Micrococcus*-12 would not. This latter strain showed an intermediate sensitivity to the lytic agent. However, it presented a greater resistance to the lysostaphin than that of *S. aureus*. The six strains were tentatively characterized as micrococci according to the routine lysostaphin test (5). The results obtained by the turbidimetric method are in general agreement with those of the Schleifer and Kloos test (4). Therefore, the method developed by these authors to estimate the sensitivity to lysostaphin seems to be appropriate for routine purposes.

On the other hand, on the basis of the G+C content of the DNA, five strains (3, 10A, 10C, 11 and 12) were characterized as micrococci. The strain *Micrococcus*-30 showed a G+C content of 55 %. This level is not related to either *Staphylococcus* (G+C 30-40 %) or *Micrococcus* (G+C 66-75 %) genera. Since the G+C content is the most definitive test to characterize a given microorganism, the strain 12 was finally identified as belonging to *Micrococcus* genus and the strain 30 was considered as *incertae sedis*.

Because of the earlier microbiological clasification (taxonomy and nomenclature) of *Micrococcaceae* members and the difficulty of differentiating between the genera *Micrococcus* and *Staphylococcus*, several micrococci strains formerly used as starter cultures for dry fermented sausages production were later identified as staphylococci (3). It is probable that some strains of both genera may be used for this aim. However, it is also probable that food hygienists would reject the utilization of staphylococci as starter cultures. Therefore, it may be more adequate to prepare the starters with micrococci definitively characterized as such. In conclusion, the strains studied in this work can be used without any trouble for dry fermented sausages production.

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TABLE 1

LYSOSTAPHIN SENSITIVITY AND DNA GUANINE + CITOSINE CONTENT OF MICROCOCCI AND *STAPHYLOCOCCUS AUREUS*

Strain	% reduction in optical density (time in min)						% G+C
	5	10	15	20	30	60	
3	0.2	4.8	5.6	6.5	8.8	16.6	67
10A	0.0	0.0	0.0	0.0	1.2	4.9	66
10C	0.0	0.0	0.0	0.6	1.7	5.8	70
11	2.4	2.4	6.5	6.8	7.6	5.9	66
12	8.0	17.3	20.1	29.0	38.9	44.3	63
30	5.9	5.6	8.4	10.6	11.1	25.9	55
S. aureus	19.7	58.3	75.0	84.2	87.5	93.4	36

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Isolation of a high frequency donor of *Rhodobacter capsulatus* by integration of the plasmid pMT1000 into the chromosome

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Summary

The temperature-independent clone of *Rhodobacter capsulatus* UA7041, carrying the temperature-sensitive plasmid pMT1000, has been obtained by selection for plasmid markers at the non-permissive temperature. The transfer to *Escherichia coli* of all drug resistance encoded by pMT1000 was of about 10^{-7} when the donor was the UA7041 strain, and of 5×10^{-4} when the donor was the temperature-sensitive strain of *R. capsulatus*.

Electrophoretic analyses showed no plasmid band typical of the autonomous pMT1000 in the UA7041 strain. Furthermore, UA7041 cells were also able to mobilize chromosomal markers of *R. capsulatus* at a frequency of about 10^{-4} per donor cell. All these results lead us to conclude that the plasmid pMT1000 is integrated in the chromosome of the UA7041 strain. The analysis of the co-transfer frequencies of *trp* and *his* markers has shown that the chromosome mobilization in the UA7041 strain of *R. capsulatus* is unidirectional from a single origin. Our data also show that the pMT1000 plasmid may be useful to the construction of chromosomal, high-frequency donor strains.

Key words: Rhodobacter capsulatus, Hfr-donor, plasmid integration, chromosome mobilization.

Resumen

A partir de una cepa de *Rhodobacter capsulatus* portadora del plásmido termosensible pMT1000 se ha aislado el clon UA7041 capaz de multiplicarse a la temperatura restrictiva en presencia de los antibióticos para los que codifica resistencia dicho plásmido. La transferencia a *Escherichia coli* de las resistencias codificadas por el plásmido pMT1000 cuando la cepa donadora era UA7041 fue de 10^{-7}, mientras que esta misma transferencia se obtuvo a una frecuencia de 5×10^{-4} cuando la cepa donadora era el clon de *R. capsulatus* sensible a la temperatura. El análisis electroforético del DNA de la cepa UA7041 no mostró la presencia de la banda correspondiente al plásmido pMT1000. Asimismo, la cepa UA7041 fue capaz de movilizar marcadores cromosómicos de *R. capsulatus* a una frecuencia de 10^{-4} por célula donadora. Este conjunto de resultados permite afirmar que la cepa UA7041 tiene el plásmido pMT1000 integrado en su cromosoma. Al estudiar las frecuencias de cotransferencia de los marcadores *his* y *trp* se ha podido establecer que la movilización del cromosoma de la cepa UA7041 de *R. capsulatus* es unidireccional y tiene lugar desde un origen único. Nuestros datos demuestran también que el plásmido pMT1000 puede ser de gran utilidad para la construcción de cepas capaces de movilizar el cromosoma bacteriano.

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The nonsulfur purple phototrophic bacterium *Rhodobacter capsulatus* (formerly *Rhodopseudomonas capsulata)* is the first phototrophic bacterium for which a useful genetic exchange mechanism has been discovered (6), and it remains the most well-developed system for genetic studies of photosynthesis. Some attempts to obtain Hfr-like strains by integration of a plasmid into the chromosome of *R. capsulatus* have been done by using the pTH10 plasmid, a derivative of the broad-host-range plasmid RP4 (12). In *Escherichia coli* K-12, pTH10 is temperature-sensitive for maintenance, being lost from cells growing at the restrictive temperature (42° C). Some Hfr-derivatives have been isolated from *Escherichia coli* strains carrying pTH10 by selecting for a temperature-independent, drug-resistance phenotype. These strains were found to present pTH10 integrated in the chromosome by means of the Tn1-mediated co-integrate formation. They behave as Hfr strains, transferring the *E. coli* chromosome from an origin at the site of insertion (4). However, studies done with pTH10 in *R. capsulatus* B10 showed neither evidence of temperature-sensitivity nor integration in the chromosome, although the plasmid was able to transfer chromosomal markers by an unknown mechanism of chromosome mobilization (12). In spite of this, some linkage between several groups of genes has been determined using this plasmid (12). Nevertheless, a high frequency of recombination (Hfr) donor with a single origin of chromosomal transfer could be very useful for genetic studies with this bacterium. We report here the isolation of a Hfr strain of *R. capsulatus* PAS100 by integration of plasmid pMT1000 in the chromosome, pMT1000 is a Tn501-loaded derivative of pMO190, a mutant of R68 described as temperature-sensitive in *Pseudomonas aeruginosa* (10).

The bacterial strains and the plasmids used are listed in Table 1. *E. coli* strains were routinely grown in LB medium or in LB-agar plates (9) at 30° C. Aerobic cultures of *R. capsulatus* were grown at 30° C with shaking in Pfennig's minimal medium (8) supplemented with Casamino Acids at 0.5 % (w/v) as the nitrogen source, and 0.2 % (w/v) succinate as the carbon source. Amino acids for auxotrophs or for the selection of chromosomal markers transfer were added to a final concentration of 20 μg/ml. The complex medium used for *R. capsulatus* was PYE (11). When needed, both media were solidified with 1.5 % (w/v) agar. Antibiotics and metal ions were added to selective media as reported (10, 12).

Isolation of spontaneous antibiotic-resistant mutants was carried out according to (1). Matings between *E. coli* and *R. capsulatus,* either as donor or recipient, were performed by the spot procedure (9, 12) on PYE agar medium. After 16-24 h, the resulting growth was scraped off from the plate, resuspended and plated either on Pfennig's minimal medium-agar (for transfer to *R. capsulatus)* or on LB-agar medium (for transfer to *E. coli),* both media containing Hg, Tc and Km. Construction of Hfr strains and selection for integration into the chromosome of the pMT1000 plasmid was done

TABLE 1
BACTERIAL STRAINS AND PLASMIDS USED IN THIS WORK

Strain or Plasmid		Relevant Genotype/Phenotype	Source or reference
Escherichia coli	MC1061	Str^r	M. Casabadan
	UA4217	MC1061 (pMT1000)	This laboratory
Rhodobacter capsulatus	PAS100	Str^r Rif^r	B. L. Marrs
	UA7034	PAS100 (pMT1000)	This work
	UA7041	As UA7034 but with pMT1000 integrated into the chromosome	This work
	LS2	*his-1 trp-1*	(11)
	UA7040	*his-1 trp-1* Nov^r	This work
Plasmids	pMT1000	Ap^r (Tn1) Tc^r Km^r Hg^r (Tn501) Tra^+	(9)*

* pMT1000 is pMO190: Tn501. pMO190 is a temperature-sensitive mutant of R68.

following Miller's strategy (7). pMT1000-mediated transfer of chromosomal markers between different *R. capsulatus* strains was done according to (12) using appropriately supplemented Pfennig's minimal medium plates for selection. Plasmid DNA was extracted as reported (3) and agarose gel electrophoresis was performed according to standard techniques (5).

pMT1000 was transferred from *E. coli* UA4217 to *R. capsulatus* PAS100 at 30° C and selected for Tc and Hg resistance. In *R. capsulatus,* pMT1000 plasmid conferred resistance to Tc, Hg and Km, but not to Ap [ß-lactamase genes seem not to be expressed in *R. capsulatus* (2)], and retained the character of thermo-sensitivity for maintenance. Thus, the plasmid was lost in cells of *R. capsulatus* growing at the non-permissive temperature (42° C). This behaviour differs from that showed by pTH10, which presents a lack of phenotypic expression of temperature-sensitivity in *R. capsulatus* B10 (12).

pMT1000 has been used in *P. aeruginosa* PAO for transposon insertion mutagenesis by means of Tn501, providing that it transposes at a frequency about ten-fold higher than does Tn1 (10), which is also present in this plasmid. For this reason, we tried to obtain the integration of pMT1000 in the chromosome of *R. capsulatus* in a similar way, either by Tn501- or by Tn1-mediated co-integration, during a process of selection for drug- resistant cells at the restrictive temperature (4). The strain harbouring the plasmid pMT1000 (UA7034) was incubated overnight at the permissive temperature (30° C) in a plasmid-selective medium containing Hg and Tc. Then, the cells were plated on a pre-warmed solid selective medium and incubated at two different non-permissive temperatures, 40° C and 42° C. After 48 hours, a few temperature-independent, drug-resistant derivatives grew at 40° C, whereas no cells grew at 42° C. One of these temperature-independent clones resistant to Tc, Hg and Km was purified and designated as UA7041. Only about 18 % of UA7041 cells retained temperature independence and drug resistance after 72 h incubation in liquid media at 30° C in the absence of selective pressure. Thus, the growth of the UA7041 strain was always performed at 40° C and in the presence of Tc, Hg and Km.

The temperature-independent derivative UA7041 was able to transfer its drug-resistances to *E. coli* MC1061 only at a frequency lower than 10^{-7} per donor cell by conjugation at 40° C on solid media. This frequency was considerably lower than that showed by the strain harbouring the autonomous pMT1000 (UA7034) at the permissive temperature (30° C) which is 5×10^{-4} per donor cell. So, it seemed very unlikely that the UA7041 clone carried a revertant of the temperature-sensitive plasmid. This result strongly suggests that the UA7041 strain carried pMT1000 integrated in its chromosome.

Furthermore, when a preparation of extrachromosomal DNA from UA7041, grown at 40° C, was examined by agarose gel electrophoresis, no plasmid band was found (not shown), as we should expect if the plasmid were not present in the autonomous state. On the other hand, a parallel preparation of extrachromosomal DNA from UA7034 grown at 30° C gave a band corresponding to the plasmid (not shown). The same result was obtained after three different plasmid extractions, and it is in agreement with the genetic evidence for integration shown above.

The results presented above support the view that pMT1000 is integrated in the chromosome in the UA7041 derivative. Therefore, we examined the ability of UA7041 to mobilize wild type chromosomal markers to the auxotrophic strain UA7040, that is novobiocin-resistant. The results obtained are shown in Table 2. The reversion rates in the receptor cells were between 8×10^{-9} for tryptophan *(trp)* and 10^{-8} for histidine *(his),* whereas the frequency of appearance of Nov^r colonies in the donor strain was about 10^{-6}. Among the genetics markers tested, *trp* and *his* were transferred at a frequency of about 10^{-5} per donor cell after 3 h in plate matings and of about 10^{-4} after 24 h. No recombinants were recovered when antibiotic-resistant chromosomal markers (Str, Rif) were assayed, probably because they are very closely linked to novobiocin, as has been suggested (12). The co-transfer frequency of unselected donors was determined by replica plating. A significant difference of the co-inheritance frequency was found when selection was first done for *trp* than for *his* (Table 2). If

TABLE 2
CHROMOSOME MOBILIZATION MEDIATED BY pMT1000 AND GENETIC LINKAGE IN *RHODOBACTER CAPSULATUS*

Selected marker	Spontaneous reversion	Frequency of recombinants per donor cell[a]	% Co-inheritance of unselected markers[b]	
			his-1	*trp-1*
his-1	1×10^{-8}	4×10^{-5}	100	62.6
trp-1	1×10^{-8}	7×10^{-5}	32.5	100

[a] Matings were carried out between UA7041 and the recipient strain UA7040. The counterselection was for novobiocin.
[b] The linkage values were obtained by screening more than 100 recombinants of each class.

Trp$^+$ clones were firstly selected, the mean co-transfer frequency for *his* yielded 32.5 %; whereas when selection was initially made for His$^+$ clones, the mean co-transfer for *trp* was 62.6 %. All these data suggest that mobilization is unidirectional from a single origin, and that the UA7041 strain could be a useful genetic tool in the construction of a linkage map in *R. capsulatus*.

Acknowledgments

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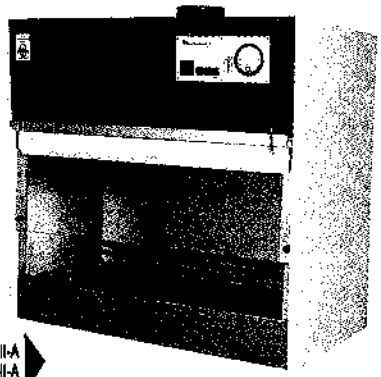

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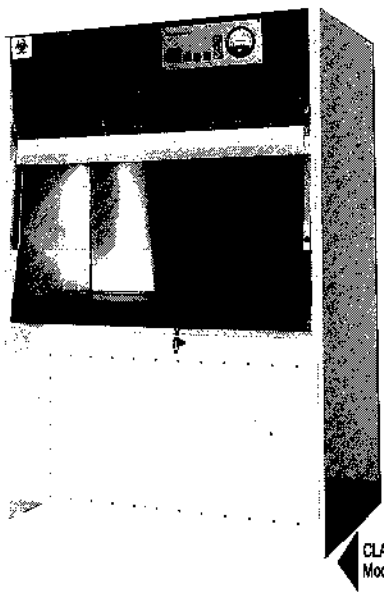

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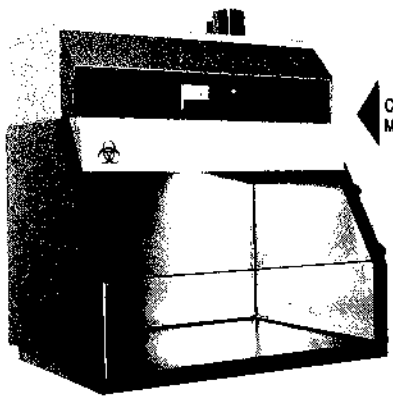

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API STAPH	(≠ 2050)	25 especies
API 20 C AUX	(≠ 2021)	43 especies
API 20 A	(≠ 2030)	60 especies

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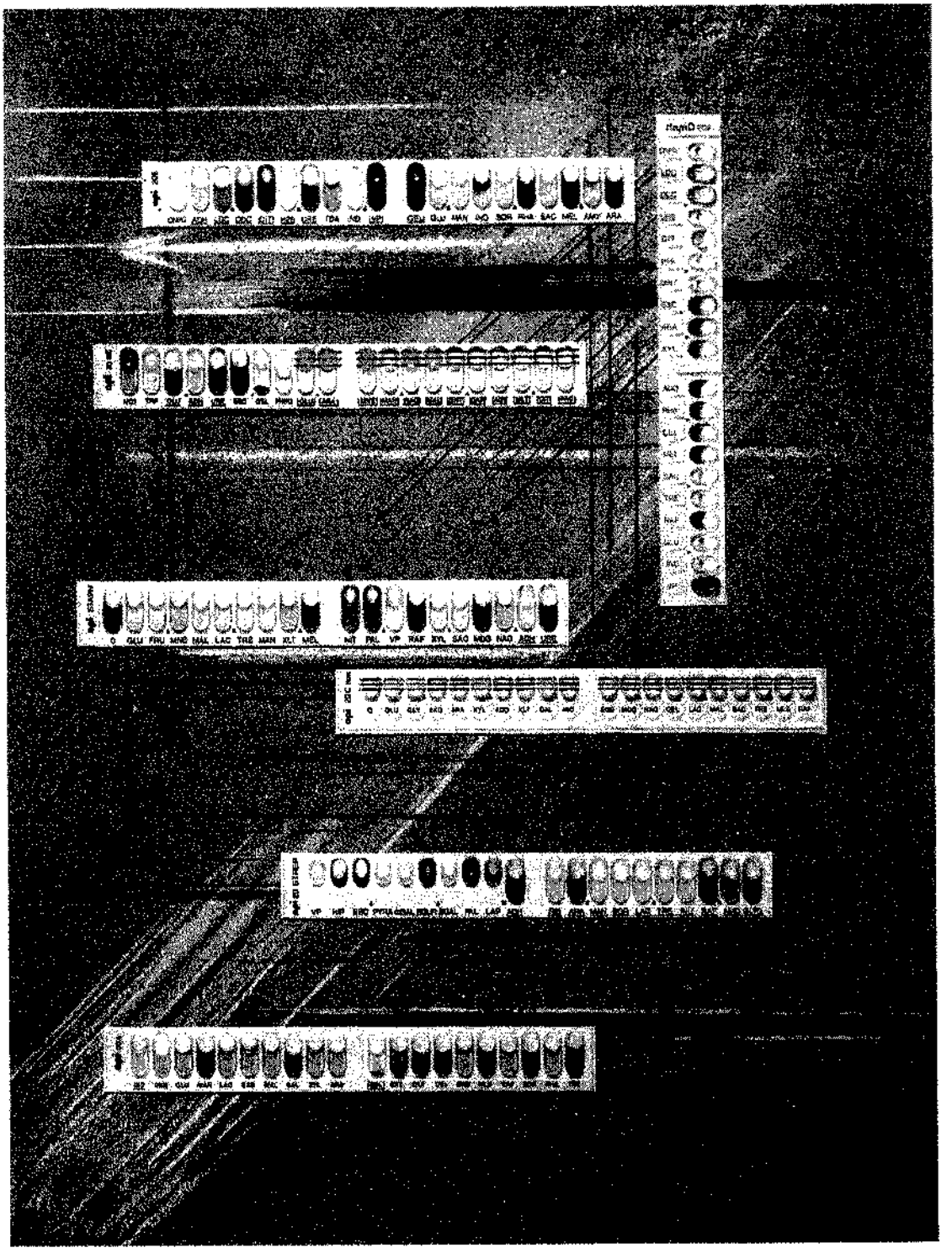

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