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Phenotypic and Genotypic Characterization of Unusual Vaginal Isolates of *Candida albicans* from Africa

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As expected by its global prevalence, the most frequently isolated species of yeast from vaginal swabs obtained from patients in Africa was *Candida albicans*, which accounted for 53 of 85 (62.4%) of the isolates from women in Madagascar and 35 of 54 (64.8%) of the culture-positive women in Angola. However, 40% of the Madagascan and 23% of the isolates from Angola, as well as two isolates obtained from one German patient, were not able to utilize the amino sugars glucosamine and *N*-acetylglucosamine as the sole carbon source. These isolates were able to form germ tubes but did not form chlamydospores. The correct identification as *C. albicans* was made possible only by using a PCR-based method of DNA fingerprinting. Only minor phenotypic and genotypic variation was observed among these strains. Whether they represent a distinct clone that is found mainly in Africa is not clear. The relevance of the amino sugar catabolic pathway in *C. albicans* is discussed in view of these results.

The predominant species of yeast isolated from vaginal specimens is *Candida albicans* (8, 11, 15, 16). The conventional methods of identifying yeasts to the species level rely on morphological characters and reactions to a panel of biochemical tests. Isolates of *C. albicans* are typically identified by their ability to form germ tubes or chlamydospores under the appropriate conditions. To identify other species of *Candida* or species of other genera of medical yeasts, as well as to confirm its identification or to biotype *C. albicans*, commercial carbohydrate assimilation systems, such as the ID 32C system, are widely available. Previous studies showed that the majority of isolates of *C. albicans* belong to a single physiological biotype, B1, which is characterized by the assimilation of xylose, adonitol, xylitol, sorbitol, methyl-D-glucoside, *N*-acetyl-D-glucosamine, sucrose, and trehalose and the inability to assimilate glycerol, L-arabinose, and melezitose (12, 25). Other minor biotypes are found only occasionally. However, some of the biochemical reactions assumed to be characteristic for a given species can be variable, depending on the isolate. Problems may arise when a reaction thought to be typical varies or when the number of variable characters for a species becomes too large (23).

With the ID 32C system, the assimilation of the amino sugars glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc) is considered to be an invariant characteristic of all isolates of *C. albicans*. This report describes strains of *C. albicans*, which were isolated from vaginal specimens in Africa, that are unable to utilize either of these amino sugars. These isolates are also unable to form chlamydospores. A PCR fingerprinting method, which had been used previously to biotype strains of *C. albicans* (18), was used in the study to identify the atypical isolates as *C. albicans.*

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MATERIALS AND METHODS

***Candida* isolates.** Vaginal swabs were taken from 258 women attending a sexually transmitted disease outpatient department in Madagascar and from 183 gynecological patients from the Medical University Luanda, Angola. None of the patients had received local or systemic antimycotic therapy during the previous month. Two additional isolates were obtained from the oral cavity and the feces of a patient with urticaria who was seen at the Dermatology Clinic of Charité Hospital. The swabs were processed by the method used routinely for the detection of yeast pathogens. Samples were inoculated onto Sabouraud glucose agar supplemented with chloramphenicol and were cultured at 37°C for 48 h. For the identification of *C. albicans*, isolates were placed in serum to test for the production of germ tubes and were incubated on rice agar for up to 10 days to induce chlamydospores. Yeast isolates that either were not *C. albicans* or that did not produce chlamydospores were identified to the species level according to their carbohydrate assimilation patterns on ID 32C strips. The test strips were stored for up to 7 days and were evaluated both manually, by using the analytical profile index, and automatically, by using the ATB reader with the corresponding identification software (BioMerieux SA, Marcy-l'Etoile, France). Thirty-one patients from Madagascar with positive cultures for *C. albicans* were examined for clinical signs of candidal vaginitis.

PCR fingerprinting. DNA was isolated by the cetyltrimethylammonium bromide (CTAB) minipreparation method (7). Briefly, a few yeast colonies were taken from an agar plate or slant and were resuspended in 300 μl of extraction buffer (100 mmol Tris-HCl per liter [pH 8.0], with 1.4 mol of NaCl per liter, 20 mmol of EDTA per liter, 2% CTAB, and 0.2% β-mercaptoethanol). The cells were weakened by freezing and thawing (three times) and were crushed with a micropestle. One volume of chloroform was added, and the mixture was vortexed and centrifuged at 13,000 × *g* for 15 min. The upper phase was removed and the DNA was precipitated with cold isopropanol (about 600 μl). After centrifugation for 10 min, the pellet was washed with 70% ethanol. The DNA was dissolved in TE buffer or in deionized water and was stored at 4°C.

The following oligonucleotides were used as single primers in the PCR experiments: 10-mer oligonucleotide AP3 (5′-TCA CGA TGC A) (24); simple repeat sequence $(GTG)_5$ (5′-GTG GTG GTG GTG GTG) (1); and T3B, which was derived from a tDNA intergenic spacer (5′-AGG TCG CGG GTT CGA ATC C) (13). Amplification reactions were performed in volumes of 50 μl containing 1 to 25 ng of template DNA; 10 mmol of Tris-HCl per liter (pH 8.0); 50 mmol of KCl per liter; 1.5 mmol of $MgCl_2$ per liter; 3 mmol of magnesium acetate per liter; 200 mmol (per liter) each of dATP, dCTP, dGTP, and dTTP (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.); and 1.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The primers AP3 or T3B were added at final concentrations of 25 pmol per 50 μl of assay mixture, and $(GTG)_5$ was used at 5 pmol per 50 μl of assay mixture. The samples were overlaid with sterile, light mineral oil (Sigma, St. Louis, Mo.) and were amplified in a Perkin-Elmer thermocycler 9600 instrument as follows: initial denaturation was for 5 min at 95°C; denaturation was for 15 s at 95°C; annealing was for 30 s at 36°C (for the AP3 primer), 50°C [for the $(GTG)_5$ primer], or 52°C (for the T3B primer); and extension was at 1.20 min at 72°C; this was followed by a final extension cycle of 6 min at 72°C. Reaction tubes were held at 4°C prior to analysis. A total of 32

TABLE 1. Identification of vaginal *Candida* isolates obtained from patients attending sexually transmitted disease outpatient clinics in Madagascar and from gynecological patients from Angola

Species	No. of isolates from the following source:	
	Madagascar (n = 258)	Angola (n = 183)
Overall yeasts	85	54
C. albicans biotype 1 or 2	32	27
C. albicans (GlcN and GlcNAc negative)	21	8
C. glabrata	9	13
C. famata	4	1
C. tropicalis	0	3
C. parapsilosis	3	0
Other species	16[a]	2[b]

[a] Nine species.
[b] Two species.

PCR cycles were run with the primers $(GTG)_5$ and T3B, whereas 45 cycles were used with the AP3 primer. Samples were concentrated to a volume of approximately 20 μl (Speed Vac; Savant, Hicksville, N.Y.) and were electrophoresed in 1.2% agarose gels (5 mm by 25 cm by 20 cm) for 5 h at 3 V cm^{-1} in 0.5× TBE buffer (0.045 mol of Tris-borate per liter, 1 mmol of EDTA per liter). Amplification products were detected after staining the gels with ethidium bromide. The stained gels were photographed, and the DNA fragments were sized and compared with the use of scanner-associated computer hardware and software (RFLPscan, version 2.01; Scanalytics CSP Inc., Billerica, Mass.).

RESULTS

Vaginal specimens indicated that 85 of 258 women (33%) from Madagascar and 54 of 183 gynecological patients (30%) from Angola were colonized with yeasts. *C. albicans* was the most frequently isolated species in both groups and accounted for 62.4% of the isolates from Madagascar and 64.8% of the isolates from Angola (Table 1). The next most frequent yeast species in both groups was *Candida glabrata* (*Torulopsis glabrata*), which was isolated from 10.6 and 24% of the women, respectively. Other species of *Candida* were cultured less often: *C. famata* (n = 5), *C. tropicalis* (n = 3), *C. parapsilosis* (n = 3), *C. kefyr* (n = 3), *C. norvegensis* (n = 2), *C. lusitaniae* (n = 2), and *C. krusei* (n = 1). These results are in agreement with those of other studies of the vaginal yeast flora (8, 11).

Twenty-nine of the *Candida* isolates from African women and the two isolates from a German woman grew very slowly and failed to produce chlamydospores on rice agar. However, they all produced germ tubes. The carbohydrate assimilation patterns of all of these strains were typical of *C. albicans* with the exception of negative utilization of the two amino sugars GlcN and GlcNAc. Consequently, the ID 32C system identified these isolates as *C. sake*. In Fig. 1, the assimilation patterns of strains that were reliably identified as *C. albicans* (profile number 73473400) were compared with those of GlcN- and GlcNAc-negative isolates thought to be *C. sake* (profile number 70423400). These chlamydospore-negative strains that were unable to metabolize the two amino sugars were subjected to the PCR fingerprinting assay.

Of the 31 Madagascan women who were investigated for clinical signs of candidal vaginitis, cultures of specimens from 16 women grew the normal biotype of *C. albicans*. Vaginal specimens from the remaining 15 of these patients had amino sugar- and chlamydospore-negative strains of *C. albicans*. Clinical vaginitis developed in 13 of the 16 patients (80%) infected with typical *C. albicans* strains and 7 of the 15 patients (47%) infected with atypical strains of *C. albicans*.

The PCR fingerprinting technique, which detects DNA polymorphisms in fungal genomic DNA by using single primers in the PCR, has been successfully applied to the epidemiological typing of clinical strains of *C. albicans* (18). However, with the individual primers T3B (Fig. 2A) or $(GTG)_5$ (Fig. 2B) or with AP 3 (data not shown), the patterns obtained with different *Candida* species are highly species specific. By using different primers, distinctive and reproducible sets of amplification products were observed for 24 *Candida* species. These products could easily be distinguished by their PCR patterns (un-

FIG. 1. Carbohydrate assimilation patterns of one Candida albicans biotype 1 strain (upper panel) and of one GlcN- and GlcNAc-negative strain (lower panel) obtained with the ID 32C system. The profile numbers are 7147 3400 in the case of biotype 1 and 7342 3400 in the case of the GlcN- and GlcNAc-negative strain.

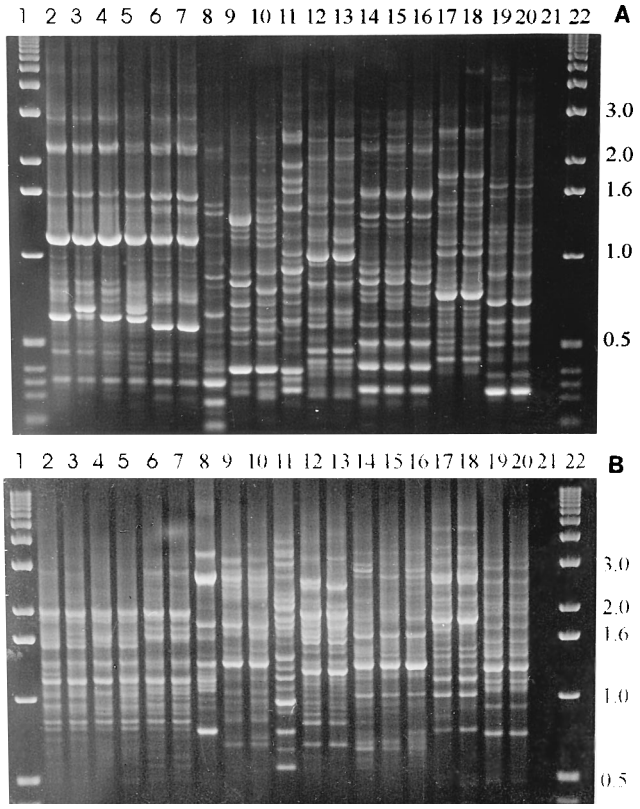

FIG. 2. PCR fingerprints of different *Candida* species including two GlcN- and GlcNAc-negative strains obtained with the primers T3B (A) and $(GTG)_5$ (B). Lane 2, *C. albicans* CBS 562 (serotype A); lane 3, *C. albicans* CBS 5983 (serotype B); lane 4, *C. stellatoidea* CBS 1905; lane 5, *C. albicans* wild-type strain of biotype 1; lanes 6 to 7, GlcN- and GlcNAc-negative strains from two different Madagascan patients; lane 8, *C. sake* CBS 159; lane 9, *C. glabrata* NCYC 350; lane 10, *C. glabrata* wild-type strain; lane 11, *C. famata* CBS 1795; lane 12, *C. kefyr* Y 601; lane 13; *C. pseudotropicalis* ATCC 4135; lane 14, *C. krusei* CBS 573; lane 15, *C. krusei* wild-type strain, lane 16, *Issatchenkia orientalis* MB 16; lane 17, *C. guilliermondii* ATCC 6260; lane 18, *C. guilliermondii* wild-type strain; lane 19, *C. parapsilosis* wild-type strain; lane 20, *C. parapsilosis* wild-type strain; lane 21, control sample without DNA; lanes 1 and 22, molecular size markers (in kilobases).

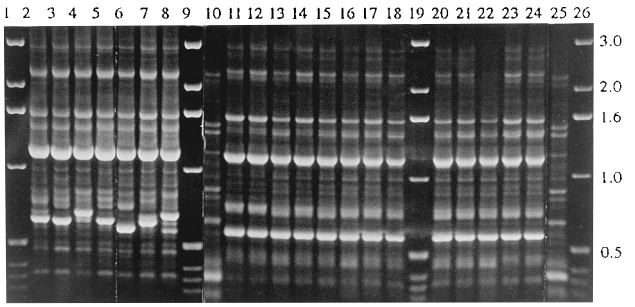

FIG. 3. PCR fingerprints obtained from GlcN- and GlcNAc-negative *C. albicans* strains with primer T3B. Lanes 2 to 5, *C. albicans* reference strains (ATCC 14053, CBS 562, CBS 5983, and CBS 1905, respectively); lanes 6 to 8, *C. albicans* wild-type strains; lanes 10 and 25, *C. sake* reference strain (CBS 159); lanes 11 and 12, GlcN- and GlcNAc-negative strains isolated from a German patient; lanes 13 to 18, GlcN- and GlcNAc-negative strains obtained from different patients in Angola; lanes 20 to 24, GlcN- and GlcNAc-negative strains obtained from different patients in Madagascar; lanes 1, 9, 19, and 26, molecular size markers (in kilobases).

published data). With primer T3B a very unique amplification profile of *C. albicans* was found (Fig. 2). If the PCR profiles of appropriate reference strains were compared with those of clinical isolates, this method might be developed to identify medically important species of the genus *Candida*.

The two GlcN- and GlcNAc-negative *Candida* isolates generated PCR fingerprints (Fig. 2A and B, lanes 6 and 7) that were very similar to those of the strains of *C. albicans* (Fig. 2a and b, lanes 2 to 5) and clearly distinguishable from those of *C. sake* (Fig. 2a and b, lanes 8). This result was true for all the GlcN- and GlcNAc-negative strains tested. Therefore, the 24 isolates from 22 African patients and the German woman, all of which failed to assimilate the amino sugars or produce chlamydospores, were concluded to be atypical strains of *C. albicans*. The GlcN- and GlcNAc-negative strains, which were characterized by very similar growth parameters and identical assimilation patterns, exhibited only slight variations in their PCR fingerprints, even among strains from different geographical regions (Fig. 3).

DISCUSSION

To our knowledge, this is the first description of a wild-type population of strains of *C. albicans* that have lost the ability to assimilate the amino sugars GlcN and GlcNAc. These isolates were also slowly growing and were unable to produce chlamydospores. Approximately 40 or 23% of all vaginal yeast isolates from women in Madagascar or Angola, respectively, belonged to this altered biotype of *C. albicans*.

The ID 32C system database of biochemical reactions indicates that the proportion of isolates of *C. albicans* that can utilize GlcN is 100% and that the proportion that can utilize GlcNAc is 99%. Therefore, these amino sugar-negative strains were misidentified by the ID 32C system as *C. sake*. By using the PCR fingerprinting method, which generated species-specific fingerprint patterns for each of the *Candida* species tested, it was demonstrated that all physiologically and morphologically atypical isolates were indeed strains of *C. albicans*.

To determine whether the strains that fail to metabolize GlcN and GlcNAc were confined to Africa, isolates in Germany were examined. Since September 1992, the Mycological Laboratory identified 5,935 isolates of *C. albicans*, which were obtained from a variety of clinical specimens from immunocompromised patients, as well as from dermatological and gynecological patients. Three hundred forty-one of these isolates (5.7%) did not produce chlamydospores and were subsequently tested in the ID 32C system. Only two of these isolates from a German woman exhibited a phenotype and genotype very close to those of the African isolates. She had no history of travel to or association with contacts from Africa.

Until now, strains unable to utilize GlcNAc as the sole carbon source have been very rare (12, 25), and no data were found in the literature concerning the ability to assimilate GlcN. Strains of *C. albicans* unable to form chlamydospores were also reported by Ginter et al. (8) in an investigation in Austria of vaginal yeast colonization among prostitutes, but it is unknown whether these strains had altered biochemical biotypes.

The catabolic pathway of GlcN and GlcNAc in *C. albicans* is well known (5, 6, 9, 19, 21). GlcNAc is transported into the cell by GlcNAc permease, where it is converted by the sequential effects of GlcNAc kinase and GlcNAc-6-phosphate deacetylase into GlcN-6-phosphate. GlcN is transported into the cell by general sugar permease and is then phosphorylated by GlcN kinase. The product of this reaction is also GlcN-6-phosphate, which is transformed to fructose-6-phosphate. The latter reaction is catalyzed by the enzyme glucosamine-6-phosphate deaminase, which is therefore necessary for the catabolism of

both of amino sugars. The enzymes of GlcNAc metabolism are inducible by GlcNAc (21). Recently, the gene encoding glucosamine-6-phosphate deaminase (*NAG1*) was cloned and extensively characterized by Natarajan and Datta (14). Their studies revealed that the *NAG1* gene is present in a single copy in the genome of *C. albicans*. A mutation in this gene may explain the inability of the GlcN- and GlcNAc-negative strains described here to assimilate the two amino sugars. Such a mutation could exist in the structural *NAG1* gene itself or in a gene that regulates its expression. Indeed, the altered assimilation pattern in these strains may be caused by the same or several different mutations.

GlcNAc supports a high growth rate of *C. albicans*, and at 37°C, GlcNAc induces the yeast-mycelium conversion of chlamydospore formation (3, 20). GlcNAc is both the inducer and the carbon source for morphogenesis. The enzymes required for the catabolism of GlcNAc are involved in the chitin synthesis during the yeast-mycelium transition (2, 4, 9). The GlcN- and GlcNAc-negative strains of *C. albicans* in the present investigation were characterized by low rates of growth. None of the strains produced chlamydospores, but they were able to form germ tubes when they were incubated in serum. These results support the suggestion of Cassone et al. (3) that GlcNAc is not directly involved in the formation of germ tubes but might trigger this process by binding to a cell surface receptor and inducing an intracellular signal that primes the cell for morphogenesis. However, it can be concluded from our data that GlcNAc is important for the process of chlamydospore formation, which occurs only in vitro (15).

Since GlcNAc is utilized only by pathogenic species of *Candida*, it has been suggested that the GlcNAc catabolic pathway may relate to virulence (14, 21). In the present study, GlcN- and GlcNAc-negative strains of *C. albicans* were able to cause candidal vaginitis. Of course, variation in virulence undoubtedly exists among strains that are positive or negative for the utilization of amino sugars. Among the patients who were diagnosed with candidal vaginitis, about 80% of those infected with typical strains of *C. albicans* developed vaginitis, but only 47% of those harboring atypical isolates had vaginitis. The relationship, if any, between the ability of *C. albicans* to metabolize amino sugars and virulence remains to be established.

C. albicans is known to be diploid and has been suggested to have a clonal population structure (10, 17, 22). Generally, the presence of several overrepresented genotypes provides evidence in support of clonality. The GlcN- and GlcNAc-negative strains demonstrate a rather unexpectedly high degree of phenotypic and genotypic similarity. When clinical isolates recovered from immunocompromised patients as well as from healthy dental clients were investigated by the same PCR fingerprinting procedure, the majority of *C. albicans* strains had individual PCR profiles. Only two patients attending the same intensive care unit were found to harbor identical *C. albicans* strains (18). The GlcN- and GlcNAc-negative isolates may represent a distinct clone of *C. albicans* that is distributed mainly on the African continent and found only rarely in other geographical areas.

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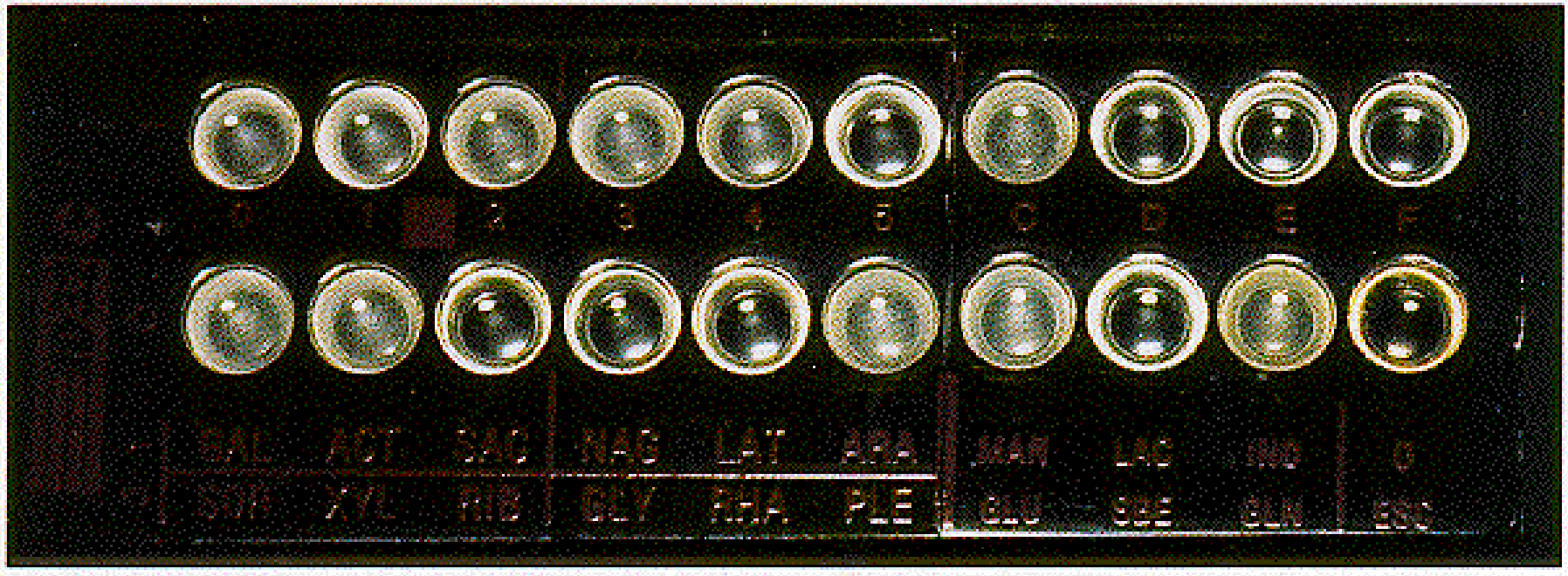
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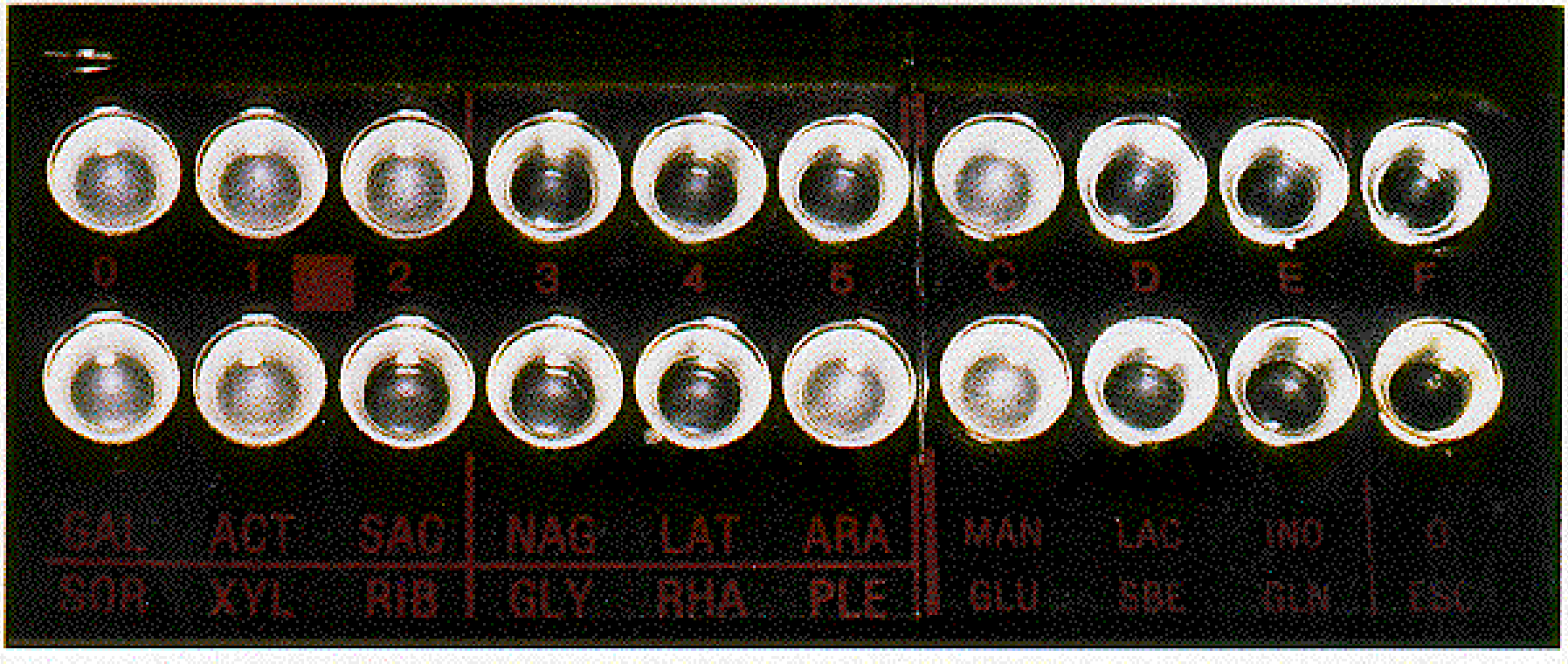
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