Induction of a Stable Morphological Change in *Propionibacterium freudenreichii*

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When cells of *Propionibacterium freudenreichii* were incubated under fasting conditions and then plated in the presence of an inhibitor of protein synthesis, a variable but significant ($>10^{-2}$) fraction of the population changed their morphology from rod to sphere, with a considerable thickening of the cell wall. This change was accompanied by metabolic and antibiotic-resistance modifications, including the synthesis of at least one new enzyme (α -glucosidase), and by the simultaneous appearance of several new species of DNA, presumably plasmids. The round cells grew faster than the parent strain and maintained their morphology indefinitely when propagated on complex medium containing glucose as the main carbon source. However, when glucose was omitted, cells returned to the rod form and regained their previous characteristics, including the absence of detectable plasmids.

INTRODUCTION

Species belonging to the genus *Propionibacterium* are described in the 8th edition of *Bergey's Manual of Determinative Bacteriology* as Gram-positive microaerophilic rods, non-motile and asporulate (Moore & Holdeman, 1974). Their morphology is variable in relation to several factors like the composition of the growth medium or the temperature of incubation. They often appear club shaped and joined in pairs forming a V, a feature characteristic of corynebacteria, but they may adopt branched elongated or coccoid forms. Since their first description (Von Freudenreich & Orla-Jensen, 1906), the metabolic properties of these bacteria have been the subject of a very large number of publications. Several reviews on the utilization of propionic bacteria in cheese making have also appeared (Hettinga & Reinbold, 1972). To our knowledge, however, and despite their economic importance, no information is available to date about their genetics.

During the course of our research on the genetic properties of propionibacteria, we occasionally observed on solid media colonies of large round cells, significantly bigger than the 'coccoid' forms. These bacteria were considered to be contaminants until we found they could be obtained in large amounts, under experimental conditions excluding any possibility of contamination or of selection of mutants. The round propionibacteria grew faster than the parent strain and maintained their morphology in complex medium containing glucose as the main carbon source. However, when glucose was replaced with lactate in the same medium, they returned to the rod form.

The procedure used to obtain stable morphological variants in high yields, their ultrastructure and some of their properties are described in this paper.

METHODS

Bacterial strains. *Propionibacterium freudenreichii* subsp. *shermanii* (ATCC 13673) was obtained from the collection of Entremont SA (France). Spontaneous Lac⁻ and chloramphenicol-resistant mutants were isolated from strain ATCC 13673 and designated LM and LM-C, respectively.

Culture media. A complex medium which allowed fast growth of *Propionibacterium* was used. The composition of BYC base was as follows: nutrient broth, 5 g l⁻¹; Casamino acids, 5 g l⁻¹; yeast extract, 3 g l⁻¹; NH₄Cl, asparagine and glutamine, 0.02 M each; NaCl, 0.01 M; MgCl₂, 1 mM; CaCl₂, 0.1 mM; Fe²⁺, Co²⁺, Mn²⁺, Zn²⁺ and Cu²⁺, 0.01 mM each. The pH was adjusted to 6.7. BYCG medium contained BYC base plus 5 g glucose l⁻¹; BYCL medium contained BYC base plus 6 g L(+)-sodium lactate l⁻¹. Solid BYCG or BYCL media contained 12 g agar l⁻¹. Glucose and lactate were not sterilized separately. MMYC medium was used by Santamaria *et al.* (1984) for the transformation of *Brevibacterium*. Probably because of a combination of high pH and low concentration of yeast extract, this medium does not give measurable growth of *Propionibacterium* after more than 24 h incubation. MET medium, containing (in 1 litre of BYC base) NaCl (5 g), sodium acetate (3 g), L-cysteine (0.5 g), glucose (3 g), sucrose (2 g) and agar (15 g), was complemented with 2 µg chloramphenicol ml⁻¹ and used in the transformation experiments described here.

In liquid medium, growth was followed at 620 nm. Except when otherwise indicated, cultures in liquid media were incubated in unstirred vials or tubes (static aerobiosis). Anaerobiosis, strictly necessary for growth of isolated colonies on solid media, was achieved using plastic jars or bags with a catalyst obtained from Merck-Clevenot France (Anaerocult).

Fermentation and antibiotic resistance profiles were determined using the API System (API 50CH, ATB Staph, ATB Strep and ATB Ana).

Transformation. *P. freudenreichii* ATCC 13673 was cultivated on BYCL medium. Cells were harvested at OD₆₂₀ 0.4 (about 7 × 10⁸ cells ml⁻¹) and resuspended in MMYC at OD₆₂₀ 0.03. At regular intervals (usually 10 or 15 min), between 0 and 4 h incubation at 30 °C in static aerobiosis, 1 ml samples of bacterial suspension (about 5 × 10⁷ cells) were removed, complemented with 8 µg DNA extracted from strain LM-C, and further incubated for 24 h at 30 °C under anaerobic conditions. From each sample, 0.1 ml was then plated on solid MET medium containing 2 µg chloramphenicol ml⁻¹, and incubated anaerobically for at least 48 h at 30 °C.

Enzyme activities. Cells were broken by two passages through a French pressure cell at about 173 MPa. A sample of the lysate was plated and the number of surviving cells was determined. The yield of broken cells was above 99% with strain ATCC 13673 and between 50 and 70% with morphological variants. These values were used in calculating the specific activities of enzymes per cell.

Lysates were centrifuged at 1000 g for 10 min, and the supernatants were used for enzyme determinations. Lactate dehydrogenases were estimated by following at 620 nm the rate of reduction of dichlorophenolindophenol (DCIP) at 25 °C, in the presence of either D(-) or L(+)-lithium lactate (Snoswell, 1966). One activity unit was defined as 1 µmol DCIP reduced min⁻¹ per 10⁹ cells. α-Glucosidase was estimated by hydrolysis of *p*-nitrophenyl-α-D-glucoside (pNPGlu) for 10 min at 37 °C in 0.1 M-potassium phosphate buffer, pH 7.0, containing 1 mM-Mg²⁺ and 1 mM-dithiothreitol. After addition of sodium carbonate up to 0.8 M, absorbance was read at 400 nm. One activity unit was defined as 1 µmol pNPGlu hydrolysed min⁻¹ per 10⁹ cells.

Detection of plasmids. Cultures (10 ml) in medium BYCG complemented with 2% (w/v) glycine were submitted to the 'second procedure' described by Yoshihama *et al.* (1985). The final DNA precipitate was dissolved in 20 µl 40 mM-Tris/HCl containing 2 mM-EDTA, and subjected to electrophoresis on 0.6% (w/v) agarose gels containing 0.5 µg ethidium bromide ml⁻¹. DNA bands were observed under UV light.

Electron microscopy. Bacteria grown in liquid medium were spun down and resuspended in fixative comprising 2.5% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 6.8, containing 5 mM-CaCl₂. After 1 h, the bacteria were washed in the same buffer, concentrated in agar (Whitehouse *et al.*, 1977) and postfixed for 1 h in 1% (w/v) osmium tetroxide dissolved in the same buffer. They were then incubated for 1 h in 1% (w/v) uranyl acetate in 0.1 M-veronal buffer, pH 6.0, dehydrated in acetone and embedded in Epon.

RESULTS

Induction of a morphological change

An experiment was devised to determine whether *P. freudenreichii* was transformable by homologous DNA after a conventional competence treatment. When following the protocol described in Methods, all the colonies growing on plates with chloramphenicol comprised round cells. The ratio of round to input cells was found to vary largely from one sample to another, without any apparent relationship with the time of sampling. However, it was constantly greater than 10⁻².

The results of one of the above experiments are given in Table 1. Controls provided the following information. (i) Omission of DNA from the competence medium had no effect. Thus, no transformation mechanism is implied in the observed phenomenon. (ii) Preincubation in the competence medium could be replaced by other types of aggression: heat shock (45 °C) in the

Table 1. Occurrence of round cells after incubation in MMYC medium and plating on chloramphenicol-containing solid MET medium

The no. of c.f.u. is expressed with respect to 1 ml of incubated suspension, which contained 50×10^6 cells ml⁻¹. All the colonies comprised round cells.

Incubation time (min)	c.f.u.	
	$10^{-6} \times$ No. of	%
110	1.60	4.6
120	1.10	3.1
130	0.65	1.9
140	4.80	13.7
150	0.16	0.5
160	2.20	6.3
170	1.60	4.6
180	0.53	1.5
190	0.70	2.0
210	6.00	17.1
210*	35.0	100

* Control plated on solid medium without chloramphenicol. All the colonies comprised rods. Percentages are expressed with respect to this control.

presence of polyethylene glycol or UV irradiation at a survival rate of about 0.1% (results not shown). (iii) Omission of chloramphenicol from the plating medium allowed treated cells to grow as normal coryneform bacteria. No colonies comprising round cells were observed.

In addition, no morphological change was detectable at any time by phase contrast microscopy of the bacteria incubated in MMYC competence medium. Round cells appeared only after growth on solid medium containing chloramphenicol at a concentration inhibitory for rod cells.

In conclusion, this treatment produced, at a high efficiency, a change in morphology, and, simultaneously, the occurrence of resistance to chloramphenicol. We were unable, however, to make the phenomenon completely reproducible. In some experiments, the yield of round cells relative to input rod cells was more than 50%, but this result, although obtained under apparently identical conditions, was obtained randomly and could not be predicted.

A dozen isolated colonies of round cells, picked at random in different experiments and designated E1, E2 etc., were propagated in BYCG medium at 30 °C. None of them reverted to the rod form when maintained in this medium. They appeared to belong to two different types. (i) Strains growing poorly on BYCG, doubling time about 10 h, maximum OD₆₂₀ about 0.3. (ii) Strains growing faster than the parent strain, up to an OD₆₂₀ greater than 1.0.

One of the strains exhibiting the best growth characteristics, strain E1, was arbitrarily selected to be described here as representative of this new form of *P. freudenreichii*. The generation time of this strain was 4.5 h under anaerobiosis, and 2 h under static aerobiosis, in BYCG medium at 30 °C; the generation time for the parent strain was 4.5 h under both conditions.

Electron microscopy

Bacteria of strain ATCC 13673, grown in BYCL medium and examined in thin section, had a rod-shaped appearance with a width of 0.5 µm (Fig. 1a). They had a homogeneous cell wall about 20 nm in thickness. Cell division occurred by the formation of a cross-wall, characteristic of all Gram-positive bacteria.

Bacteria incubated in MMYC medium and plated on solid medium containing chloramphenicol as described in Methods exhibited important morphological modifications. Their shape was spherical, with a diameter of about 1 µm. During exponential growth, the wall thickness was rather regular and was 40 nm, twice that of rod-shaped bacteria (Fig. 1b, c). In some bacteria, thickened wall portions were found near the septa, which were formed in the equatorial plane of

Fig. 1. *P. freudenreichii* ATCC 13673 growing in BYCG medium. (a) Control cells. (b, c) Exponentially growing cells after induction of morphological change. These spherical cells have a diameter twice that of rod-shaped bacteria. Their wall is thicker and shows some irregularity in thickness (arrows). Bars, 0.5 μm .

Fig. 2(a, b). Spherical bacteria in stationary phase. The cell wall thickness is very irregular and large dense aggregates seem to detach from the bacterial surface and accumulate in the culture medium (arrows). Bars, 0.5 μ m.

Table 2. *Antibiotic resistance profiles*

Concentrations are in $\mu\text{g ml}^{-1}$. Resistance (growth) or inhibition are indicated by + or - respectively, on the left side for the lowest, and on the right side for the highest concentration.

	Concn		Strain				Concn		Strain		
	Low	High	13673	E1	R3		Low	High	13673	E1	R3
Penicillin	0.25	16	+ -	- -	+ -	Minocycline	4	8	- -	- -	- -
Ampicillin	4	16	+ -	- -	+ -	Chloramphenicol	2	8	- -	+ -	- -
Ticarcillin	16	128	- -	- -	- -	Erythromycin	1	4	- -	+ +	- -
Oxacillin	2	-	+	-	+	Josamycin	2	8	- -	+ -	- -
Cefoxitin	4	16	+ +	- -	- -	Pristinamycin	2	4	- -	- -	- -
Cefoperazone	4	32	+ -	- -	+ -	Lincomycin	2	8	+ -	- -	+ -
Cefotaxime	4	32	+ -	- -	+ -	Clindamycin	2	8	- -	- -	- -
Latamoxef	4	32	+ -	- -	+ -	Metronidazole	4	32	+ +	+ +	+ +
Gentamicin	4	8	+ +	- -	+ +	Vancomycin	4	32	- -	- -	- -
Netilmicin	4	8	+ +	- -	+ +	Fusidic acid	2	16	- -	- -	- -
Dibekacin	4	8	+ +	- -	+ +	Cotrimoxazole	2	8	+ +	- -	+ +
Amikacin	8	16	+ +	- -	+ +	Foxfomycin	6	32	+ +	- -	+ +
Doxycycline	2	8	- -	- -	- -	Trimethoprim	2	8	+ +	- -	+ +

Table 3. Carbohydrate fermentation profiles

The no. of + is indicative of the estimated intensity of reaction.

	Strain				Strain		
	13673	E1	R3		13673	E1	R3
D-Arabinose	—	—	—	D-Raffinose	—	—	—
L-Arabinose	—	—	—	Inulin	—	—	—
D-Ribose	—	—	—	Glycerol	+	+	+
D-Xylose	—	—	—	Erythritol	++	—	++
D-Lyxose	—	—	—	Adonitol	+++	—	+++
Galactose	+	+	+++	Xylitol	—	—	—
D-Glucose	++++	++++	++++	D-Arabitol	—	—	—
D-Fructose	—	++++	—	Dulcitol	—	—	—
D-Mannose	++++	+	+++	Inositol	++++	—	++++
L-Sorbose	—	—	—	Mannitol	—	—	—
D-Tagatose	—	—	—	Sorbitol	—	—	—
Rhamnose	—	—	—	Starch	—	—	—
D-Fucose	—	—	—	Glycogen	—	—	—
L-Fucose	—	—	—	Methyl β -xyloside	—	—	—
Cellobiose	—	—	—	Methyl			
				α -D-mannoside	—	—	—
Maltose	—	+++	—	Methyl			
				α -D-glucoside	—	—	—
Lactose	++++	+++	++++	Amygdalin	—	—	—
Melibiose	—	—	—	Arbutin	—	—	—
Saccharose	—	++++	—	Aesculin	+	+	+
Trehalose	—	++++	—	Salicin	—	—	—
β -Gentiobiose	—	—	—	N-Acetyl-			
				glucosamine	—	—	—
D-Turanose	—	—	—	Glucanate	++	—	+
Melezitose	—	—	—	2-Oxogluconate	—	—	—

the cell. When the separation of the two daughter cells did not occur before the subsequent division, the new septa were generally seen in a plane perpendicular to the first septum.

In stationary phase (Fig. 2*a, b*) the bacteria retained their spherical shape but their diameter was less uniform (Fig. 2*a*: 0.7–1.2 μ m). In addition, the walls of many bacteria were very thick in some regions and large aggregates of dense material were found in the extracellular medium. These seemed to correspond to large pieces of wall released from the bacterial surface.

Antibiotic resistance profiles

Antibiotic sensitivities were determined for strains ATCC 13673 and E1. As shown in Table 2, besides the slight augmentation in chloramphenicol resistance which allowed the isolation of round forms, only one new resistance (to erythromycin) appeared. A weak resistance to josamycin also occurred. The main change in the antibiotic profile was, however, the suppression of resistance to a number of drugs tested (15 out of 26). Round cells were sensitive to low concentrations of most aminoglycosides, which may mean that their permeability to these positively charged molecules had been enhanced.

Fermentation profiles

The most noticeable variations in the physiological activities of the spherical isolates were the loss of the ability to ferment some polyalcohols (erythritol, adonitol and inositol), and the acquisition of the capacity to actively ferment disaccharides containing an α -glucosidic linkage (maltose, saccharose and trehalose), and D-fructose (Table 3). Resistance and fermentation profiles of the other round strains isolated were also determined. Only slight differences were observed from one strain to another.

Lactate dehydrogenases

P. freudenreichii ATCC 13673 had a high D(–)-lactate dehydrogenase activity (specific activity 5.6 units, K_m 12.5 mM). L(+)-Lactate dehydrogenase activity was not measurable using

Fig. 3. Agarose gel electrophoresis of DNA extracted by a 'boiling' method. Lane 1, round variant E2; 2, molecular size markers [plasmids pUC8 (2.7 kb) and pHV33 (7.3 kb)]; 3, rod revertant R1; 4, round variant E1; 5, parent strain ATCC 13673.

Table 4. *Reversion of round cells to the rod form*

Samples of an exponentially growing culture of strain E1 were transferred into BYC base with (a) or without (b) glucose and incubated under static aerobiosis. After the indicated times, samples were withdrawn, diluted according to their optical density to obtain an expected 300 to 500 c.f.u. per plate, and plated on either solid BYCG (a) or solid BYC (b). Colonies were counted after 48 h incubation at 30 °C. Mean values of five determinations are given.

	Time (h)					
		(a)			(b)	
	20	48	115	45	65	115
Expected no. of colonies	300	400	460	460	480	460
Round cells	302	392	450	452	387	338
Rods	0	0	0	0	77	119
Percentage of rods	0	0	0	0	17	26

the protocol described in Methods. However, using a test plate assay (I. Caprini, A. Naud & J. Legault-Démare, unpublished) based on the reduction of nitro blue tetrazolium in the presence of phenazine methosulphate, a slow reaction was observed. Thus, both D(-)- and L(+)-activities are present in this strain. A similar result has been obtained by Molinari & Lara (1960) with *P. pentosaceum*. In strain E1, only the L(+)-enzyme was detected by the test plate assay; the reaction rate was of the same order of magnitude as that observed with the parent strain.

α -D-Glucosidase

The ability of strain E1 to ferment α -glucosides, revealed by the API test system, correlated with α -D-glucosidase activity in cellular extracts. The specific activity of strain E1 was 0.4 units, while it was a hundred times less (0.004) in the parent strain. This latter value is within the range of experimental error and consequently is not significant.

Detection of plasmids

Several bands attributable to plasmid DNA were found in extracts of round cells. Most of these extracts contained three bands which seemed to correspond to molecular species common to all the clones tested. [Figure 3 shows the results obtained with two round strains, E1 and E2 (lane 4 and 1, respectively).] In contrast, the parent strain ATCC 13673 did not contain detectable plasmid DNA (Fig. 3, lane 5).

Reversion to the rod form

Strain E1, as well as the other fast-growing clones obtained during this study, maintained their round form indefinitely when periodically subcultured on BYCG medium from isolated colonies. However, when BYC base, or BYCL medium (containing L-lactate instead of glucose as the main carbon source) were inoculated with strain E1, rod cells progressively appeared. Table 4 shows the results of one of the reversion experiments.

'Revertant' clones were picked up at random, and their resistance and fermentation profiles were determined. As shown in Tables 2 and 3, the revertant clone R3 obtained from strain E1 was identical to the parent strain, except for a slight difference in galactose fermentation, and in the resistance to cefoxitin. Again, minor variations in fermentation profiles were observed between several revertant clones isolated from the same round strain.

Reversion from round to rod shape was accompanied by the disappearance of plasmid DNA bands on agarose gels (Fig. 3, track 3). Thus, the presumptive plasmid DNA was either lost or reintegrated into the bacterial chromosome when the cells regained their previous morphological and physiological characteristics. Experiments using labelled plasmid DNA as a probe are in progress to determine whether it is still present in revertant cells.

One of the revertants obtained from strain E1 was resubmitted to the procedure described in Methods. Round cells were obtained in yields similar to those observed for the first time with the parent strain ATCC 13673.

DISCUSSION

The experiments presented here show that *P. freudenreichii* may exist in two stable shapes – as coryneform bacterium or a large thick walled coccus. High efficiency transition from the first state to the other can be triggered by imposing a combination of two different treatments on the bacteria. (i) Preincubation under conditions which do not allow growth of *Propionibacterium*, or UV irradiation. The result of this stress may be the occurrence of a temporary 'induced' state of a proportion of the population, during which the potential modification would be expressed only if a selective treatment were applied. Such a treatment (ii) was obtained by plating 'induced' cells on solid medium containing chloramphenicol (or erythromycin, results not shown) at a concentration sufficient to inhibit the growth of rods. When stressed cells were either resuspended in BYCG medium or plated on solid medium without antibiotic, they all grew as normal rods and presumably returned to an 'uninduced' state.

This hypothesis does not give a complete explanation of the phenomenon. As stated in Results, the spontaneous appearance of round *P. freudenreichii* is not a rare event. In addition, the fact that a precise yield could not be predicted using our procedure shows that some factors governing the morphological change remain to be investigated.

The role played by chloramphenicol remains to be elucidated. Previous studies of other bacteria demonstrated that synthesis of cell wall material is independent of protein synthesis (Hancock & Park, 1958), so that amino acid starvation or translation inhibitors both produce a thickening of peptidoglycan layers (Higgins & Shockman, 1970). However, this is only an apparent analogy between the two situations: in the case of *P. freudenreichii* cell growth was not prevented as colonies were formed, and, once established, the round morphology and the thicker wall were maintained indefinitely in the absence of inhibitor.

The role played by the main carbon source is also difficult to understand. As shown in Results, the 'induced' cells maintain their rod shape when plated on solid BYCG medium without chloramphenicol. Thus, glucose is not able by itself to provoke the appearance of spheres. In

contrast, even after many generations without chloramphenicol, the omission of glucose is sufficient to induce the reversion of spheres to the rod form. Due to the complex nutritional requirements of *Propionibacterium*, a more detailed analysis of the implication of carbon sources in the observed phenomenon is not yet possible.

Several spherical mutants of *E. coli*, usually obtained through nitrosoguanidine mutagenesis, have been described. Mutations mapped in genes *cya* (Kumar, 1976), *envB* (Westling-Haggstrom & Normark, 1975) and *rodA* (Matsuzawa *et al.*, 1973) are pleiotropic; in addition to shape modification, they confer unpredictable enhanced or reduced resistance to a number of mutagenic, antibiotic or surface-active agents. Conditional morphological mutants have been obtained from other Gram-negative bacteria (Satta *et al.*, 1981; Fujiwara & Fukui, 1972), and from *Bacillus* spp. (Rogers *et al.*, 1968, 1970, 1971; Boylan & Mendelson, 1969). In some cases, phenotypic changes in the morphology of mutants have been demonstrated to depend on medium composition (Rogers & McConnell, 1970; Rogers *et al.*, 1976).

In this respect, *P. freudenreichii* behaves like a 'natural' mutant: in contrast with all the previously published cases, no mutagenesis of any kind was used, and the high yield of shape modification observed with the protocol described here completely excludes the intervention of a mutational mechanism. The changes observed in resistance and fermentation profiles may be tentatively attributed to the obviously important modifications of the structure of the envelope: the loss of the ability to ferment some polyols may be due to the inactivation of a common membrane-linked transport mechanism, viz. the phosphoenolpyruvate phosphotransferase system(s) (Dills *et al.*, 1980). The appearance of a new enzyme may also be explained by an increased permeability to an inducer of the synthesis of the protein. The inducibility of α -glucosidase in *Propionibacterium* has, however, not yet been demonstrated. The synthesis of α -glucosidase might be also prevented by insertion of a plasmid or a prophage into the chromosome of rod cells, at a region of homology located inside the enzyme locus. Thus, expression of the enzyme activity would be possible only after excision of the foreign DNA. Such a mechanism might explain the results reported in Fig. 3: several bands attributable to plasmid DNA were found in extracts of round cells only. Further work is necessary to ascertain the structure of the DNA contained in these bands, but their presence seems to reveal a mechanism of DNA rearrangement accompanying shape modification.

Another case of morphology change perhaps more akin to our observations is offered by the growth cycle of *Arthrobacter* (Clark, 1972). These bacteria normally evolve from rod to coccus while approaching the stationary phase. The round form of *A. crystallopoietes* can be maintained stably in glucose-salt medium, but reverts to the rod form after addition of some amino acids (Ensign & Wolf, 1964). The two cases are strikingly similar: no mutagenesis is implied and both *Arthrobacter* and *Propionibacterium* in the rod state are typical pleiomorphic coryneforms with a variable Gram stain. We are not aware of differences in fermentation properties, resistance profiles or in the occurrence of plasmids between the two forms of *Arthrobacter*.

We think that the results presented here may contribute to the study of cell shape control and perhaps add new information to the taxonomy of coryneform bacteria.

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