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**Review**

# Genetics and molecular biology of propionibacteria

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**Abstract** — Research in the genetics and molecular biology of propionibacteria is currently making much progress. In order to develop efficient DNA transfer systems for the genus *Propionibacterium,* dairy and environmental propionibacteria were screened for the presence of suitable plasmids as a first step. Following nucleotide sequence analysis, potential replication functions were identified on several *Propionibacterium* plasmids such as pLME106/pRGO1, p545 and pLME108. Furthermore, *ppnA,* the gene encoding the propionicin SM1, was detected on pLME106. Three of these plasmids which had been fused with antibiotic resistance selection markers (*ermE*, *cml*, *hygB*) originating from bacteria with high G+C DNA content were recently successfully used as *Escherichia coli* – *Propionibacterium* shuttle vectors. DNA restriction/modification systems observed in propionibacteria have to be taken into account since successful DNA transformation at high rates (up to $10^8$ *Propionibacterium* transformants/μg of DNA) succeeds only with plasmid DNA originating from propionibacteria with the same restriction/modification system(s) as the strain to be transformed, and not from *E. coli* hosts. The basis for an integrating vector has been set up after identification of a potential attP site and an adjacent integrase gene from a *Propionibacterium* phage/prophage system. Finally, approximately 30 gene sequences with attributed coding functions from propionibacteria are available on databases.

***Propionibacterium* / plasmid / bacteriophage / DNA transformation / genetics**

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**Résumé — Génétique et biologie moléculaire des bactéries propioniques.** Pour développer des systèmes de transfert d'ADN pour le genre *Propionibacterium*, la présence de plasmides a été recherchée dans des souches laitières et environnementales. En analysant la séquence de l'ADN, des fonctions probablement responsables de la réplication ont été identifiées sur des plasmides de *Propionibacterium* : pLME106/pRGOl, p545 et pLME108. De plus, *ppnA*, le gène codant pour la bactériocine SM1, a été identifié sur pLME106. Ces plasmides ont été fusionnés avec des gènes de résistance aux antibiotiques (*ermE*, *cml*, *hygB*) provenant de bactéries dont l'ADN est riche en nucléotides G+C. Ces vecteurs de clonage ont été utilisés avec succès comme vecteur navette *Escherichia coli – Propionibacterium*. Des systèmes de restriction/modification dans les bactéries propioniques ont été suspectés du fait que le nombre des *Propionibacterium* transformants était très haut ($10^8$ $\mu g^{-1}$ d'ADN) seulement si l'ADN provenait de souches de *Propionibacterium* et non d'*E. coli*. Un vecteur de clonage intégratif pourra être construit dans l'avenir par l'identification d'un site attP et d'un gène d'intégrase sur le génome d'un bactériophage de *Propionibacterium*. Enfin à peu près 30 gènes du génome des bactéries propioniques ont été séquencés et figurent dans des banques de données.

***Propionibacterium* / plasmide / bactériophage / ADN transformation / système génétique**

## 1. INTRODUCTION

Propionibacteria are well established as traditional starter cultures in the production of typical Emmental type cheeses. In addition, other functions of propionibacteria such as probiotic and nutraceutical properties are gaining interest (see contributions of Ouwehand et al. and Hugenholtz et al. in this issue). In contrast to numerous studies on the physiology and technological applications of propionibacteria, genetic investigations have so far been limited to a few recent studies which provided already a considerable basis for the genetics and molecular biology of propionibacteria. In order to develop the tools necessary for genetic engineering and biotechnological applications, molecular biologists have turned the focus of their attention to *Propionibacterium* plasmids and bacteriophages and their usage in vector construction and development of transformation protocols. In addition, the nucleotide sequences of whole plasmids, part of bacteriophages and single chromosomal segments of different *Propionibacterium* species were determined, in order to characterise genes and their functions. These topics, including new results, will be reviewed in this article.

## 2. PLASMIDS FROM PROPIONIBACTERIA

### 2.1. Detection and prevalence of *Propionibacterium* plasmids

Several research groups have screened dairy propionibacteria for the presence of plasmids. The first were Rehberger and Glatz [35–38], who found plasmids in 21 out of 50 investigated *Propionibacterium* strains (41%). Based on their size, 11 different plasmids were distinguished. 7 plasmids were further characterised by using restriction endonuclease analyses, DNA hybridisations and curing experiments. Strains typically harboured one or two plasmids. The results have been reviewed [47].

Out of 30 *Propionibacterium* strains (isolates from Swiss type cheese), Perez Chaia et al. [34] reported 8 strains harbouring one or two plasmids (27%). Panon [32] screened 53 strains and found 20 strains harbouring each one or two plasmids (38%). It is noteworthy that a plasmid was isolated for the first time from a *P. thoenii* strain. Gautier and Rouault [10] found 4 strains harbouring one plasmid out of 50 strains (8%). Ogata et al. didn't detect any plasmids in 8 strains [31].

Fessler et al. [7, 8] examined 373 *Propionibacterium* strains which were all isolates from raw milk. 30% of these strains contained plasmids. Of the plasmid harbouring strains, 83% contained only one plasmid, 13% had two plasmids and the rest probably harboured more than two plasmids. Interestingly, all the *P. thoenii* strains which were studied did not contain any plasmids. 89% of the isolated plasmids were larger than 16 kb. The small plasmids ranged from 1.2 to 16 kb. Plasmids of the size of 6–8 kb were often isolated from *P. jensenii* strains.

At the Laboratory of Food Microbiology, ETH Zurich, 357 *Propionibacterium* strains (isolates from raw milk and cheese) were screened for the presence of plasmids [3]. Thirty-one strains contained one or two plasmids (9%), some of which were further characterised [3, 4, 24–26, 42]. A 6.9-kb plasmid could be isolated from *P. thoenii* ("*sanguineum*") NCIMB 8902 (Stierli, unpublished).

In order to find small plasmids suitable for vector constructions, Kiatpapan et al. [17] investigated 50 *Propionibacterium* strains from three different culture collections for the presence of plasmids but they did not report their screening results.

Jore et al. [16], (van Luijk, unpublished) examined 75 *Propionibacterium* strains for the presence of plasmids. Only six strains harboured small plasmids, 3.6–10 kb in size. One of these strains was *P. acidipropionici* ATCC 4875, whose plasmid had been described before [36]. The other five strains all harboured a second larger plasmid (>20 kb). Another 20 strains harboured only one large plasmid (>20 kb).

To summarise, small plasmids (<20 kb) have not been found very often in *Propionibacterium* strains; when present, small plasmids were often accompanied by another larger plasmid. Only one megaplasmid (approx. 250 kb) isolated from a *P. thoenii* strain has been described so far [22]. Concerning *P. thoenii* strains only two more plasmids have been isolated from this species at all [32] (Stierli, unpublished).

In most studies, mainly *P. freudenreichii* strains were screened for the presence of plasmids, since most of the isolates from raw milk and cheese belong to this species.

### 2.2. Determination of the nucleotide sequences of *Propionibacterium* plasmids

Several *Propionibacterium* plasmids have been sequenced. Plasmid pLME108 from *P. freudenreichii* DF2 was sequenced by Dasen [3, 4]. Its size is 2051 bp and it has a G+C content of 64% in the DNA. The nucleotide sequence of pLME106 from *P. jensenii* DF1 was determined by Stierli [26, 42]. This sequence is 100% identical to the nucleotide sequence of plasmid pRGO1 from *P. acidipropionici* E214 [36], determined by Kiatpapan et al. [17, 18]. The size of plasmid pLME106/pRGO1 is 6868 bp, the G+C content is 65%. Jore et al. [16] determined the nucleotide sequence of p545 from *P. freudenreichii* LMG 16545. Its size is 3555 bp and its G+C content 63%.

### 2.3. Replication of *Propionibacterium* plasmids

Plasmid p545 and plasmid pLME106/pRGO1 are both assumed to replicate by the theta replication mechanism. The two putative replication proteins are translationally coupled. A larger ORF (302, 303 aa respectively), named ORF1, is followed by a smaller ORF2 (85 aa, 115 aa respectively). Such a coupled arrangement has been described before for plasmid pMB1 isolated from *Bifidobacterium longum* strain B2577 [39], and for plasmids from *Corynebacterium* and *Mycobacterium* for instance. It is remarkable, that ORF1 and ORF2 from pLME106/pRGO1

exhibit significantly higher identity to several *Brevibacterium linens, Rhodococcus* sp., *Corynebacterium glutamicum* and *Mycobacterium fortuitum* plasmid replicons [5] than to ORF1 and ORF2 from p545 from *P. freudenreichii* LMG 16545.

Plasmid pRGO2 [36], which has been isolated from several *P. jensenii* strains, is assumed to replicate by the theta replication/mode, too. The region that comprises the earlier described identical order of enzyme restriction sites conserved between plasmids pRGO1 and pRGO2 contains the two ORFs responsible for the theta replication of pRGO1/pLME106 as well as ORF5 and ORF6 (respectively ORF8 and ORF9 in pLME106), whose functions are unknown, but which have been assumed to play a role in replication of plasmid pRGO1 [17]. Plasmids pRGO5 and pRGO7, which were described to be homologous in some parts to pRGO1 and pRGO2 (based on hybridisation experiments), might contain these kind of *rep* genes, too. On the other hand, p545 and pRGO1 did not show any positive signals in hybridisation experiments [16] at high stringency conditions, but still revealed 35.3% identity in a 249 amino acid overlap of ORF1.

The Rep protein of plasmid pLME108 from *P. freudenreichii* DF2 [3, 4] showed 42.1% amino acid identity with the Rep protein of plasmid pAP1 from *Arcanobacterium pyogenes* [2]. Specific amino acid motifs needed for the replication by the rolling circle (RC) mechanism were found on pLME108 and led to the conclusion that this plasmid replicates by the RC mode.

In conclusion, both the theta replication mode and the rolling circle replication mode are proposed for *Propionibacterium* plasmids. Furthermore, the assumed *rep* region of pLME106/pRGO1 seems functional in all 4 dairy *Propionibacterium* species since the plasmid has been detected in representatives of all 4 species [36, 42] (Stierli, unpublished). Moreover, both putative theta replication regions of p545 and pLME106/pRGO1 have been utilised successfully for the construction of *Propionibacterium–E. coli* shuttle vectors (see Sect. 3.2.).

### 2.4. Functions encoded by *Propionibacterium* plasmids

#### *2.4.1. Curing of Propionibacterium strains of their plasmids*

In order to cure *Propionibacterium* strains of their plasmids, only one method has been successful. The treatment of strains with acriflavine has been described by two groups [10, 36]. However, Gautier and Rouault [10] could not elucidate the encoded functions of any plasmid even though they had screened for many functional properties such as carbohydrate metabolism, proteolytic activity, production of inhibitory substances, drug resistance and inorganic ion tolerance. Based on curing experiments Rehberger and Glatz [36, 38] assumed that plasmid pRGO5 is responsible for the formation of cell aggregates of *P. jensenii* P38. In addition, the authors attributed the ability of *P. freudenreichii* subsp. *"globosum"* P93 to ferment lactose to plasmid pRGO3 [35]. Following a comparable approach, Miescher et al. [26] did not succeed in curing *P. jensenii* strain DF1 from its plasmid pLME106 by acriflavine treatment.

#### *2.4.2. Propionicin SM1: the first function proposed for a Propionibacterium plasmid*

Miescher [25], Miescher et al. [26] and Miescher Schwenninger et al. [27] described the first proposed function of a *Propionibacterium* plasmid. Propionicin SM1 (PpnA) from *P. jensenii* DF1, whose gene had been located on *P. jensenii* DF1 plasmid pLME106 (*ppnA, orf4*), is a heat-stable bacteriocin that is strongly bactericidal against *P. jensenii* DSM 20274. Due to its N-terminus the propionicin SM1 must

be synthesised as a 207-amino acid prepeptide before excretion from the cell as an active protein consisting of 180 amino acids. According to DNA sequence analysis, the N-terminal leader peptide had a length of 27 amino acids and seemed to be proteolytically cleaved off before or at secretion. Five other *Propioni- bacterium* strains which harboured plasmid pLME106 – *P. thoenii* NCIMB 8902 and four other *P. jensenii* strains (DF4, DF8, DF9 and DF10) – also showed bactericidal activity against *P. jensenii* DSM 20274.

## 2.5. Other putative open reading frames of plasmids pLME108, pLME106/pRGO1 and p545

### *2.5.1. pLME108 from P. freudenreichii DF2*

Apart from the Rep protein of pLME108, a second open reading frame (ORF2) was determined on pLME108. It showed no significant homologies to known sequences of the GenEMBL and Swissprot databases. Therefore, its function remains unknown. Construction of a *Propionibacterium – Escherichia coli* shuttle vector containing the replicon of pLME108 is in progress.

### *2.5.2. p545 from P. freudenreichii LMG16545*

No more open reading frames, apart from the two ORFs being part of the replicon, were determined on plasmid p545. A shuttle vector based on its replicon was successfully constructed [16] (see Sect. 3.2.).

### *2.5.3. pLME106 from P. jensenii DF1 and pRGO1 from P. acidipropionici E214*

Apart from the above described Rep region (ORF1 and ORF2) and propionicin SM1 (ORF4 in pLME106), another seven putative ORFs were determined on pLME106 [26]. Due to their low G+C content ORF4 and ORF5 were not considered by Kiatpapan et al. [17]. ORF5 encodes a putative protein of unknown function and resides downstream of *ppnA*. It was suggested that pLME106/pRGO1 is a composite plasmid, which must have gained *ppnA* and *orf5* from another organism [29]. ORF3, encoding a putative protein of unknown function, was not described by Kiatpapan et al. [17, 18] either.

ORF6 of pLME106 (ORF3 in pRGO1) encodes a protein with a 51.1% identity to a DNA invertase-like protein from a *Rhodococcus equi* virulence plasmid (EMBL acc. no. af116907). The three open reading frames ORF5, ORF8 (ORF5 in pRGO1) and ORF10 revealed similarities with ORF25, ORF27 and ORF26 from the *Corynebacterium striatum* plasmid pTP10 [43] which seem to form an operon of unknown function on the *Corynebacterium* plasmid. On plasmid pLME106, ORF5 has probably been separated from ORF10 and ORF8 by the insertion of the replication genes and *ppnA*. The residual putative open reading frames ORF7 (ORF4 in pRGO1) and ORF9 (ORF6 in pRGO1) are preceded by “good” ribosome binding sites and are in agreement with the codon preferences for *Propionibacterium* genes.

Plasmid pLME106/pRGO1 has been isolated from all four dairy *Propionibacterium* species [36, 42] (Stierli, unpublished). *P. thoenii (“sanguineum”)* strain NCIMB8902 also harbours pLME106 (Stierli, unpublished) and in addition a second, larger plasmid. A spontaneous creamy coloured mutant strain of *P. thoenii* NCIMB8902 still harbours the large plasmid but lacks pLME106, and in the absence of the *ppnA* gene, no longer shows bactericidal activity against *P. jensenii* DSM20274. Furthermore, the mutant strain doesn’t show β-hemolytic activity in contrast to the wildtype strain and many other red *P. thoenii* and *P. jensenii* strains (Stierli, unpublished).

## 3. GENE TRANSFER SYSTEMS FOR PROPIONIBACTERIA

### 3.1. Development of genetic tools for propionibacteria

The development of genetic tools will facilitate an increase of fundamental and application-oriented knowledge of *Propionibacterium*. Genetic investigations of these important dairy bacteria is dependent on the availability of an efficient gene transfer system for *Propionibacterium*.

Transfer of exogenous DNA between bacteria requires either natural competence or conjugation processes. For a more controlled introduction of DNA into bacteria, the cells have to be made competent by treatment with chemical or physical agents. Although DNA transfer methods, such as conjugation and polyethyleneglycol-mediated protoplast transformation, have been developed for several Gram-positive bacteria, no examples of their successful application in *Propionibacterium* are known.

Gautier et al. [9] describe electro-transfection of *Propionibacterium freudenreichii* by using *Propionibacterium* bacteriophage DNA, ruling out the possibility that the thick cell wall of *Propionibacterium* constitutes an insuperable barrier to incoming DNA. However, the DNA used in these experiments was isolated from a *Propionibacterium* phage. Given the narrow host range of phages and their lytic nature, this is not an approach that will lead to a general method for obtaining stable genetic transformants. In this respect, a host-vector system based on an autonomously replicating plasmid vector will be more generally applicable.

Attempts to transform *Propionibacterium* with plasmids originating from other Gram-positive bacteria, including plasmids known to have a broad host-range, failed [16, 17, 24]. One of the barriers that was expected to play an important role is the existence of restriction/modification systems in *Propionibacterium*. Restriction/modification systems are widespread in bacteria and it is generally believed that these systems protect bacteria from foreign DNA entering the cell. Restriction/modification systems can be plasmid located or chromosomally derived.

To exclude that the replicon on which the vector is based is not recognised by *Propionibacterium* and that the vector can thus not replicate in the host strain, the use of a *Propionibacterium* based replicon seems to be a prerequisite for construction of a cloning vector. As pointed out in chapter 2, the occurrence of several endogenous *Propionibacterium* plasmids and the complete nucleotide sequences of a number of such plasmids have been published. The availability of these plasmid sequences allows the selection of putative non-essential regions where selective marker genes can be integrated, and thus permits the construction of vectors with which a gene transfer system for *Propionibacterium* can be developed.

### 3.2. Electroporation based transformation of propionibacteria

Recently, two reports on an efficient electrotransformation system for *Propionibacterium* have been published [16, 17]. Kiatpapan et al. [17] describe the development of an *E. coli – Propionibacterium* shuttle vector pPK705, based on part of the pRG01 plasmid, containing the replication region of this plasmid, and the *E. coli* cloning vector pUC18. A hygromycin B (*hygB*) gene from *Streptomyces hygroscopicus* was used as a selective marker. When plasmid pPK705 was prepared from a *Propionibacterium* transformant, and transferred to *Propionibacterium*, transformation efficiencies of up to $10^6$ to $10^7$ transformants per μg DNA were obtained, a thousand-fold higher than when plasmid DNA had been prepared from *E. coli*. This suggests the presence of a restriction/modification

system in *Propionibacterium*. This shuttle vector was used for successful transformation of strains belonging to *P. freudenreichii* subsp. *freudenreichii*, *P. freudenreichii* subsp. *shermanii* and *P. pentosaceum* (this last species name is no longer valid [46], and several *P. pentosaceum* strains have been reclassified as *P. acidipropionici*). Since plasmid pRG01 has been detected in all four dairy propionibacterial species (as pointed out in Sect. 2.3.) a broader host-range might be expected for pPK705. However, different restriction/modification systems in these species may influence the transformability of the various strains. The pPK705 vector was subsequently used for construction of expression vectors. Cloning of the ALA (5-aminolevulinic acid synthase) gene (*hemA*) from *Rhodobacter sphaeroides* into these expression vectors resulted in successful overproduction of 5-aminolevulinic acid [19].

Jore et al. describe another efficient transformation system for *Propionibacterium* [16]. Reproducible transformation of *P. freudenreichii* was achieved with shuttle vectors based on plasmid p545 from *P. freudenreichii*. The erythromycin resistance gene from *Saccharopolyspora erythreae* (*ermE*) [1, 44] and the chloramphenicol resistance gene from *Corynebacterium striatum* (*cml*) [43] were used as the selection markers. Also in this study restriction/modification in *Propionibacterium* was observed. With plasmid DNA prepared from a *Propionibacterium* transformant, transformation efficiencies of up to $10^8$ transformants per µg DNA could be achieved, a frequency $10^6$ to $10^7$-fold higher than when DNA was prepared from *E. coli*. This large increase in efficiency suggests the presence of more than one restriction/modification system. Only *P. freudenreichii* strains proved transformable (with similar efficiencies in all strains tested), whereas – using the same protocol – *P. jensenii*, *P. acidipropionici* and *P. thoenii* did not yield any transformants.

Successful transformation of *Propionibacterium* was also achieved with 2 different vectors based on plasmids pLME106 and pLME108, as presented by Stierli at the 3rd International Symposium on Propionibacteria in Zurich, 2001 (Stierli, unpublished). A similar transformation protocol was followed as described by Jore et al. [16].

### 3.3. Prerequisites for high efficiency transformation of propionibacteria

Analysis of type and specificity of the restriction/modification system(s) in one of the *P. freudenreichii* host strains used, only revealed that no type II restriction enzymes were present [16]. The vast majority of known restriction/modification systems are of the type II which is explained by the relative ease with which their activities can be detected biochemically. The only restriction enzyme so far isolated from Propionibacterium (designated *P. intermedium*), is *Pnt*I, a type II restriction enzyme that has CGATCG as its recognition sequence [48].

Both reports on transformation of *Propionibacterium* [16, 17] confirm the prior assumption that the lack, until recently, of a reliable gene transfer system, is due to the presence of restriction/modification systems in *Propionibacterium* and that the choice of an appropriate selection marker and the choice of the replicon in the vector are of the utmost importance. The latter was hampered by the scarcity of relatively small plasmids in *Propionibacterium*, suitable for vector construction [16, 32, 34, 36]. Indications that the G+C-content of DNA of the selection marker chosen is of importance, are supported by the observation that the use of a number of relatively A+T-rich marker genes, including erythromycin and chloramphenicol resistance genes, utilised in transformation systems of other bacteria, proved unsuccessful in *Propionibacterium* [16, 17, 24]. Both successful systems published so far,

are based on the use of G+C-rich selection marker genes (*hygB*, *ermE* and *cml,* respectively). In how far the high G+C-content of DNA is essential for efficient expression of genes in *Propionibacterium*, remains speculative. Miescher et al. found that a part of plasmid pLME106 containing the propionicin SM1 encoding gene, has a significantly lower G+C– content (51%) than the rest of the plasmid [26].

## 4. MOLECULAR BIOLOGY OF BACTERIOPHAGES FROM DAIRY PROPIONIBACTERIA

### 4.1. Demonstration of lysogenic strains of *Propionibacterium freudenreichii* subsp. *shermanii*

Two types of bacteriophages have been isolated originating from dairy propionibacteria of Swiss type cheeses [11]. One type belongs to group B1 phages (Bradley's classification), the other is a filamentous phage. Based on their restriction/hybridisation pattern all the B1 type phages are closely related and are occurring as prophages [11]. It was shown by the authors that during the manufacture of the Emmental cheese, the bacteriophage replication occurred essentially during the ripening process in the warm room, either in endogenous propionibacteria or in starter strains. However, at that time, their mode of replication (virulent or temperate) was not elucidated. Since free phages have been rarely found in raw milk, the lysogeny phenomenon was suspected to be one of the ways of entry during the manufacture. Indeed, a recent study from the same laboratory [14] demonstrated for the first time lysogeny in dairy propionibacteria. Hervé and co-workers hybridised the chromosomal DNA of 76 propionibacteria strains with the entire genome of nine bacteriophages isolated from industrial Emmental cheeses. The DNA of 25 strains hybridised strongly with one or several genomes of 9 different bacteriophages. The *P. freudenreichii* subsp. *shermanii* TL146 strain was selected to perform an extended study. The prophage 146 present in the genome of strain TL146 was able to infect the strain *P. freudenreichii* subsp. *shermanii* TL110. Thus, Hervé and co-workers could show that the prophage Ø146 was spontaneously inducible ($10^6$ pfu·mL$^{-1}$) or by treatment with mitomycin C or UV irradiation at 254 nm ($10^9$ pfu·mL$^{-1}$). Prophages present in two other strains could be induced by such treatments, too. TL146C, a prophage cured derivative of *P. freudenreichii* subsp. *shermanii* strain TL146, was isolated and then relysogenised by the same temperate bacteriophage. Based on comparative hybridisation analysis of the genomes of TL146, TL146C and the relysogenised derivative convincing evidence of lysogeny among dairy propionibacteria could be demonstrated [14, 15].

### 4.2. Integrative functions of the phage Ø110E1t infecting *P. freudenreichii* subsp. *shermanii* TL110

For the purpose of constructing an integrating vector, integrative functions such as the attP site and integrase of temperate *Propionibacterium* bacteriophages were investigated in an approach by Hervé [15]. According to the Campbell model the attP site was assumed to be present for a temperate *Propionibacterium* phage and absent for the prophage (splitting attP into two new sites attL and attR). By comparative genome analysis of free and integrated phages, Hervé [15] could localise the potential attP site of the bacteriophage Ø110 E1t on a 1978-bp fragment containing putative integrative functions (EBI acc. no. AJ320254). Nucleotide sequence analysis strongly suggested the presence of the attP site since a sequence of 28 bp already described for the mycobacteriophage

L5 (infecting both *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*) was also found on this fragment of bacteriophage Ø110 E1t [15, 33]. For that mycobacteriophage, this sequence corresponds to the 3'-end of the $tRNA^{Gly}$ gene which is the integration site of this phage [33]. The high conservation of the tRNA genes in the bacterial world, suggests that the prophage Ø110 E1t also integrates into one of these genes.

In addition, a recombinase gene was expected near the attP site as it is described so far in all specific recombination systems. From structural homology and from the knowledge of bacteriophage lambda integrase, a putative integrase gene was localised next to the potential attP site [15]. The encoded sequence was attributed to an enzyme belonging to the tyrosine recombinase family.

## 5. CHARACTERISATION OF GENES FROM PROPIONIBACTERIA

Despite the industrial importance of classical (dairy) propionibacteria relatively few of their genes have been characterised. The high G+C-content of the DNA from these bacteria and the lack of a suitable gene transfer system may have played a role in this respect.

Of the *Propionibacterium* sequences published in public databases, many represent the 16S, 23S and 5S ribosomal RNA genes and intergenic spacer regions from the different *Propionibacterium* species. This interest in ribosomal RNA sequences can be explained by the fact that comparisons of 16S rRNA genes are reliable taxonomical tools for species differentiation.

Table I lists the *Propionibacterium* protein-encoding genes that have hitherto been sequenced, and to which a function has been assigned, based either on homology to other, functional genes, or on a functional analysis of the *Propionibacterium* gene. Mostly genes coding for uncommon enzymes involved in the peculiar physiology [46] of *Propionibacterium* have been sequenced. These include enzymes such as pyrophosphate-dependent phosphofructokinase [20] and enzymes involved in propionic acid fermentation (methylmalonyl-CoA transcarboxylase subunit 1.3S, 5S and 12S sequences ([30] and public databases; see Tab. I), and methyl-malonyl-CoA mutase subunits [23]), siroheme formation (*hem YHBXRL*

**Table I.** List of protein encoding genes from *Propionibacterium* that have been completely sequenced. *P. acidi.* = *P. acidipropionici*; *P. freud. sherm.* = *P. freudenreichii* subsp. *shermanii*.

| Gene product | Gene | Organism | Plasmid/ Reference |
|---|---|---|---|
| *Central metabolism* | | | |
| pyrophosphate-dependent phosphofructokinase | *$PP_i$-PFK* | *P. freud.* | [20] |
| transcarboxylase : 1.3 S subunit | | *P. freud. sherm.* | [30] |
| 5 S subunit | | *P. freud. sherm.* | [NCBI database acc. no. M11738] |
| 12 S subunit | | *P. freud. sherm.* | [NCBI database acc. no. L06488] |
| methylmalonyl-CoA mutase: α subunit | *mutA* | *P. freud. sherm.* | [23] |
| β subunit | *mutB* | *P. freud. sherm.* | [23] |

**Table I** *(suite)*

| **Gene product** | **Gene** | **Organism** | **Plasmid/ Reference** |
|---|---|---|---|
| *Porphyrin biosynthesis* | | | |
| uroporphyrinogen (III)- methyltransferase | *cobA* | *P. freud.* | [40] |
| ATP dependent transport protein | *cbiO* | *P. freud.* | [40] |
| δ-aminolevulinic acid dehydratase | *hemB* | *P. freud.* | [12, 13] |
| ferrochelatase | *hemH* | *P. freud.* | [12] |
| glutamate 1-semialdehyde 2,1-aminomutase | *hemL* | *P. freud.* | [12, 29] |
| putative regulatory protein | *hemR* | *P. freud.* | [12] |
| putative transport protein | *hemX* | *P. freud.* | [12] |
| protoporphyrinogen oxidase | *hemY* | *P. freud.* | [12] |
| *Cellular processes* | | | |
| propionicin T1 | *pctA* | *P. thoenii* | [6] |
| putative immunity protein | *orf2* | *P. thoenii* | [6] |
| propionicin SM1 | *ppnA* | *P. jensenii* | [25, 26, 42] |
| *Other functions* | | | |
| 60 $kg \cdot mol^{-1}$ heat shock protein | *GroEL* | *P. acnes* | [NCBI database acc. no. AF222061] |
| 70 $kg \cdot mol^{-1}$ heat shock protein | *dnaK* | *P. acnes* | [NCBI database acc. no. AF222062] |
| hyaluronate lyase | | *P. acnes* | [41] |
| proline iminopeptidase | *pip* | *P. freud. sherm.* | [21] |
| lipase | *gehA* | *P. acnes* | [28] |
| *Plasmid-encoded replication proteins* | | | |
| putative rolling circle replicase | *rep* | *P. freud.* | pLME108 [3, 4] |
| putative theta replicase | *repA* | *P. acidi.* | pRG01 [17] |
| | *repA* | *P. jensenii* | pLME106 [26, 42] |
| | *repA* | *P. freud.* | p545 [16] |
| replication protein | *repB* | as for *repA* | as for *repA* |

[12, 13, 29] and vitamin $B_{12}$ synthesis (*cobA* and *cbiO* [40]). Also genes unique to *Propionibacterium* have been characterised, such as the plasmid-encoded propionicin SM1 encoding gene *ppnA* [26], and the propionicin T1 encoding gene *pctA*

[6]. The latter is part of an operon together with its putative immunity protein encoding gene (*orf2*) [6].

All genes described here so far have been isolated from dairy *Propionibacterium* strains. The number of genes sequenced from the cutaneous strains of *Propionibacterium* is even lower, and includes a lipase [28] and a hyaluronate lyase encoding gene (public databases; see Tab. I). A last category of sequenced genes to which a function has been assigned (mainly based on their homology to other such genes) are those located on plasmids, encoding replication functions (Tab. I).

Aside from these gene sequences which are available in public databases, two Dutch companies (DSM Food Specialties and Friesland Coberco Dairy Foods), announced at the 3rd International Symposium on Propionibacteria in Zurich (2001), that they had successfully sequenced the complete 2.6-megabase genome of *Propionibacterium freudenreichii* subsp. *freudenreichii* (type strain ATCC 6207).

## 6. CONCLUSIONS

The lack of fundamental knowledge of the physiology and the regulatory networks present in *Propionibacterium* has seriously hampered an improved and extended industrial exploitation. The availability of gene transfer systems such as reported by Jore et al. [16], Kiatpapan et al. [17, 19] and of Stierli (unpublished) will undoubtedly facilitate studies on the genetics of these bacteria, and will allow the engineering of genetically modified *Propionibacterium* strains. The elucidation of some molecular aspects of *Propionibacterium* phages will contribute to both further development of integrative vectors and to the understanding of phage mediated lysis of propionibacteria during the cheese ripening process. On the other hand, new and challenging prospects are presented by the recent developments in genomics (transcriptomics, proteomics and metabolomics) [45]. Furthermore, a public access to the genome data (although so far not announced) of *Propionibacterium freudenreichii* would greatly improve metabolic and regulatory knowledge on *Propionibacterium*. Application of new genomics technologies in combination with the conventional, more reductionistic scientific approaches (focussing on individual molecules) is expected to become of significant importance in *Propionibacterium* research. These technologies are expected to become a valuable tool for the improvement of industrial applications.

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