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Comparison of the Cell Surface Barrier and Enzymatic Modification System in *Brevibacterium flavum* and *B. lactofermentum*

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Abstract To investigate impediments to plasmid transformation in *Brevibacterium flavum* BF4 and *B. lactofermentum* BL1, cell surface barriers were determined by measuring growth inhibition whilst enzymatic barriers were determined by comparing DNA methylation properties. *B. lactofermentum* was more sensitive to growth inhibition by glycine than *B. flavum*. Release of cellular proteins during sonication was more rapid for *B. lactofermentum* than for *B. flavum*. Plasmid DNA (pCSL17) isolated from *B. flavum* transformed recipient McrBC$^+$ strains of *Escherichia coli* with lower efficiency than McrBC$^-$. *McrBC* digestion of this DNA confirmed that *B. flavum* contain methylated cytidines in the target sequence of *McrBC* sequences but *B. lactofermentum* contained a different methylation pattern. DNA derived from the *B. lactofermentum* transformed recipient EcoKR$^+$ strains of *E. coli* with lower efficiency than EcoKR$^-$, indicating the presence of methylated adenosines in the target sequence of EcoK sequences. The present data describe the differences in the physical and enzymatic barriers between two species of corynebacteria and also provide some insight into the successful foreign gene expression in corynebacteria.

Keywords: *Brevibacterium flavum*, *Brevibacterium lactofermentum*, identification, transformation

INTRODUCTION

Non-pathogenic *Corynebacterium* bacteria, including species such as *Brevibacterium flavum*, *B. lactofermentum* and *Corynebacterium glutamicum*, are Gram-positive food grade microorganisms widely used for industrial production of amino acids such as glutamic acid, phenylalanine, tryptophan, aspartic acid, threonine and lysine [1,2]. There are several advantages to using *Corynebacterium* species for the production of food additives and other materials of use for human and animal consumption. *Corynebacterium* species have been used for over 50 years in industry such that the primary metabolism of selected species has been well characterized. Moreover, *Corynebacteria* do not produce endotoxins. Corynebacterial lytic bacteriophages are relatively rare, and metabolic regulatory mechanisms are relatively simple when compared to *E. coli* [3,4].

Thus, in order to further increase production amino acid production by corynebacteria, recombinant DNA techniques have been developed for strain improvement, the introduction of foreign genes, and for the production of functional, recombinant proteins [5]. Although several foreign genes including α-amylase [6] and protease [7] have been transformed into *Corynebacterium* species successfully, many report poor efficiency of transformation [8]. Barriers to efficient transformation include restriction and modification enzyme(s), as well as the structure of the cell wall itself, which acts as a physical barrier to DNA transformation into the cell [9]. In order to obtain a high transformation frequency, the DNA to be transformed must be correctly methylated to avoid restriction by the host. The present work aimed to investigate the effect of the presence of glycine and/or isonicontic acid hydrazide (INH) on cell growths and to examine the enzymatic barriers to transformation of *Corynebacterium* species with specific reference to methyltransferase (MTase) activity.

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MATERIALS AND METHODS

Bacterial Strains, Plasmid, and Growth Conditions

Corynebacteria strains used were: *B. flavum* [10], *B. lactofermentum* BL1 [8], *C. glutamicum* AS019 [11]. *E. coli* strains, LE392 [12] and N4830 [13], were used as well. The plasmid used was pCSL17, a 7.2-kb *E. coli*-corynebacteria shuttle vector [14]. Corynebacteria strains were grown routinely in Luria-Bertani broth (LB) supplemented with 0.5% (w/v) glucose (LBG) and incubated

at 30°C. *E. coli* cells were grown in LB at 37°C. Cells were inoculated into 100 mL of LB in 250-mL culture flasks, with an initial optical density at 600 nm of 0.1~0.2. Cell growth rates were compared in two ways: μ value (specific growth rate, μ, h^{-1}) and period of lag growth phase. The specific growth rates of corynebacteria were expressed as a percentage relative to the growth in the LBG. In the presence of high concentrations of the two chemicals in the growth medium, growth of some strains were retarded for several hours before starting growth. Therefore, inhibition of growth due to the presence of the cell wall modifier in the medium were also compared by measuring the time of cell lag phase, which was defined as the time taken from after inoculation to before initial exponential growth phase.

Protein Release During Sonication

Cultures of corynebacteria from overnight cultures were incubated at 30°C (37°C for *E. coli* cells) and 200 rpm. Various volumes of LBG (LB for *E. coli* cells) were harvested (750 mL for A_{600} 0.43~0.48; 375 mL for A_{600} 0.77~0.87; 150 mL for A_{600} 1.10~1.29) at different growth stages. After sonication (power, 340 Watt) with a Branson sonifer (model 450), 0.5 mL of samples were taken at different sonication times and 50 μL of samples was used for protein analysis. Protein concentration was estimated using the method described by Bradford [15] in which a BioRad protein assay kit was used.

DNA Isolation and Restriction Enzyme Analysis

Plasmid DNA was isolated using an alkaline lysis method [16], and further purified using CsCl-EtBr density gradient centrifugation [12]. Restriction endonucleases were purchased from Boehringer Manheim GmbH Biochemica (Germany), and NEB (Beverly, MA, USA). Restriction enzyme digests of DNA were resolved using 0.8% or 1.5% (w/v) agarose gels as described previously [12].

DNA Transformation Procedures

E. coli strains were transformed as described by Sambrook *et al.* [12], following $CaCl_2$ treatment. Transformants were confirmed by plating LB containing 50 μg/mL kanamycin. The presence of plasmid DNA in transformants was confirmed using agarose gel electrophoresis of alkaline lysates. Transformation efficiency was calculated as colony-forming units (cfu) per μg of plasmid DNA. Cell counts were performed in triplicate and average numbers were taken.

RESULTS AND DISCUSSION

Characterization of Cell Surface Barriers in *B. flavum* BF4 and *B. lactofermentum* BL1

Cell growth of BF4 and BL1 in different concentrations of glycine and INH was compared to identify any differences in sensitivity to these additives. In the absence of glycine in the growth medium, the specific growth rates of the strains were: 0.50~0.55 (BF4), 0.65~0.75 (BL1) and 1.20~1.26 (*E. coli* LE392). As seen in Fig. 1, BF4 was less inhibited in the presence of glycine than BL1. At above 2~3% glycine, cell growth of BL1 was completely inhibited for more than 10 h before growth commenced. Cell grown in the presences of INH showed increasing inhibition of growth as the concentration of INH increased although the lag period was not greatly affected (Fig. 2). Similar kinetics of growth inhibition were seen for BF4 and BL1. The relative specific growth rates for BF4 and BL1 were inhibited 50% and 40% at 4

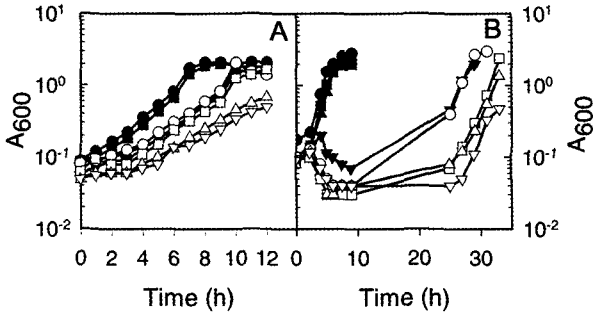

Fig. 1. Effect of glycine in the medium on growth of *B. flavum* BF4 (A) and *B. lactofermentum* BL1 (B). For *B. lactofermentum* BL1, at concentrations above 2% glycine, absorbances were not measured during the period 9 to 24 h of incubation. Symbols indicate glycine concentration (%, w/v) in LBG medium: For *B. flavum* BF4, (●) 0, (■) 1, (▲) 2, (▼) 3, (○) 4, (□) 5, (△) 6, (▽) 7% glycine. For *B. lactofermentum* BL1, (●) 0, (■) 1, (▲) 1.5, (▼) 2, (○) 2.5, (□) 3, (△) 3.5, (▽) 4% glycine.

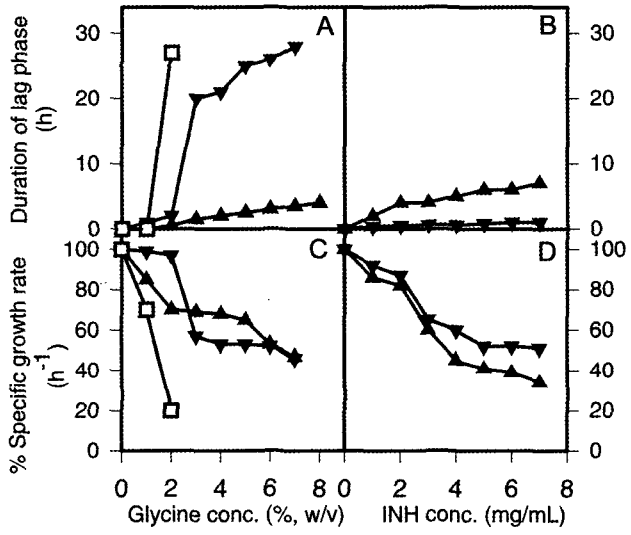

Fig. 2. Effect of glycine or INH in the medium on the duration of lag phase (A,B) and specific growth rates (C,D) of the bacteria tested. Specific growth rates are presented as percentages relative to growth rates seen in LBG. Symbols: ▲, *B. flavum* BF4; ▼, *B. lactofermentum* BL1; □, *E. coli* LE392.

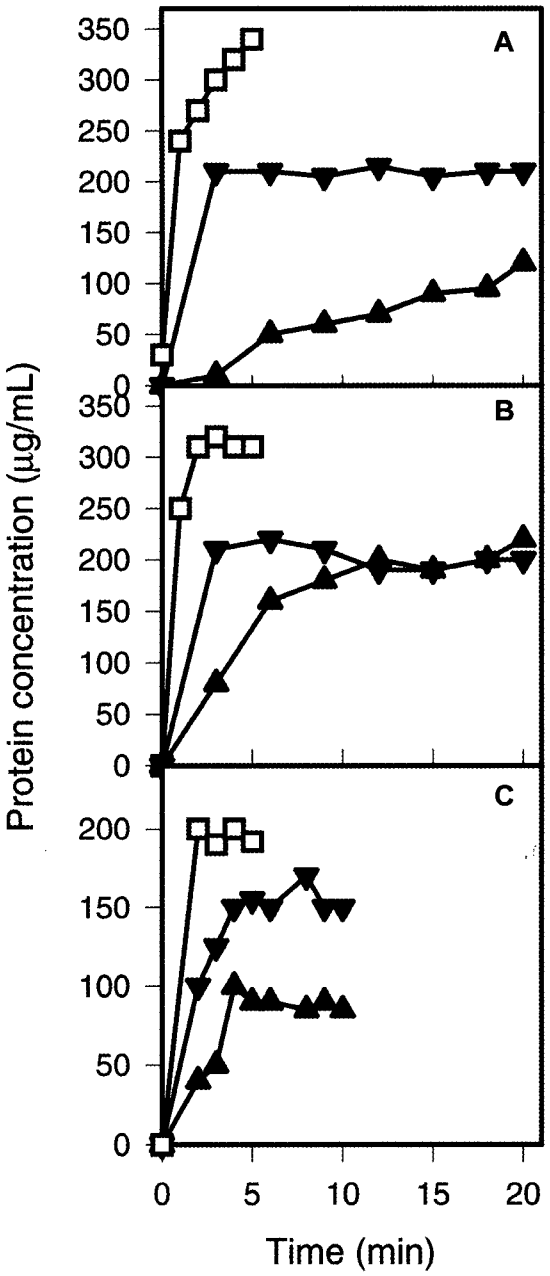

Fig. 3. Relationship between the amount of protein levels from bacterial strains and increasing sonication time. A, A_{600} 0.43~ 0.68; B, A_{600} 0.77~0.87; C, A_{600} 1.10~1.29. Symbols; ▲, *B. flavum* BF4; ▼, *B. lactofermentum* BL1; □, *E. coli* LE392.

mg of INH/mL. The cell wall structure of corynebacteria is quite complex, containing peptidoglycan, arabinogalactan, free mycolic acids, covalently-bound mycolic acids, proteins, peptides, and an ion channel [17,18]. This complexity may partially explain why these bacteria do not easily produce protoplasts because the linked lipid and sugar-rich layers would interfere with the action of lysozyme on the peptidoglycan. In general, glycine has been shown to be effective in both Gram-positive and Gram-negative bacterial cell wall synthesis, particularly in peptidoglycan biosynthesis [11,19]. INH affects mycolic acid synthesis in mycolic acid-containing bacteria. Thus, the presence of the two chemicals in the growth medium may describe the physical status of the cell surface structure of corynebacteria. The concentration of glycine required to significantly inhibit cell growth rates of BF4 and BL1, was generally between 1~6% (w/v), which is similar to that reported for Gram-positive bacteria, including *Bacillus subtilis*, *Streptomyces* species, and *C. glutamicum* [20,21]. The amount of INH required to cause inhibition of *C. glutamicum* species strains was very high compared to other mycolic acid-containing bacteria. At 0.1 mg/mL, INH inhibits *Mycobacterium*, *Nocardia*, *Rhodococcus* [22]. However, there were distinct differences in sensitivity to INH and glycine between BF4 and BL1, where the two strains exhibited different degrees and kinetics of inhibition. It has been argued that *B. flavum* and *B. lactofermentum* should be reclassified as *C. glutamicum* [23], but variations in sensitivity to INH in the present study may indicate physiological differences, which would support distinction between the three strains.

In order to investigate the rigidity of the cell wall structure, cells were harvested from *E. coli* LE392, BF4, and BL1 at different growth phases and the protein release during sonication were determined. Since the number of cells in the culture increased with longer fermentation time, the number of cells used for sonication was adjusted by reducing the volume of cultures harvested as growth progressed. After centrifugation of culture fluids, cells were re-suspended with the same amount of buffer and used for sonication. With increasing sonication time, the amount of protein released from *E. coli* cells increased but the time taken to completely disrupt cells was much less than for BF4 and BL1 (Fig. 3). This trend was seen throughout all cell growth phases tested, indicating that corynebacteria have much stronger cell surface structures than *E. coli*. Protein release from BL1 was quicker than that seen for BF4, which is consistent with its high growth sensitivity to glycine.

Characterization of Enzymatic Restriction Barriers in *B. flavum* BF4 and *B. lactofermentum* BL1

RM systems in *E. coli* have been extensively studied and it has been found that there are at least six restriction systems, including EcoK and two modification systems, *Dam* MTase and Dcm MTase, in addition to McrA, McrBC and Mrr [12,24]. Among the restriction systems in *E. coli* only the EcoK endonuclease (ENase) degrades unmethylated DNA, while the others all restrict specifically methylated DNA. In order to investigate the restriction and modification system(s) in BF4 and BL1, a pCSL17 plasmid DNA was chosen because this DNA can be transformed into *B. flavum*, *B. lactofermentum*, and several strains of *E. coli*. Therefore several different pCSL17 DNAs, which have different methylation patterns on DNA, could be prepared depending on the host. Transformation efficiency of *B. flavum*-derived plasmid DNA was compared with that of *E. coli* LE392-derived plasmid

Table 1. Transformation efficiencies of *E. coli* recipient strains with different restriction backgrounds using *E. coli*-, *B. lactofermentum*- and *B. flavum*- derived pCSL17 DNA.

E. coli recipient	Restriction system[a]									Reference for *E. coli* strains
	Transformation efficiencies for DNA from[b]				*EcoK*		*McrA*	*McrBC*	*Mrr*	
	LE392[c]	BF4[c]	N4830[c]	BL1[c]	R	K				
MC1061	1.7×10^5	4.5×10^4	1.2×10^5	2.2×10^4	–	+	–	–	+	[12]
HB101	2.3×10^4	6.9×10^4	1.9×10^4	1.9×10^4	–	–	+	–	–	[12]
LE392	1.5×10^4	5.0×10^2	1.8×10^5	1.2×10^5	–	+	–	+	+	[12]
ED8654	3.1×10^5	1.0×10^2	2.1×10^5	6.5×10^4	–	+	–	+		[12]
CSR603	3.0×10^2	< 100	2.1×10^5	<100	+	+	+	+	+	[25]
JM101	3.2×10^4	< 100	7.5×10^4	<100	+	+	+	+	+	[12]

[a]Transformation efficiency was calculated as the number of transformants per mg DNA used. The number presented was obtained from the average values of two experiments.

[b]Information on the restriction background of the strains used is from Raleigh *et al.* [26] and Waite-Rees *et al.* [27]. '+' indicates the presence of activity; '-' indicates lack of activity. A blank indicates that no information was available.

[c]0.1 μg of pCSL17 DNA derived from either *E. coli* LE392 , N4830, *B. flavum* BF4, and *B. lactofermentum* BL1 were mixed with 200 μL of recipient cell.

DNA for several different recipient *E. coli* strains with a range of ENase and DNA MTase backgrounds. A similar approach was also applied to *B. lactofermentum* (Table 1). *B. flavum*-derived pCSL17 transformed McrBC$^-$ strains of *E. coli* (MC1061 and HB101) with similar efficiency to *E. coli* LE392-derived pCSL17 DNA. However, the efficiency of transformation of McrBC$^+$ strains of *E. coli* (LE392, ED8654, CSR603, and JM101) using *B. flavum*-derived pCSL17 was at least 100-fold lower than for *E. coli* LE392-derived pCSL17 DNA. Since the McrBC$^+$ background restricts uptake of foreign DNA with methylated cytidines located at specific GC sites, this data suggests that *B. flavum* has a MTase which methylates cytidines, at least some of which are located in McrBC restriction enzyme recognition sites. However, *B. lactofermentum*-derived pCSL17 had transformed McrBC$^+$ strains of *E. coli* (LE392, ED8654) at high efficiency suggesting that *B. lactofermentum* does not methylate cytidines residues at GC sites. These data indicate that methylation patterns in *B. lactofermentum* strain BL1 are different from those of *B. flavum* strain BF4.

McrBC is a methylation-dependent ENase which recognises the sequence $Pu^mC(N_{40\text{-}2,000})Pu^mC$ (where Pu indicates A and G bases and N indicates either G, C, T, or A) and acts upon DNA containing methylcytidine on one or both strands of the DNA. *McrBC* acts on methylated DNA. According to the supplier's notes (Biolabs), the enzyme produces smeared DNA, rather than a sharp banding pattern. *McrBC* ENase digestion of pCSL17 plasmid DNA showed that *B. flavum* contained methylated cytidine in the GC sequence, which is consistent with the results for transformation experiments in Table 1 (Fig. 4). In contrast, plasmid DNA from *B. lactofermentum* BL1 was not degraded by *McrBC* ENase. DNA derived from the *B. lactofermentum* had transformed recipient EcoKR$^+$ strains of *E. coli* (CSR603 and JM101) with lower efficiency than EcoKR$^-$ (MC1061, HB101, LE392, and ED8654), indicating the presence of methylated adenosines in the target sequence of *EcoK*. Although this approach could not be used to determine which DNA bases in *B. lactofermentum* BL1 were methylated, it is interesting to note that DNA derived from the BL1 was affected by the presence of *EcoK* systems in recipient strains of *E. coli*. The *EcoK* ENase recognized the sequence of AACNNNNNNGTGC (where N is a purine or pyrimidine base) [24] and cleaved DNA unless one of the first two adenosines is methylated. This indicated that this bacterium contained a different modification system to that found in *B. flavum*.

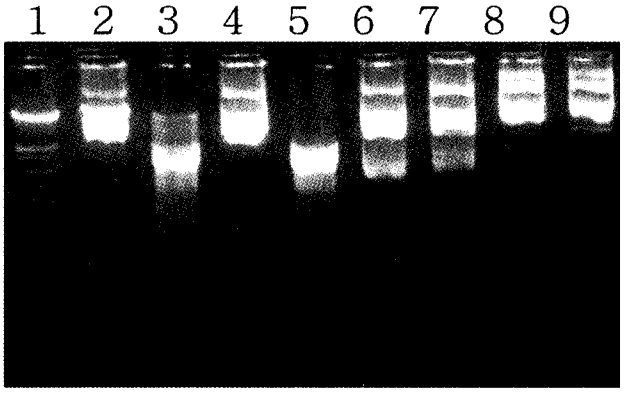

Fig. 4. Agarose gel electrophoresis of three corynebacteria- and *E. coli*-derived pCSL17 DNA treated with *McrBC* restriction endonucleases. *C. glutamicum* AS019, *B. flavum* BF4, *B. lactofermentum* BL1, and *E. coli* LE392 samples in order were digested with *McrBC* enzyme. Lanes: 1, DNA digested with *Eco*RI/*Hin*dIII (11 fragments: 564, 831, 947, 1375, 1584, 1904, 2027, 3530, 4268, 4973/5148, 21226 bp); 2,4,6,8, undigested; 3,5,7,9, digested with *McrBC* enzyme.

In conclusion, the present data describes the differences in the physical and enzymatic barriers in two species of corynebacteria. The results of this study give some insight into successful foreign genes expression in corynebacteria.

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