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Colony switching in an alpha-amylase-producing strain of *Bacillus subtilis*

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Four colony variants (two stable and two unstable phenotypes) were observed in *Bacillus subtilis* α-10, an α-amylase-hyperproducing strain. The stable variants lost the ability to produce α-amylase, while the unstable ones reverted to the typical morphology after restreaking. Unstable expanding sectors appeared in typical colonies, and their appearance was influenced by the culture origin and age.

Keywords: colony switching; *Bacillus subtilis*; morphological variants; alpha-amylase; transposable elements

Introduction

The frequent occurrence of two or more colony types has been reported in different microorganisms such as *Neisseria gonorrhoeae* [13], *Candida albicans* [12], *Xanthomonas campestris* [3] and *Bacillus subtilis* [4]. This switching often affects the production of important metabolites [4,6,8]. In most of the cases, spontaneous mutation has been claimed as the cause of this phenomenon. However, the frequency of variation is too high and the heredity of the changes too variable to be adequately explained by this concept [9]. In this paper we describe the occurrence of both stable and unstable colony variations in *Bacillus subtilis* α-10, an α-amylase-hyperproducing strain, and their relationship with the enzyme production and culture conditions. The possible genetic mechanism involved is discussed.

Materials and methods

Strain: *Bacillus subtilis* α-10 is an α-amylase-hyperproducing mutant [1].

Cultivation conditions and media: Growth was carried out in a rotary shaker at 150 rpm and 37° C, in 250-ml Erlenmeyer flasks containing 30 ml of medium. The culture medium contained (g L^{-1}): starch (from corn) 15.0; yeast extract 6.0; sugarcane molasses (as reducing sugars added to the medium) 10.0; NH_4HPO_4 10.0; $MgSO_4 \cdot 7H_2O$ 0.3. For solid cultures, nutrient agar-starch medium (Oxoid, UK) was used. It contained (g L^{-1}): peptone 5.0; yeast extract 2.0; Lab-Lenco powder 1.0; NaCl 5.0; and precooked corn starch 10.0.

Determinations: Growth was determined by viable counts on nutrient agar-starch plates. α-Amylase activity was estimated by an iodine-binding assay: 1% starch was submitted to hydrolysis with 1 ml of the culture supernatant fluid for 10 min at 60° C and pH 6.0, as previously described [1]. One unit of activity is defined as the amount of enzyme hydrolysing 1 mg of starch per min under the assay conditions. Plasmid extraction was done in chloramphenicol-amplified cultures, by the alkaline lysis method described by Maniatis *et al* [5], and the preparations were examined using agarose gel electrophoresis.

Assessment of variation frequency from liquid cultures: A flask containing 30 ml of medium was inoculated with cells from a colony showing typical morphology after 48 h growth, and grown for 24 h. Three milliliters of this culture were transferred to a flask containing fresh medium, and this subculturing was repeated every 24 h for 13 days. After each transfer, the culture was plated and analysed for the presence of the variant phenotypes. The experiment was carried out in duplicate.

Results

Occurrence of morphological colony variants

The typical colony phenotype of strain α-10 on nutrient agar-starch plates is opaque, slightly irregular borders, white-cream colonies. However, variant colonies exhibiting non-typical morphologies appeared frequently when the cultures were plated on the solid medium (Table 1). Two of them kept the variant phenotype after 20 passes in solid medium ('stable variants': α-IV and A), while the other two were highly unstable, reverting to the original morphology after restreaking. The rate of appearance of variants was variable, ranging from 3×10^{-1} to 5×10^{-3}.

Sectors of the two stable alternative phenotypes were detected rarely inside typical (wild type) α-10 colonies. Besides this, expansive sectors, at the periphery of the colonies, were observed at a high frequency in the plated cultures. The morphology of these sectors resembled that of one of the non-stable colony variants (variant VI, Table 1), and also reverted to the typical morphology after restreaking or plating. The cell morphology of samples picked from

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Table 1 Colony types of *Bacillus subtilis* α-10 on nutrient agar-starch plates

Colony type	Characteristics after 48 h of growth	Reversion to original type
α-10 (original)	Smooth, opaque, dark-cream, slightly irregular borders, 5–6 mm diameter	–
α-IV	Smooth, brilliant, regular borders, dark-cream, 3–4 mm diameter	No
A	Rough, opaque, cream-brownish, irregular borders, 6–7 mm diameter	No
α-5	Smooth, dark-cream, slightly irregular borders, opaque, 1–2 mm diameter	Yes
α-VI	Smooth, dark-cream, slightly irregular borders, opaque, 7–8 mm diameter	Yes

sectors, colony centres and variant colonies was identical to that of the typical colony.

Study of the stable variants

The biochemical and morphological characteristics of the two stable variants (α-IV and A) were similar to the original type and showed the characteristic pattern of *Bacillus subtilis*, according to Buchanan and Gibbons [2] (Table 2). Although total cell growth was similar in the variants and the original type (data not shown), the production of α-amylase activity in liquid cultures was greatly reduced in the α-IV variant (44 Units ml^{-1} *vs* 444 Units ml^{-1} in the original strain), and completely lost in the A variant. This coincidence between colony switching and a decreased ability to synthesize specific metabolites has been observed in various microorganisms [4,6,8].

To test whether plasmid loss could be involved in the morphological switching, the wild type and variant strains were analysed for the presence of plasmids. One plasmid, with the same molecular weight (23 kb) was detected in both the original and variant clones, suggesting that no plasmid loss had occurred.

The frequency of occurrence of variants from serial liquid cultures starting from a typical colony was investigated. After eight transfers, the α-IV variant was detected at a frequency of 2% with respect to the total number of colonies. This proportion increased through successive transfers, reaching 18% of the total colonies after 13 serial subcultures. Variant A appeared after 11 serial transfers, reaching a frequency of 15%.

The frequency of switching was further analysed by dilution and plating of one typical colony. Among 13317 colonies, the α-IV variant appeared at a frequency of 1.1%, indicating that cells having the potential to express the α-IV phenotype were present in the typical colony.

Both α-IV and A variants were stable: serial subcultures in liquid medium starting from a single variant colony were carried out for both variants. After 18 transfers the cultures were plated on nutrient agar-starch plates. From 18034 colonies examined for α-IV, and 19175 colonies examined for the A subculture, none showed reversion to the typical colony morphology.

Unstable expanding sectors

Table 3 shows the frequency of formation of unstable variant sectors obtained by dilution and plating of flask and slant cultures of the original α-IV strain. Flask cultures exhibited a markedly lower tendency to produce sectoring colonies than slant cultures. The tendency to form sectors decreased with the culture age. This points to an influence of the culture origin on the potential for production of variant sectors.

In a further experiment, a typical *B. subtilis* colony was picked, suspended in 1 ml of 0.9% NaCl and dilutions were plated on nutrient-agar starch medium. Two percent of colonies were sectored, 97.5% were of the typical morphology

Table 2 Biochemical and morphological characteristics of the original clone (α-10) and the variants α-IV and A, in comparison with the type species *Bacillus subtilis*

Characteristic	α-10	α-IV	A	*B. subtilis*[a]
Gram stain	+	+	+	+
Spore production	+	+	+	+
Motility	+	+	+	+
Production of pellicle in nutrient broth	+	+	+	+
Catalase	+	+	+	+
Starch hydrolysis	+	+	+	+
Casein hydrolysis	+	+	+	+
Gelatin liquefaction	+	+	+	+
Acid from:				
Glucose	+	+	+	+
Arabinose	–	–	+/–	–
Xylose	+	+	+	+
Manitol	+	+	+	+
Growth temperature (°C)				
Minimum	15	15	15	5–20
Maximum	50	50	55	45–55

[a]According to Buchanan and Gibbons [2]

Table 3 Frequency of occurrence of unstable variant sectors in colonies derived from wild type *Bacillus subtilis* α-10 cultures

Type of culture	Growth time (days)	Sectoring colonies (%)
Flask	1	5.4
Flask	1	1.4
Slant	1	75
Slant	3	47
Slant	7	52
Slant	28	50
Slant	46	30
Slant	60	30

and 0.5% were of the stable variant morphologies. One typical and one sectored colony were picked, suspended and diluted as before. The sectored colony cell suspension yielded 15% sectored colonies (180/1200 colonies examined), and 84% typical colonies. Only 2.5% (25/1002 colonies examined) of colonies from the typical colony cell suspension were sectored; the rest had typical colony morphology. This indicates that the sector cells show a higher tendency to form sectored colonies, when diluted and plated, than the typical colony cells.

Discussion

Stable colony variants

The production of stable morphological colony variants reported here coincides with previous observations for *Bacillus subtilis* and other microorganisms [3,4,6]. However, the high and variable rate of occurrence of these variants suggests that spontaneous mutation could not properly account for this phenomenon [9].

Sectors corresponding to the two stable alternative phenotypes were detected inside typical (wild type) α-10 colonies. Sectoring such as this, showing stable novel colony types, has also been observed in *Pseudomonas* [9] and *E. coli* [10] carrying a transposable element.

As in previous reports on colony-switching affecting production of important metabolites [4,6,8], our observations indicate that the occurrence of morphological variants in the α-10 strain of *B. subtilis* brings about the decrease or loss of the α-amylase activity in the stable variant clones. This relationship could arise from some genetic change that influenced simultaneously a series of genes. In *Bacillus subtilis*, the loss of enzyme activity was postulated by Tichy *et al* [14] to be related to the loss of a plasmid by the variant clone. In our case, however, the enzyme-associated morphological change was not connected with plasmid loss, since the native plasmid detected in the original clone was still present in the variants.

The frequency of occurrence of stable variants increased upon successive transfers of shaker cultures, indicating the enrichment of the cultures in the variant cells after the switch had taken place. In contrast, both variants remained stable when transferred in a similar manner. This suggests that, although the changes take place at a relatively high frequency, the nature of the genomic rearrangement allows a good stability after the variation has been established.

Unstable variants and sectors

The appearance of unstable variants which revert to the original type after restreaking or plating is a new observation for this species, although this phenomenon has been reported for other microorganisms such as *Candida albicans* [12].

Among the unstable types of variations, an interesting observation was the frequent appearance of unstable, expansive sectors at the colony borders. The formation of such radially expanding sectors suggests that the new phenotype started to be expressed in one cell at the colony edge, and then the change was transmitted and expressed in all the progeny cells, ie that some kind of hereditary change had occurred in the progenitor of each sector. This type of unstable variant sector had not been previously reported for *Bacillus subtilis*, but a similar phenomenon has been observed in *C. albicans* [12], and in *E. coli* colonies harbouring a Mudlac element [8].

Our results suggest that culture origin and age influence the frequency of unstable variant sector formation, when the cells are plated on an agar surface. This result is similar to that observed in *Neisseria gonorrhoeae* [13] and *C. albicans* [7], where the frequency of occurrence of variant colonies was affected by propagation history and medium composition.

Although the sector cells reverted to the parental type when restreaked, these revertants showed an increased tendency to throw off the same type of sectors again. This behaviour is similar to that observed in *E. coli* and *Salmonella* (JA Shapiro, personal communication, Dept of Biochemistry & Molecular Biology, University of Chicago), where it was established that the sectors resulted from a genetic duplication, which was favoured by a rearrangement in the ancestor of the sector.

The occurrence of both stable and unstable colony variants in this strain also is reminiscent of that reported in maize, where insertion of transposable elements causes inherently stable as well as unstable insertional mutations [11]. All these facts suggest the possibility that transposable elements could be involved in the observed colony morphology variations in this *B. subtilis* strain.

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