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Conditions affecting cell surface properties of human intestinal bifidobacteria

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Abstract

The cell surface properties of human intestinal bifidobacteria have been characterized for 30 strains isolated from a fecal sample. Strain identification to the species level was obtained by restriction analysis of the amplified 16S rRNA gene and confirmed by DNA/DNA reassociation experiments. The isolates were grouped in four genetically homogeneous clusters whose members belonged to *Bifidobacterium bifidum*, *Bifidobacterium adolescentis*, *Bifidobacterium longum* and *Bifidobacterium pseudocatenulatum* species. Cell surface properties of *Bifidobacterium* strains were evaluated by determining the level of hydrophobicity, adhesion to hydrocarbons and contact angle measurements, and their autoaggregation ability. The results showed high and homogeneous level of hydrophobicity in all tested strains when contact angle measurements values were considered. On the contrary, autoaggregation assays and bacterial adhesion to hydrocarbons detected interesting differences in cell surface properties among the tested *Bifidobacterium* strains. The highest levels of autoaggregation, detected in *B. bifidum* and *B. adolescentis* strains, were strictly dependent on the pH of the medium. Moreover, protease treatment experiments suggested that proteins had a key role in the autoaggregating ability of *B. bifidum* and *B. adolescentis* strains.

Introduction

Bifidobacteria are one of the dominant bacterial groups of human gut (Sgorbati et al. 1995). Their physiological activity is thought to exert a range of beneficial effects on host health, such as protection against potentially pathogenic bacteria (Bernet et al. 1993; Gibson and Wang 1994; Fujiwara et al. 1999), reduction of serum cholesterol levels (Tahri et al. 1995), vitamin production (Noda et al. 1994) and enhancement of immune response (Kado-Oka et al. 1991).

The utilization of bifidobacteria, already widespread in fermented dairy products, is now extending also to the production of numerous probiotic food supplements, commercially available as tablets, powders or capsules (Temmerman et al. 2003). Moreover, a growing number of physicians coprescribe probiotics to their patients to prevent antibiotic-related diarrhoea and, more recently, bifidobacteria were also successfully used in therapy for *Helicobacter pylori* eradication (Hamilton-Miller 2003; Gill 2003). Nevertheless, the mechanisms by which probiotic strains benefit humans are generally not well understood (Elmer et al. 1996; Miller 2001). This lack of knowledge has raised a lively discussion on the actual need to use probiotics as therapy or prophylaxis for certain pathological conditions (Edmunds 2001; Miller 2001). Therefore, it is important to clarify

the complex interrelationships that can establish between humans and the so-called probiotic microorganisms.

The ability to adhere to intestinal wall is considered an indispensable pre-requisite of probiotic strains in order for them to colonize the human gastro-intestinal tract and then to exert health-promoting effects (Pedersen and Tannock 1989; Alander et al. 1999). Adhesion ability has been consequently proposed as one of the main selection criteria for potential probiotic strains (Fogh et al. 1977). Nevertheless, *in vivo* studies of bacterial adhesion are not easy to perform while *in vitro* model systems, mainly based on the use of human enterocyte-like Caco-2 cells (Fogh et al. 1977), are expensive and time-consuming. Reliable indirect methods to evaluate the adhesion ability of lactic acid bacteria and bifidobacteria have thus been investigated. Several authors described a good correlation between adhesion ability and cell surface hydrophobicity (Wadstrom et al. 1987; Marin et al. 1997; Del Re et al. 2000). However, these results have not been confirmed in other studies by other authors. It was reported that the methods employed can sensitively influence the relative hydrophobicity level measured, and that microbial adhesion involves an interplay of many physical–chemical and structural factors rather than cell surface hydrophobicity only (Marin et al. 1997; Pérez et al. 1998; van der Mei et al. 1991, 1995; Busscher et al. 1995). For these reasons autoaggregation ability was proposed as a more effective, easier and reproducible tool for preliminary selection of strains with potential adhesive features (Del Re et al. 1998, 2000; Pérez et al. 1998).

The present work was carried out on bifidobacterial strains isolated from a single faecal sample collected from a healthy woman, with the aim of investigating the chemical–physical elements involved in surface properties of bifidobacteria and on the conditions affecting them.

Materials and methods

Subject, strains and culture conditions

Faecal sample was collected from a healthy adult woman. The subject had no history of gastrointestinal complaint or antibiotic therapy within the previous two months.

The specimen was collected in sterile plastic universal container and processed within 2 h in an anaerobic cabinet (H_2 10%, CO_2 5%, N_2 85%, v/v). Faeces were homogenized, serially 10-fold diluted in physiological solution (150 mM NaCl) and spread in duplicate on pre-reduced Beerens agar medium (Beerens 1990). After incubation for 72 h at 37 °C, in the same anaerobic cabinet, all the colonies grown at the highest dilutions were picked. Unless otherwise stated, bacteria were grown anaerobically at 37 °C until stationary phase (24 – 36 h) in MRS medium (Difco Laboratories, Detroit, Mich., USA) supplemented with 0,05% of L-cystein hydrochloride. Type strains were purchased by DSMZ collection (*Bifidobacterium bifidum* DSMZ 20456, *Bifidobacterium longum* DSMZ 20219, *Bifidobacterium adolescentis* DSMZ 20438 and *Bifidobacterium pseudocatenulatum* DSMZ 20083). Type strains and new isolates were maintained at –20 °C in MRS broth added with glycerol (15% v/v) and reactivated by two consecutive subcultures in the same medium broth.

Bacterial identification

The identification of the isolates at genus level was assessed on the basis of their morphological properties and positive response to fructose-6-phosphate phosphoketolase test (F6PPK) (Scardovi 1986).

Randomly amplified polymorphic DNA (RAPD) experiments with primers OPI17 (5′-CG AGGGTGGTGATC –3′), OPI02-mod (5′-GCTC GGAGGAGAGG-3′), AP2 (5′-AGTCAGCCAC-3′) and AP5 (5′-TGTTCCACGG-3′) were performed as previously described (Manachini et al. 2002) in order to evaluate the presence of multi-isolates. For checking reproducibility, experiments were repeated at least three times, employing independently isolated DNA samples.

The identification at species level was carried out by amplified ribosomal DNA restriction analysis (ARDRA) digestion of the 16S rRNA gene by *Sau*3AI and *Bam*HI restriction enzymes (Ventura et al. 2001).

DNA–DNA homology experiments with type strains were carried out between the type strain of each bifidobacterial species and one strain arbitrarily chosen from each ARDRA-cluster. Hybridisation was performed as previously

described (Manachini et al. 1998). The extent of DNA reassociation was calculated on the basis of renaturation rates determined spectrophotometrically, with a "Gilford Response" spectrophotometer equipped with an Advance Kinetics Graphic Version 1.3 thermoprogrammer (Ciba Corning Diagnostics Corp., OH), and according to the procedures of Seidler and Mandel (1971) and Kurtzman et al. (1979). The reaction was carried out under optimal conditions (25 °C below the Tm) in 5× SSC buffer containing 20% dimethylsulfoxide (DMSO).

Bacterial adhesion to hydrocarbons

The bacterial adhesion to hydrocarbons (BATH) test was performed according to Rosenberg et al. (1980) with some modifications. Cells were washed once with phosphate-buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.2) and resuspended in the same buffer to an absorbance (A) of about 0.5 at 600 nm, then an equal volume of hexadecane or xylene was added. The two phase system was thoroughly mixed by vortexing for 3 min. The aqueous phase was removed after 1 h of incubation at room temperature and its A_{600} was measured. Affinity to hydrocarbons was reported as adhesion percentage according to the formula: $\mathrm{Ad}\% = [(A_0 - A)/A_0] \times 100$, where A_0 and A are absorbance before and after extraction with organic solvents, respectively. Adhesion percentages were obtained from at least three independent experiments.

The influence of pH on BATH test was assessed on cells washed with PBS and resuspended in phosphate buffer (pH range from 5.0 to 10.0) or citrate buffer (pH range from 2.0 to 5.0).

Contact angle measurement (CAM)

Contact angle was measured by sessile drop technique as previously described by Daffonchio et al. (1995). Cells were washed with PBS, resuspended in the same buffer and filtered on a micropore filter (cut-off 0.45 μm). Bacterial layers were dried at room temperature and the determinations were done on the plateau of the drying time after 2 h (van der Mei et al. 1991). Hydrophobicity was measured by determining the contact angle of 1 μl of saline (100 mM NaCl) or pure water on the dry bacterial layer at 20 ± 3 °C. Contact angle values were obtained from at least ten measurements on two different filters. Contact angles higher than 45° indicated hydrophobic cells (Daffonchio et al. 1995).

Autoaggregation assay

Autoaggregation ability was measured according to Del Re et al. (1998) as the autoaggregation percentage. Briefly, bacterial cultures at stationary phase were shaken and maintained at 15 °C. At different times after shaking (30, 60, 90, 120, 150 min), 1 ml of the upper suspension of the culture was transferred to another tube where the optical density ($\mathrm{O.D._{600nm}}$) was measured. Autoaggregation percentage was expressed as: 1 – (O.D. upper suspension/O.D. total bacterial suspension) × 100.

The influence of pH on autoaggregation was tested at values ranging from 3.0 to 11.0. Optical density was measured after 1, 3 and 5 h of incubation at room temperature after shaking. The pH was modified adding NaOH 1 N or HCl 1 N directly to the broth cultures. For each strain, pH of the culture at the stationary phase (between 4.1 and 4.3 units) was included.

Enzymatic treatments of bacterial cells

All the enzymatic treatments, were carried out on cells washed with PBS or by direct addition of the enzymes to the cultures. Proteinase K treatment (0.5 g l^{-1}, 120 min at 37 °C) and trypsine treatment (1 g l^{-1}, 120 min at 37 °C) were carried out at pH 7.0, while pepsin (1 g l^{-1}, 120 min at 37 °C) at pH 3.0. All proteases were purchased by Sigma (St Louis, MO, USA).

Cell-wall-associated protein extraction

Cells from 0.5 l of liquid culture were harvested by centrifugation and processed according to Mattarelli et al. (1993) with the only modification

regarding the use of French Press (12,000 psi) for breaking cells. Cell wall proteins were examined by SDS-PAGE using the method of Laemmli (1970).

Results

Taxonomic identification and DNA fingerprinting

Thirty isolates, representing the predominant bifidobacterial population in the tested faecal sample, were obtained from Beerens plates. All the isolates were anaerobic, Gram-positive, non spore-forming, pleomorphic rods, giving a positive response to F6PPK. In order to identify multiple isolates, RAPD analysis was performed with four different primers. The results obtained showed the presence of 25 different genotypes among a total of 30 analysed isolates. A computer evaluation of similarities and clustering of *Bifidobacterium* isolates was carried out on all RAPD patterns obtained. The derived dendrogram grouped the detected patterns into four main branches separated at a similarity level of 0.01 (Figure 1). The highest level of genotypic polymorphism was detected in the clusters A and B, with ten and nine genotypes, grouped at a similarity level of 0.66 and 0.72, respectively. Three genotypes, grouped at a similarity level of 0.92, were detected in cluster C. Four isolates grouped into three genotypes were assigned to cluster D.

ARDRA analysis performed with the restriction enzymes *Sau*3AI and *Bam*HI revealed that all the isolates within each cluster were genetically closely related, showing identical restriction patterns (data not shown) and suggesting that each RAPD cluster could be considered as a *Bifidobacterium* species (Ventura et al. 2001). In this context, the 16S ribosomal DNA digestion of all isolates and of *Bifidobacterium* type strains allowed us to ascribe the 30 isolates to 4 different bifidobacterial species: strains grouped in cluster A to *B. bifidum*, cluster B to *B. longum*, cluster C to *B. adolescentis* and cluster D to *B. pseudocatenulatum*.

To confirm the exact identification at species level, DNA–DNA reassociation experiments between type strains and one strain, arbitrarily chosen from every species-specific cluster, were carried out. The levels of DNA–DNA reassociation were in agreement with the ARDRA results, strengthening the preliminary species identification. Strain NAB 1 (cluster A) showed a DNA relatedness value of 91.0% with *B. bifidum* DSMZ 20456; NAL 4 and NAP 34 of 98.2 and 85.9% with *B. longum* DSMZ 20219 and *B. pseudocatenulatum* DSMZ 20438, respectively and NAA 28 of 77.9% with *B. adolescentis* DSMZ 20083. Reassociation values lower than 22% were detected between strains belonging to different ARDRA groups (Table 1).

Hydrophobicity of the strains

The adhesion percentages of bifidobacteria strains to the tested hydrocarbons are shown in Figure 2a. The results indicate that *B. bifidum* strains showed the highest adhesion ability with both tested hydrocarbons, in contrast to the low affinity of *B. pseudocatenulatum*. With regard to *B. longum* and *B. adolescentis* species, different behaviour among strains was observed; moreover most strains showed different affinities depending on the hydrocarbon employed.

The results obtained from the contact angle measurements (CAM) (Figure 2b), indicated that all the tested strains were hydrophobic, with values ranging from 56.1° (DSMZ 20083 strain) to 94.2° (NAB 12 strain). Two *B. bifidum* strains, NAB 12 and NAB 15, were found to be the most hydrophobic with contact angle higher than 90° (94.2,° and 92.6°, respectively). A high degree of hydrophobicity (greater than 80°) was also found not only within *B. bifidum* species (NAB 1, NAB 7 and NAB 13 strains), but also among *B. longum* (NAL 8, NAL 10 and NAL 18) and *B. adolescentis* strains (NAA 28 and NAA 29). Recently, Vadillo-Rodrìguez et al. (2004) reported that the contact angles of three out of six *Lactobacillus* strains changed when measured with solutions of different ionic-strength. Particularly, two strains without surface layer proteins showed a lower contact angle when measured with 10 mM KCl than when measured with 100 mM KCl; in contrast, a surface layer positive strain of *Lactobacillus acidophilus* reduced its contact angle upon increase of the ionic strength. In our experiments, no significant differences were found in the contact angles of five representative strains selected from each *Bifidobacterium* species, when pure water was employed instead of saline, even after treating cells with proteases (Table 2).

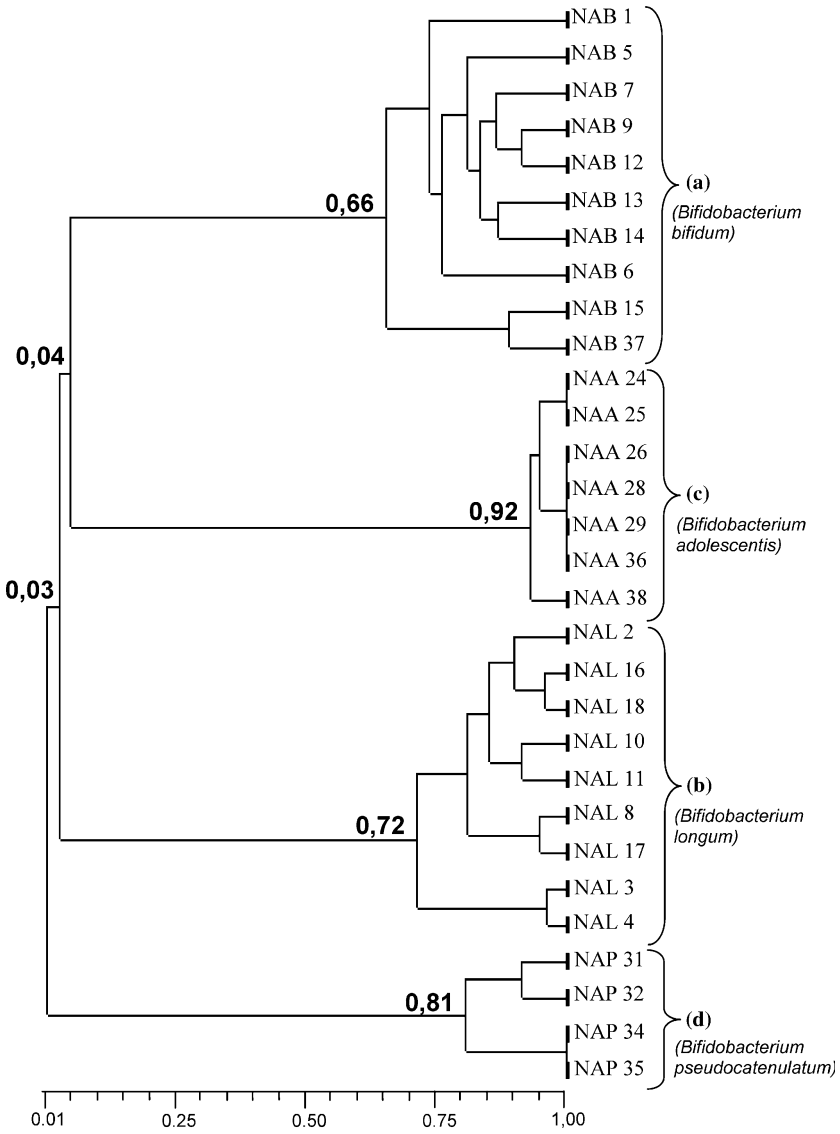

Figure 1. UPGMA dendrogram derived from similarity coefficients calculated by the Jaccard method (S_j, shown on the scale at the bottom), showing the relationship among bifidobacteria strains isolated from human intestine, analysed by RAPD using primers OPI17, OPI02-mod, AP2, and AP5. The species assignment according to ARDRA and DNA–DNA homology analysis is shown in parenthesis.

Autoaggregation ability

Depending on the rate and the level of sedimentation, five autoaggregation phenotypes were found. Particularly, all the *B. bifidum* strains were found to be strongly autoaggregating (autoaggregation percentage ≥89%), forming a precipitate and resulting in a clear supernatant (Figure 3). However, two different typologies of aggregation curves, with regard to the aggregation rate, were evident. NAB1, NAB14 and NAB37 strains showed an autoaggregation percentage of about 75% after only 30 min of incubation (phenotype Ia), while all the other *B. bifidum* strains, including the type strain, autoaggregated slowly and, at the same time, only about 30% of the cells were sedimented (phenotype Ib). The aggregation curves II and III relate only to *B. adolescentis* strains: four strains (NAA 28, NAA

Table 1. Levels of DNA–DNA reassociation (%) among *Bifidobacterium* type strains and representative isolates of each RAPD cluster.

Strains	1	2	3	4	5	6	7	8
1 – *B. bifidum* DSMZ 20456	100							
2 – *B. longum*DSMZ 20219	n.d.	100						
3 – *B. adolescentis* DSMZ 20438	n.d.	n.d.	100					
4 – *B. pseudocatenulatum* DSMZ 20083	n.d.	n.d.	n.d.	100				
5 – NAB 1 (Cluster A)	**91.0**	7.8	4.1	8.3	100			
6 – NAL 4 (Cluster B)	15.7	**98.2**	14.2	9.6	18.2	100		
7 – NAA 28 (Cluster C)	21.8	12.3	**77.9**	20.6	11.4	12.5	100	
8 – NAP 34 (Cluster D)	19.5	6.9	17.4	**85.9**	8.2	13.8	21.7	100

n.d. = not determined.

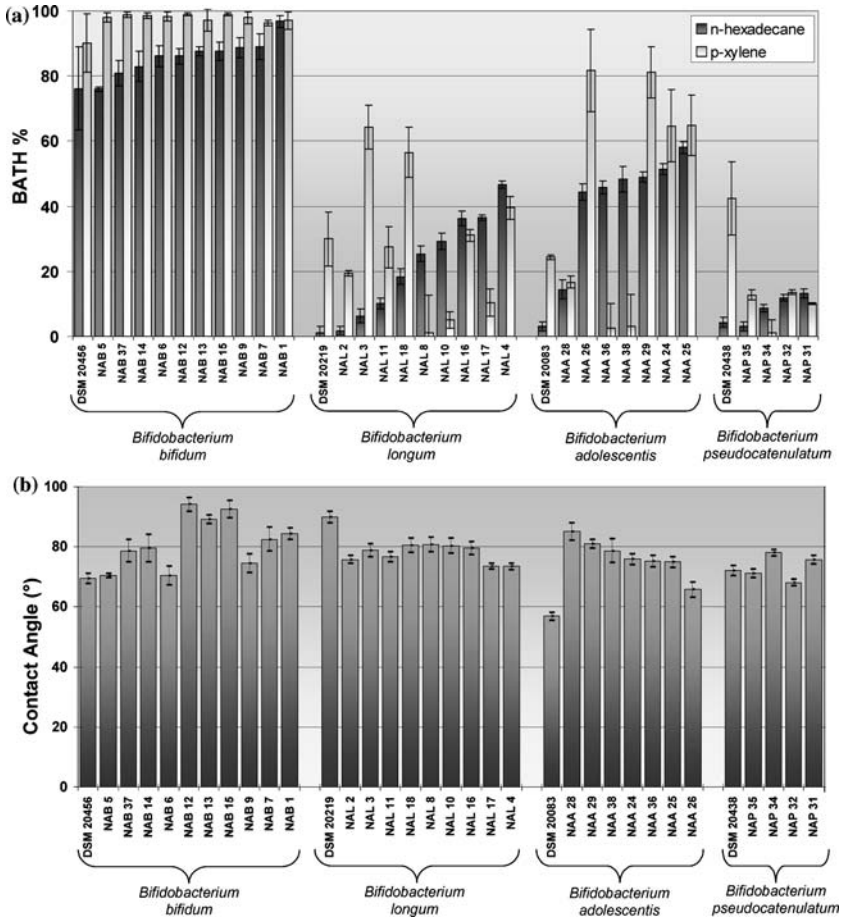

Figure 2. Percentages adhesion to hydrocarbons and water contact angles of the isolates as measured through BATH test (a) and CAM test (b), respectively. Bars represent standard deviations ($n \geq 3$ for BATH test and $n \geq 10$ for CAM).

29, NAA 36 and NAA 38) showed a moderate autoaggregation ability (about 62%), while the others (NAA 24, NAA 25 and NAA 26) showed a lower value (about 32% after 3 h of incubation). The last aggregation phenotype includes all the strains ascribed to *B. longum* and *B. pseudocatenulatum* species together with the other three type strains, which did not show autoaggregation ability.

Table 2. Comparison of the contact angles (degrees) obtained from saline or pure water drops on the cell layers.

Strain	Contact angle (°)	
	SALINE (100 mM NaCl)	PURE WATER
B. bifidum NAB 1	82.3 ± 3	86.6 ± 3.1
B. adolescentis NAA 28	79.4 ± 3	80.7 ± 1.8
B. adolescentis NAA 26	62.8 ± 1.1	63.3 ± 2.7
B. pseudocatenulatum NAP 32	73.7 ± 3.3	75.1 ± 1.8
B. longum NAL 8	81.1 ± 2.1	82.7 ± 2.3
B. bifidum NAB 1 after pepsin treatment	49.1 ± 0.5	47.7 ± 1.2
B. adolescentis NAA 28 after pepsin treatment	78.6 ± 2.4	74.8 ± 3.7
B. adolescentis NAA 26 after pepsin treatment	58.0 ± 0.5	56.7 ± 1.6
B. bifidum NAB 1 after proteinase K treatment	25.4 ± 2.1	27.9 ± 2.8
B. adolescentis NAA 28 after proteinase K treatment	45.1 ± 1.3	41.6 ± 4.2
B. adolescentis NAA 26 after proteinase K treatment	46.5 ± 4.3	52.0 ± 4.2

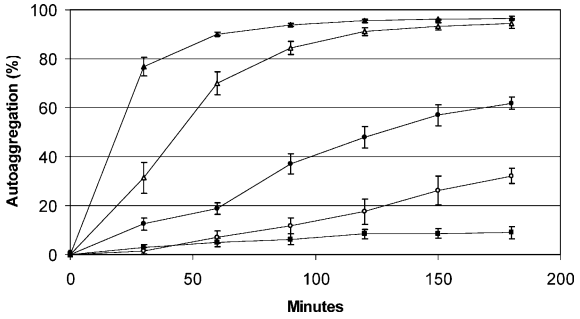

Figure 3. Main typologies of autoaggregation curves observed in the isolates in relation to the speed and maximum level of aggregation. ▲: phenotype Ia (NAB 1, NAB 14, NAB 37); △: phenotype Ib (NAB 5, NAB 6, NAB 7, NAB 9, NAB 12, NAB 13, NAB 15, *B. bifidum* DSM 20456); ●: phenotype II (NAA 28, NAA 29, NAA 36, NAA 38); O: phenotype III (NAA 24, NAA 25, NAA 26); ■: phenotype IV (NAL 2, NAL 3, NAL 4, NAL 8, NAL 10, NAL 11, NAL 16, NAL 17, NAL 18, NAP 31, NAP 32, NAP 34, NAP 35, *B. adolescentis* DSM 20438, *B. longum* DSM 20219, *B. pseudocatenulatum* DSM 20483).

Effect of pH on surface properties

The effect of pH value on autoaggregation and adhesion ability to hydrocarbons was investigated in six strains arbitrarily chosen as representatives of each aggregation phenotype and each isolated species. The tested strains were: *B. bifidum* NAB 1 (autoaggregation phenotype Ia), *B. bifidum* NAB 5 (phenotype Ib), *B. adolescentis* NAA 28 (phenotype II), *B. adolescentis* NAA 26 (phenotype III), *B. longum* NAL 8 and *B. pseudocatenulatum* NAP 32 (phenotype IV). The results obtained after an incubation of 5 h from shaking (Figure 4), showed strong autoaggregation ability by *B. bifidum* strains only at pH values below 4.2, no autoaggregation was present when the pH values ranged from 4.2 to 9.0 and at pH values over 10.0 the autoaggregation ability was partially restored (20 – 30%). The influence of pH on the autoaggregation process of *B. adolescentis* NAA 28 was evident too. In the pH range from 5.0 to 8.0 the aggregation percentage was reduced from 58% to about 25 – 30%, while at pH values over 9.5 and below 4.2 it reached the highest values. The behaviour of strains representing the phenotypes III and IV (NAA 26, NAL 8 and NAP 32) did not seem to be affected by any pH values, as shown in Figure 4 for NAA 26. The aggregation ability at different pHs was not influenced by the incubation time from the shaking step. In fact, all the tested strains showed similar aggregation features after 1 and 5 h of incubation. Moreover, on passing from an aggregation not permissive pH to a permissive pH, the effect of pH was perfectly reversible.

Nevertheless, no significant effects were detected concerning to the influence of pH on the ability to adhere to hexadecane of the six representative strains (data not shown).

Changes in surface properties after protease treatments

In order to obtain more information regarding the surface/wall constituents involved in the aggregation mechanism, the cells of NAB 1 (aggregation phenotype I), NAA 28 (phenotype II) and NAA 26 (phenotype III) strains were treated with three different proteases: pepsin, trypsin and proteinase K. All proteases caused the loss of autoaggregation

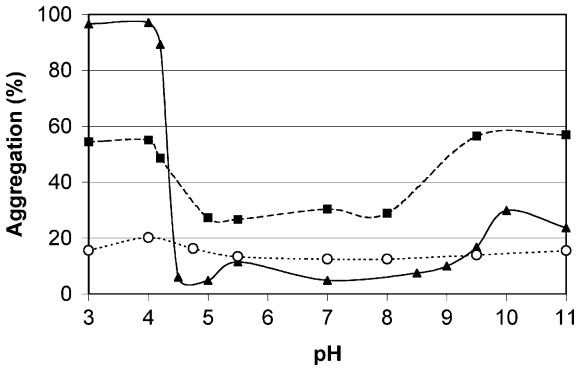

Figure 4. Influence of pH on autoaggregation level reached by the isolates after 5 h of aggregation. ▲: NAB 1 (representatives of autoaggregation phenotypes I); ■: NAA 28 (phenotype II); ○: NAA 26 (phenotype III). Every curve represents the average of at least two different experiments.

ability of the aggregating strains (NAB1 and NAA 28) resulting in turbid growth cultures and disappearance of microscopic clusters (Figure 5). These results suggest that one or more of the surface components involved in autoaggregation properties of these organisms are proteins.

Adhesion to hexadecane was influenced by protease treatments too. Affinity of *B. bifidum* NAB 1 for hexadecane after treatment with pepsin and proteinase K was strongly reduced by 67% and 62%, respectively. When trypsin was used only a slight effect was observed (reduction of 15%). The adhesion ability of NAA 28 and NAA 26 strains was strongly affected by pepsin and trypsin treatments and completely erased by proteinase K (Table 3).

Protease treatments influenced also the contact angle values of the three tested strains. A particularly evident loss of hydrophobicity was observed in *B. bifidum* NAB 1, with reductions of the contact angle of 40% and 69% after pepsin and proteinase K treatments, respectively (Table 3). The contact angles of *B. adolescentis* NAA 26 and NAA 28 were significantly reduced only after treating the cells with proteinase K (43 and 26%, respectively, Table 3).

Physiological growth phase and growth temperature vs. autoaggregation processes in B. bifidum strains

A correlation between the growth temperature or the physiological growth phase and the autoaggregation ability in *B. bifidum* strains was observed. All strains autoaggregated only at the end of the exponential growth phase and when cells grew at temperature values higher than 30 °C. Nevertheless, the adhesion of the strains to hexadecane seemed not to be affected, suggesting that the determinants involved in autoaggregation and adhesion could be different. Changes in surface properties at different growth temperatures were previously reported in *Bifidobacterium globosum* strains (Mattarelli et al. 1999). To verify this hypothesis cell wall protein extracts, from cells grown at different temperatures and harvested at the beginning and at the end of the exponential phase, were analysed by SDS-PAGE. Comparison of the electrophoretic patterns obtained, revealed no significant difference (data not shown). On the basis of the pH dependent aggregation phenotypes (Figure 4), the pH values of cultures, grown at different temperatures and collected at different phase of growth, were measured and lowered to pH 4.2 as this was the value observed in the broth cultures grown at 37 °C at the end of growth exponential phase. In these acidic conditions, autoaggregation and adhesion abilities were restored in all the cultures. These results indicated that the lack of autoaggregation was not due to the lack of determinants but to the insufficient acidification of the broth, thus suggesting that the pH value plays an important role in these processes (Table 4a, b).

Discussion

Strains belonging to four different *Bifidobacterium* species were isolated from a fecal sample collected from a healthy woman. By RAPD analysis several possible multiple isolate clusters were identified. Nevertheless, phenotypic surface properties highlighted significant differences among them. Particularly, *B. adolescentis* NAA 26, NAA 28, NAA 29 and NAA 36 strains, completely indistinguishable by RAPD analysis performed with the four primers employed, exhibited markedly different features in relation to autoaggregation ability and affinity to hydrocarbons. Moreover, different adhesion ability to hydrocarbons was observed between *B. pseudocatenulatum* NAP 34 and NAP 35 strains. Thus, the results obtained support the conclusion that even very closely genetically

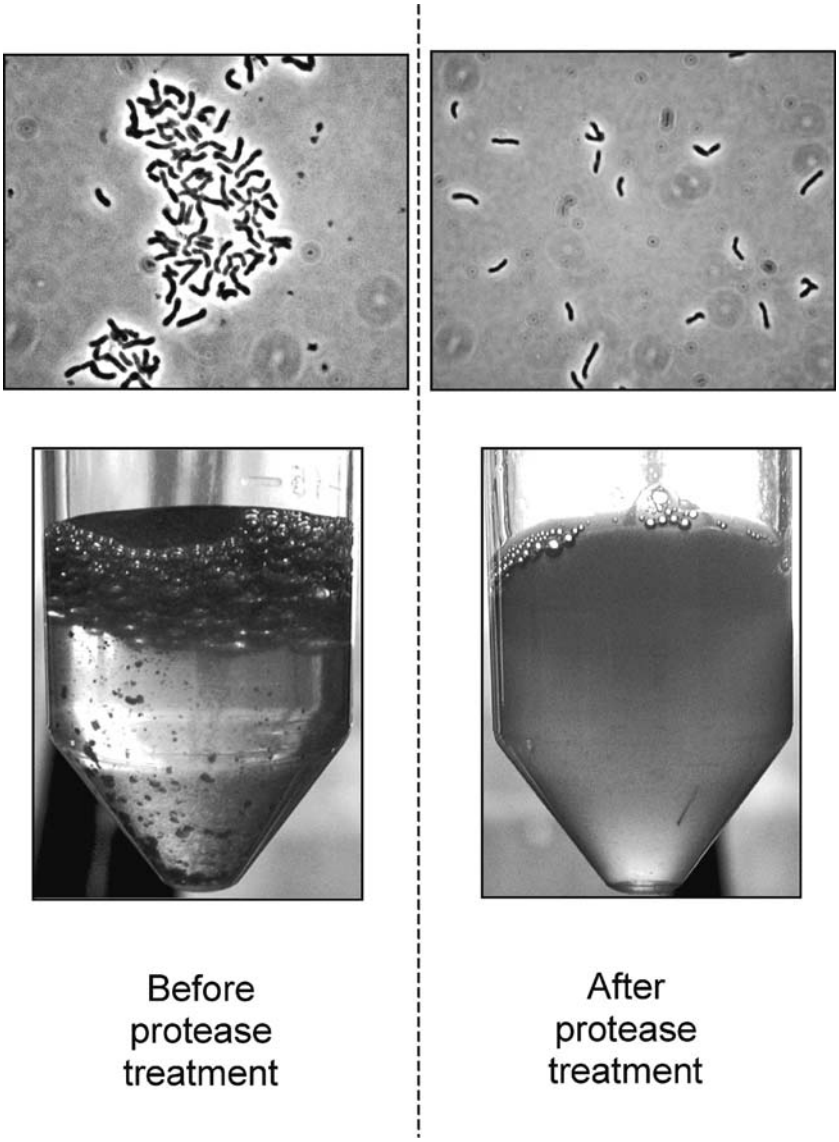

Figure 5. Microscopic and macroscopic effect of protease treatments on the aggregation of *B. bifidum* NAB 1 cells.

related *Bifidobacterium* strains can reveal significantly different phenotypes.

The BATH test was performed with hexadecane and xylene, two of the hydrocarbons routinely used because of their different chemical nature, respectively aliphatic and aromatic. Our data, as previously described (Marin et al. 1997), suggest that BATH values partially depend on the chemical nature of the hydrocarbon employed. Most *B. longum* and *B. adolescentis* strains showed a noticeably different adhesion ability in relation to the hydrocarbon used (Figure 2a).

It is noteworthy that all tested strains were considered hydrophobic in relation to contact angle measurements (CAM). These data are in contrast with the great heterogeneity observed in hydrophobicity when measured through affinity to hydrocarbons (BATH). The BATH test has been extensively used for measuring cell surface hydrophobicity in lactic acid bacteria (Marin et al. 1997; Kos et al. 2003; Vinderola et al. 2004) and bifidobacteria (Op den Camp et al. 1985; Pérez et al. 1998; Gòmez Zavaglia et al. 2002). Nevertheless, it was demonstrated that the most commonly used

Table 3. Effects of protease treatments on affinity to hexadecane and on contact angles (degrees). Percents in parentheses represent the reductions of BATH and CAM values after protease treatments.

Strain	BATH% (hexadecane)	Protease treatment (reduction%)			CAM	Protease treatment (reduction%)	
		Pepsin	Proteinase K	Trypsin		Pepsin	Proteinase K
B. bifidum NAB 1	96.9 ± 3.0	29.1 ± 7.3 (67%)	33.2 ± 3.6 (62%)	74.3 ± 11.2 (15%)	82.3 ± 3°	49.1 ± 0.5° (40%)	25.4 ± 2.1° (69%)
B. adolescentis NAA 28	14.4 ± 4.8	3.6 ± 0.9 (75%)	< 0 (~100%)	5.3 ± 1.7 (63%)	79.4 ± 3°	78.6 ± 2.4° (1%)	45.1 ± 1.3° (43%)
B. adolescentis NAA 26	44.4 ± 3.8	8.1 ± 0.9 (82%)	< 0 (~ 100%)	4.7 ± 1.8 (89%)	62.8 ± 3.9°	58.0 ± 0.5° (8%)	46.5 ± 4.3° (26%)

hydrocarbons, in the conditions in which the BATH test is performed, are negatively charged (Medrzycka 1991; Busscher et al 1995), probably because of the adsorption of negative ions from the aqueous phase. Since most bacterial cells at pH 7.0 have a negative charge, repulsive electrostatic interactions can then interfere with the adhesion of cells to the hydrocarbon molecules (Busscher et al 1995). These interactions are not present in CAM, in which the affinity of the cells with water is directly monitored and depends only on the cell wall constituents, thus justifying the higher hydrophobicity detected with this method. Our data seem therefore to support the hypothesis that BATH test measures a complicated interplay of several factors involved in microbial adhesion to surfaces, such as van der Waals forces and electrostatic interaction, and thus it would seem an unsuitable method to determine cell surface hydrophobicity (Geertsema-Doornbusch et al. 1993; van der Mei et al. 1993; Busscher et al. 1995). Adhesion to intestinal epithelial cells is a multifactorial process too; it includes specific (adhesins) and non-specific physical interactions (Freter 1992; Rojas and Conway 1996; Pérez et al. 1998). Del Re et al. (2000) observed that only *B. longum* strains showing high affinity to hexadecane, as measured by BATH test, were able to adhere to Caco-2 cells, while no correlation between adhesion ability and hydrophobicity, determined by hydrophobic interaction chromatography and salting-out aggregation test, was found. These results would seem to strengthen still further the fact that BATH measures not only hydrophobicity but more than one factor involved in adhesion to the intestinal epithelium.

Several other authors have reported a positive correlation between autoaggregation ability and adhesion to enterocytes in bifidobacteria (Del Re et al. 1998; Petr and Rada 2000). Moreover adhesion ability was not observed in the absence of autoaggregation even if strains showed a highly hydrophobic surface (Pérez et al. 1998). For this reason, autoaggregation assay has been proposed as a suitable tool, more effective than surface hydrophobicity measurement, for a preliminary screening of strains potentially able to adhere to intestinal epithelium. Among the 34 strains examined, even if all were hydrophobic as measured by CAM, only strains belonging to *B. bifidum* species showed a strong autoaggregation

Table 4. Features of *Bifidobacterium bifidum* NAB 1 cultures in relation to growth temperature (a) and growth phase (b). In the last column the autoaggregation ability of the strain after acidification of the broth to pH 4.5 is shown.

Growth Temperature	Broth culture O.D.[a]	Broth culture pH[b]	BATH (Hexadecane)	Autoaggregation Phenotype (AAG)	AAG After Acidification
a					
25 °C	1.7	5.1	91.84 ± 5.01	–	+
30 °C	2	4.5	92.92 ± 4.22	+	+ +
37 °C	2	4.2	96.86 ± 3.05	+ +	+ +
41 °C	2	4.2	89.91 ± 6.53	+ +	+ +
b					
Stationary	2	4.2	96.86 ± 3.05	+ +	+ +
Early Exp	0.7	5.8	87.06 ± 5.82	–	+ +

[a]Spectrophotometer accuracy ±5%.
[b]pH meter accuracy ±0.1.
The values are the average of at least three different determinations.

ability (more than 75% in 3 h) and four out of seven *B. adolescentis* aggregated more than 50% (Figure 1). These data further suggest that autoaggregation is the result of several chemical–physical determinants and that hydrophobicity is only one of them.

By plotting percentages of autoaggregation vs. BATH values (Figure 6a, b), it has been possible to verify that a wide range of biodiversity exists in relation to cell surface properties among bifidobacterial strains isolated from the same intestinal ecosystem, with the exception of *B. bifidum* strains. These data are in agreement with Pérez et al. (1998) and Del Re et al. (2000) who described a clear heterogeneity in surface features of bifidobacteria isolated from infant and adult faeces.

Autoaggregation of *B. bifidum* NAB1 and *B. adolescentis* NAA28 strains appeared to be strongly affected by pH. Since the loss of autoaggregation of cells at higher pH values was reversible, it seems that cell surface determinants were not washed out even at extreme pHs. Such a behaviour in relation to pH, particularly evident for *B. bifidum* NAB1, could be due to a different

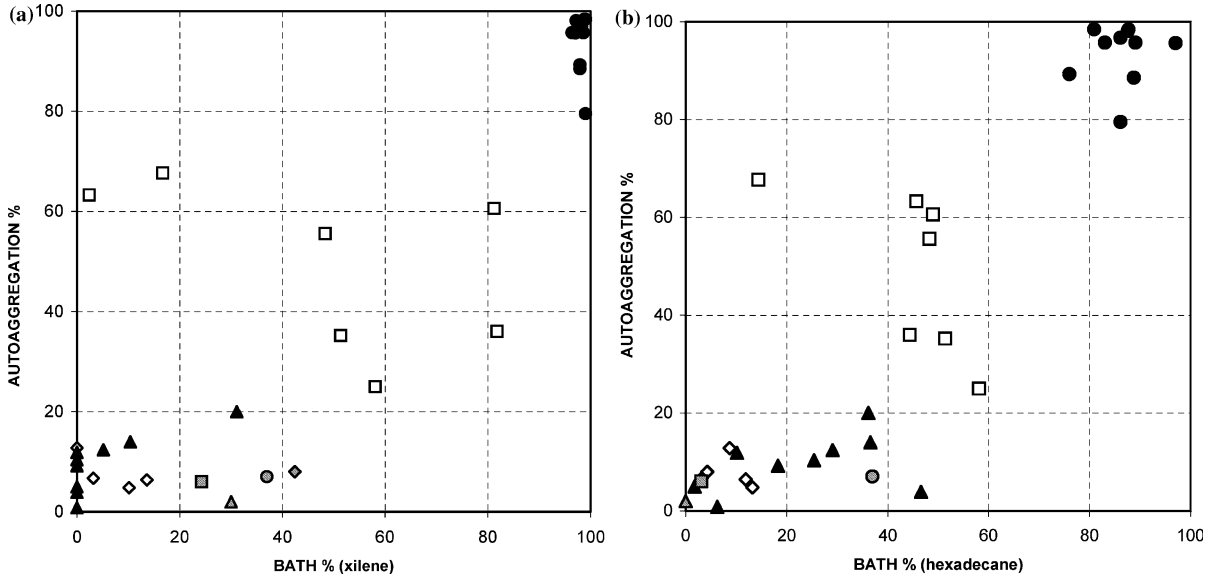

Figure 6. Autoaggregation index of the strains as a function of adhesion to hexadecane (a) and xylene (b). Values are the average from at least two experiments. ●: *Bifidobacterium bifidum* strains; □: *B. adolescentis* strains; ▲: *B. longum* strains; ◇: *B. pseudocatenulatum* strains; type strains are represented with grey symbols.

dissociation degree of the acidic and basic groups of the aminoacids, modulated by pH, resulting in a conformational change of proteins and in their agglutination. Pérez et al. (1998) reported weaker pH mediated changes in the surface properties of two strains ascribed to *B. bifidum* and *B. adolescentis* species, suggesting that the increase in autoaggregation could be related to the reduction of the electrostatic repulsion due to the decrease of pH values.

To investigate if proteins were involved in the aggregation mechanism of the aggregating *B. bifidum* and *B. adolescentis* strains, enzymatic treatments with three different proteases (pepsin, trypsin and proteinase K) were performed. Protease treatments erased the aggregation ability of the strains and significantly reduced adhesion to hexadecane and contact angles, suggesting that proteins could be, at least, one of the cell wall determinants involved in the aggregation process.

Proteinase K treatment significantly affected the contact angles of the investigated strains. It would seem that there exists a correlation between the reduction of the contact angle after protease treatment and autoaggregation ability. In fact, the CAM value of *B. bifidum* NAB1, the most autoaggregating strain, was the most drastically reduced while the contact angles of the moderate autoaggregating *B. adolescentis* NAA28 and low autoaggregating *B. adolescentis* NAA26 were less reduced in relation to their autoaggregating ability. The protease treatments confirmed also that proteins have a key role in the surface hydrophobicity of these strains.

Further studies are now in progress to identify and characterize bifidobacterial cell-wall proteins with the aim to understand their role in cell autoaggregation mechanism.

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