Environmental Microbiology (2000) **2**(1), 51–58

Modification of the aggregation behaviour of the environmental *Ralstonia eutropha*-like strain AE815 is reflected by both surface hydrophobicity and amplified fragment length polymorphism (AFLP) patterns

Peter Bossier,[1†] Eva M. Top,[1] Geert Huys,[2] Karel Kersters,[2] Christophe J. P. Boonaert,[3] Paul G. Rouxhet[3] and Willy Verstraete[1*]
[1]*Laboratory for Microbial Ecology and Technology, University of Gent, 653 Coupure L., 9000 Gent, Belgium.*
[2]*Laboratory for Microbiology, University of Gent, 35 K. L. Ledeganckstraat, 9000 Gent, Belgium.*
[3]*Unite de Chimie des Interfaces, Catholic University of Louvain-la-Neuve, 2/18 Place Croix du Sud, 1348 Louvain-la-Neuve, Belgium.*

Summary

After inoculation of the plasmid-free non-aggregative *Ralstonia eutropha*-like strain AE815 in activated sludge, followed by reisolation on a selective medium, a mutant strain A3 was obtained, which was characterized by an autoaggregative behaviour. Strain A3 had also acquired an IncP1 plasmid, pLME1, co-aggregated with yeast cells when co-cultured, and stained better with Congo red than did the AE815 strain. Contact angle measurements showed that the mutant strain was considerably more hydrophobic than the parent strain AE815, and scanning electron microscopy (SEM) revealed the production of an extracellular substance. A similar hydrophobic mutant (AE176R) could be isolated from the AE815-isogenic *R. eutropha*-like strain AE176. With the DNA fingerprinting technique repetitive extragenic palindromic-polymerase chain reaction (REP-PCR), no differences between these four strains, AE815, A3, AE176 and AE176R, could be revealed. However, using the amplified fragment length polymorphism (AFLP) DNA fingerprinting technique with three different primer combinations, small but clear reproducible differences between the banding patterns of the autoaggregative mutants and their non-autoaggregative parent strains were observed for each primer set. These studies demonstrate that, upon introduction of a strain in an activated sludge microbial community, minor genetic changes readily occur, which can nevertheless have major consequences for the phenotype of the strain and its aggregation behaviour.

Received 18 August, 1999; revised 22 October, 1999; accepted 26 October, 1999. †Present address: CLO-Gent, Department of Sea Fisheries, Ankerstraat 1, B-8400 Oostende, Belgium. *For correspondence. E-mail willy.verstraete@rug.ac.be; Tel. (+32) 9 264 59 76; Fax (+32) 9 264 62 48.

Introduction

Activated sludges can vary rapidly with respect to floc formation and cell aggregation properties (Urbain *et al*., 1993; Bossier and Verstraete, 1996). Often, these shifts result from population changes within the complex microbial community (Kampfer *et al*., 1996). However, it is possible that certain key bacteria also undergo natural genetic alterations. The relation between changes in cell aggregative behaviour and mutational events has not yet been thoroughly investigated in environmental strains.

The cell surface hydrophobicity (CSH) of a bacterium is an important factor in its interaction with the environment and may influence the ecological niche it will occupy. Yet, the CSH is not always a steady characteristic. Microorganisms change the CSH in response to environmental and physiological conditions (Bar-Or, 1990), such as medium composition and growth phase. It is now assumed that microorganisms can modify, within certain limits, the hydrophobicity of the cell envelope as a function of environmental demands. Changes in CSH can be caused by, e.g. differential lipopolysaccharide (LPS) epitope, exopolysaccharide (EPS) or protein expression. Rough mutant strains of *Salmonella typhimurium* producing LPS with truncated O-specific side-chains were found to be more hydrophobic than the wild-type smooth strains producing intact EPS (Hermansson *et al*., 1982). In a variety of strains, EPS capsules reduce the CSH, while fimbriae production renders cells more hydrophobic (Rosenberg and Doyle, 1990).

Bacteria have been shown to change surface characteristics, leading towards increased adherence (Darfeuille-Michaud *et al*., 1992; Savarino *et al*., 1994) or specific aggregation (Andrup *et al*., 1993), through the acquisition of a plasmid. In a genetically unspecified way, *Aeromonas salmonicida* (Fernández *et al*., 1995) species have been shown to change surface characteristics through the production of a 50 kDa protein (A-layer) after passage through

fish. Strains producing the A-layer were more hydrophobic and autoaggregated.

In this paper, we show that an increase in CSH in a *Ralstonia eutropha*-like strain was associated with cell aggregation and better staining with Congo red. These phenotypic changes concurred with chromosomal mutational events that could be detected with the amplified fragment length polymorphism (AFLP) DNA fingerprinting technique. These changes were provoked by simple introduction of the strain to an activated sludge community.

Results

Isolation of the autoaggregative strain A3

Bacterial strains have been shown to change cell surface characteristics through the acquisition of a plasmid (Darfeuille-Michaud *et al*., 1992; Andrup *et al*., 1993; Savarino *et al*., 1994) or, in the case of a pathogenic bacterium, through contact with its host (Fernández *et al*., 1995). In this study, attempts were made to isolate autoaggregative variants of the environmental *R. eutropha*-like strain AE815 after contact with an activated sludge community. Initially, attention was focused on the possibility that the plasmid-free strain AE815 would obtain this aggregative phenotype through the acquisition of plasmid-encoded genes by conjugation. The autoaggregative strain A3 was isolated as follows. The *R. eutropha*-like strain AE815 (10^8 cfu) was mixed with 4 mg of activated sludge from a hospital wastewater purification station. After centrifugation, the pellet was resuspended into 30 μl of LB, spotted onto a LB plate and left to incubate overnight at 28°C. By selective enrichment in E-medium, supplemented with kanamycin, rifampicin and cycloheximide, the strain AE815 was recovered from this mixture (Bossier and Verstraete, 1996). Subsequently, strains that were able to co-aggregate with added yeast cells were enriched by co-precipitation (Bossier and Verstraete, 1996) as a result of their ability to settle as flocs. Bacteria in the settled flocs were then plated on E-medium with the same antibiotics, and single colonies were tested for their ability to co-aggregate with yeast cells in a co-culture in YEPD medium. In total, 31 out of 50 isolates were able to co-aggregate with yeast cells. The 31 isolates could be grouped into two different classes. The first group of strains (26 isolates) co-aggregated with yeast cells within 2 h of co-culturing (as observed for a *Comamonas testosteroni* strain A20; Bossier and Verstraete, 1996). The second group of strains (five isolates) only formed aggregates with yeast cells after overnight co-culturing. They also autoaggregated when grown in LB. One particular strain from the second group, A3, was investigated further.

Strain A3 had the same antibiotic resistance pattern and the same BIOLOG substrate utilization pattern as strain AE815 (data not shown). Moreover, the repetitive extragenic palindromic-polymerase chain reaction (REP-PCR) fingerprint of strain A3 was the same as those of AE815 and other derivatives of the original *R. eutropha*-like strain CH34 (Fig. 1). These results indicate that strain A3 was derived from strain AE815. Apart from the autoaggregative phenotype, strain A3 also stained darker red on LB plates containing Congo red, compared with strain AE815 (Fig. 2). In *Azospirillum brasilense* (Katupitiya *et al*., 1995) and *Aeromomas salmonicida* (Dalsgaard *et al*., 1994), autoaggregation and Congo red staining were found to be linked phenotypically as well. The strain A3 typically produced smaller colonies on an LB plate compared with strain AE815. Interestingly, prolonged incubation very often resulted in the production of rough colonies (data not shown).

Cell surface characterization

The physicochemical origin of the described features of the A3 strain were investigated. The cell surface hydrophobicity (CSH) of strains AE815 and its variant A3 was compared by measuring water contact angles. The water contact angles of two independent measurements (microbial cells harvested from two independent cultures) at t_0

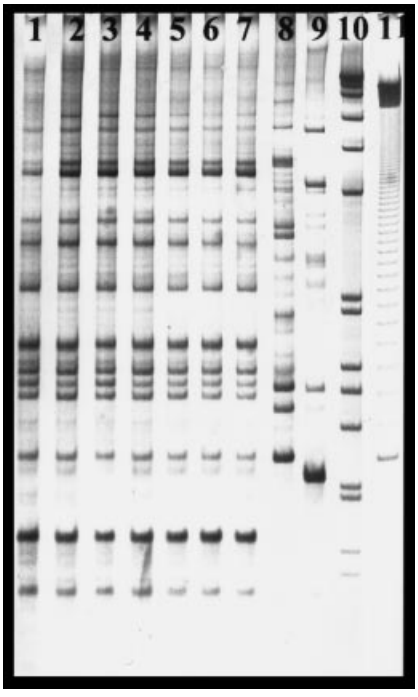

Fig. 1. REP-PCR patterns of the different *Ralstonia eutropha*-like strains. As a comparison, two other strains were included. Lanes: 1, AE815; 2, A3; 3, AE815(RP4); 4, A3(RP4); 5, AE176; 6, AE176R; 7, AE176 W; 8, *Ralstonia eutropha* JMP228; 9, *Comamonas testosteroni* I2, 10, 1 kb ladder; 11, 50 bp ladder.

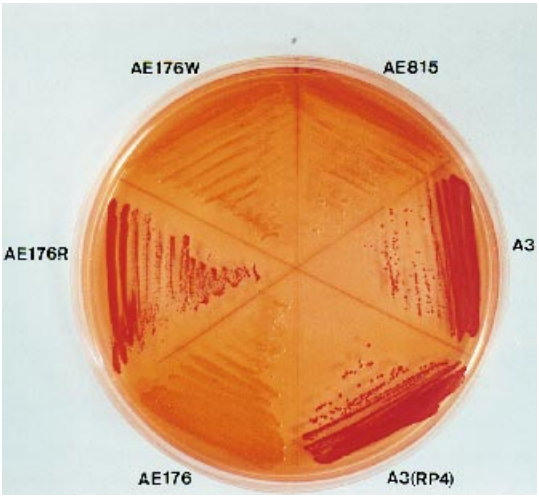

Fig. 2. Congo red staining of strains AE815 and AE176, their isogenic autoaggregative variant strains A3, A3(RP4) and AE176R and the isogenic non-aggregative variant AE176W.

were $31.9 \pm 3.8°$ and $24.4 \pm 2.6°$ for strain AE815 and $53.2 \pm 6.8°$ and $87.2 \pm 7.2°$ for strain A3. The water contact angles generally increased slightly with drying time and reached a plateau after 10–15 min, subsequent to mounting on the microscope slide. At 60 min (t_{60}), the water contact angles were $36.7 \pm 4.0°$ and $25.8 \pm 2.1°$ for strain AE815 and $72.3 \pm 6.2°$ and $87.7 \pm 5.1°$ for strain A3. This indicated that strain A3 had a considerably more hydrophobic cell surface than the original strain AE815. Scanning electron microscope (SEM) analysis showed that A3 produced an extracellular substance (Fig. 3) that appeared as long fibres connecting individual cells.

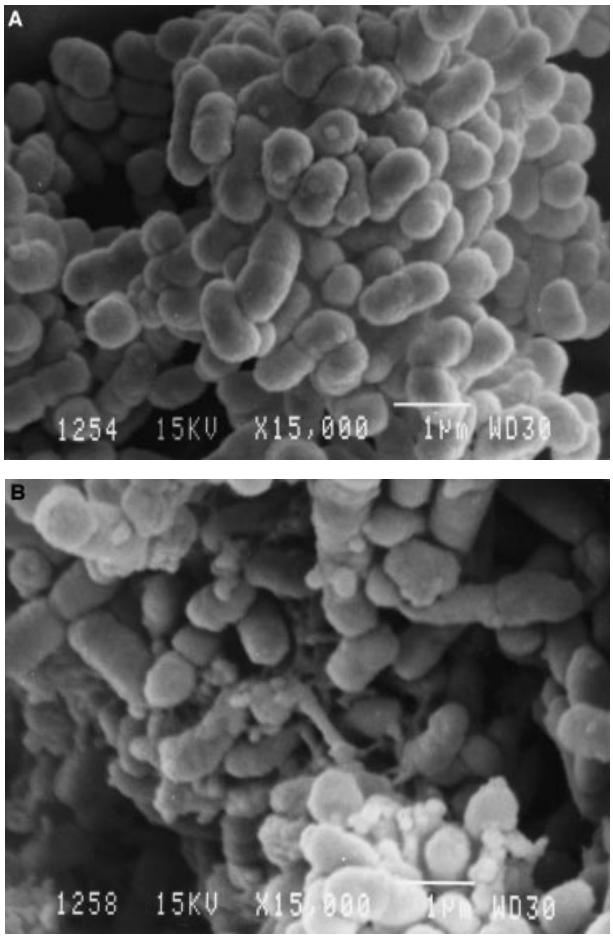

Fig. 3. SEM images of strains AE815 (A) and A3 (B). In the preparation of strain A3, an extracellular substance can be seen linking individual cells.

Genetic analysis of strain A3

Strain A3 contained a plasmid, designated pLME1 (result not shown), which was not present in strain AE815. The A3 strain probably acquired the plasmid through conjugation with microorganisms of the activated sludge community. It was investigated whether the phenotypic features (namely autoaggregation and staining with Congo red) of the strain A3 were encoded by the plasmid. PCR amplification with specific primers for IncP plasmids (*trfA*1) (Götz *et al*., 1996) and subsequent electrophoresis and Southern hybridization with the *trfA1* fragment of RP4 showed that plasmid pLME1 yielded a fragment of the same size and with highly similar sequence to RP4. These data indicated that plasmid pLME1 belonged to the IncP incompatibility group. This incompatibility between the two plasmids allowed us to cure strain A3 from its plasmid pLME1 by introducing the incompatible plasmid RP4. *Escherichia coli* CM120(RP4) was used as donor, and tetracycline-resistant transconjugants A3(RP4) were isolated. These transconjugants A3(RP4) were effectively cured from the plasmid pLME1, as demonstrated by plasmid extraction and *Eco*RI restriction digest analysis (data not shown). Nevertheless, the strain A3(RP4) retained the phenotypic characteristics of strain A3, namely autoaggregation and staining by Congo red (Fig. 2), which suggests that the acquired phenotypic characteristics were not encoded by plasmid pLME1.

A conjugation between strain A3 and another derivative of the *R. eutropha*-like strain CH34, namely strain AE176, was performed. Using liquid E-medium supplemented with lysine and Ni, AE176 cells were selectively enriched. From this enrichment culture, AE176 cells (as verified by their lysine auxotrophy) that stained dark red in the presence of Congo red (Fig. 2) and autoaggregated were isolated. Such a strain was designated AE176R. AE176 cells with the same phenotypic characteristics as AE176 (namely non-aggregative and no staining with Congo red), designated AE176W, were also obtained (Fig. 2). Both strains AE176R and AE176W harboured the plasmid pLME1.

Again, the plasmid could be cured from these two strains by conjugation with CM120(RP4) without affecting their phenotype. The latter observation again suggests that the plasmid pLME1 as such does not carry the genetic information coding for the phenotypic characteristics observed with the autoaggregative strains A3 and AE176R. It also provided the insight that contact with activated sludge was not a prerequisite for the acquisition of the described phenotypic characteristics, as the autoaggregative strain AE176R was isolated from a conjugation mixture of A3 and AE176 cells.

AFLP analysis

The small strain collection was analysed further using the DNA fingerprinting technique AFLP. This revealed that the acquisition of the autoaggregative phenotype was associated with the absence of specific bands in the AFLP pattern. In comparison with strain AE815, strain A3 was lacking three AFLP fragments (Fig. 4), using primer combination A04/T01. A similar difference was also noted between the AFLP patterns of strains AE815(RP4) and A3(RP4), which do not contain plasmid pLME1. This indicated that plasmid pLME1 was not responsible for the observed changes in the respective AFLP patterns. Interestingly, the AFLP pattern of the latter two strains showed one additional RP4-specific band. The AFLP pattern of the autoaggregative strain AE176R was, in comparison with the pattern of strains AE815 and AE176W (the latter two strains have identical patterns), only lacking one of the three bands that were absent in the pattern of strain A3. Identical results were obtained when the PCR amplification reactions were repeated on the same gDNA or when the whole procedure was repeated starting with gDNA isolated from other cultures (data not shown). Differences in AFLP patterns between the aggregative and non-aggregative strains were also detected with two other primer combinations (A01/T01 and A01/T03), which were amplifying a different subset of DNA restriction fragments. These primer combinations did not yield RP4-specific bands (result not shown). Taken together, these results show that the A3 and AE176R strains differed from their respective control strains AE815 and AE176W by chromosomal mutations, as revealed by the changes in AFLP patterns. On the other hand, considering that the observed changes between the four strains (namely AE815, A3, AE176R and AE176W) are minor, it can be concluded that all strains are clearly isogenic. The AFLP results also suggested that, in strains A3 and AE176R, similar, but not identical, chromosomal changes had occurred, as reflected by the disappearance of one identical AFLP fragment. The latter mutations concurred with the acquisition of the same phenotypic characteristics by strains A3 and AE176R, namely staining with Congo red and autoaggregation.

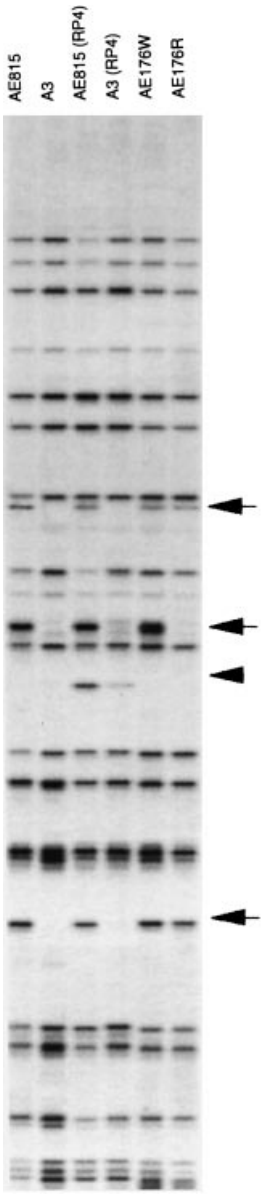

Fig. 4. AFLP banding patterns of strains AE815, A3, AE815(RP4), A3(RP4), AE176R and AE176W generated with the PCR primer combination A04/T01. Arrows indicate AFLP bands missing in the pattern of A3 or AE176R with respect to AE815 or AE176W. Arrowhead indicates RP4-specific band.

Discussion

This study indicates that the acquisition of the autoaggregative phenotype of strains A3 and AE176R concurred with chromosomal changes, which could be detected by the AFLP technique.

Genotypically, all strains (non-aggregative as well as aggregative) are very similar, as differences in their DNA fingerprinting patterns could only be detected with the AFLP technique and not with the REP-PCR technique, which can be used to distinguish strains at the subspecies

level (de Bruijn, 1992). Given the fact that these differences in AFLP patterns were detectable with three different primer combinations, it is unlikely that the change in AFLP pattern is the result of a single point mutation. Rather, the observed differences can be explained by either the occurrence of various point mutations, affecting the restriction and/or amplification steps in the AFLP procedure, or specific recombination events. Because the disappearance of an AFLP band did not concur with the appearance of a single smaller or larger AFLP band, it is also unlikely that small deletions or insertions (e.g. up to 10 nucleotides) are responsible. The fact that, for each of the three primer sets used, the AFLP patterns of the two autoaggregative strains showed similar changes in relation to the control strains seems to indicate that these events do not occur randomly, but that a non-random mechanism is at work at one or more loci. The relation between chromosomal mutational events, other than point mutations, and changes in cell surface composition has been studied in some detail in pathogenic bacteria (Rainey *et al*., 1993; Moxon *et al*., 1994), but is poorly documented in environmental strains. In a *Pseudomonas atlantica* strain, EPS production was found to be modulated by an insertion or deletion of an IS element (Bartlett *et al*., 1988). Our data confirm and extend the available literature, indicating that, in environmental strains also, the occurrence of mutational events in the bacterial chromosome can be important and is associated with changes in the expression of cell surface constituents. A possible relation between the acquired plasmid pLME1 in the autoaggregative variants A3 and AE176R and the observed phenotypic changes could not be demonstrated. The fact that this plasmid could be removed again without affecting the phenotype of these two strains seems to indicate that the plasmid itself does not code for the phenotypic changes.

A change in the composition and/or abundance of cell surface determinants, associated with autoaggregation, was evidenced in the current study by a differential staining with Congo red and by SEM analysis. In *Azospirillum brasilense* (Katupitiya *et al*., 1995) and *Aeromomas salmonicida* (Dalsgaard et al., 1994), where autoaggregation and Congo red staining were found to be phenotypically linked, it was not clear what cellular or extracellular components were responsible for the differential staining with Congo red. Congo red is most frequently used to stain polysaccharides with a β-1,3 or β-1,4 covalent bond, although outer membrane proteins that bind Congo red have been described (Smalley *et al*., 1995) as well. In *Salmonella enteritidis* and *E. coli*, Congo red binding and autoaggregation were linked to the ability to produce some specific aggregative fimbriae (Collinson *et al*., 1992; 1993). In summary, it is likely that the production of an extracellular compound(s) of unknown nature, modulating the staining with Congo red, is responsible for the increased CSH in strain A3.

A variety of mechanisms has been described for the formation of activated sludge flocs (Zita and Hermansson, 1994). Electrostatic interactions, as well as the production of exopolymeric material, are considered to be important determinants. In addition, the ability of free-living cells to attach to activated sludge flocs is strongly correlated with their CSH (Zita and Hermansson, 1997), while the internal hydrophobicity of a floc has been found to determine its ability to settle to a very large extent (Urbain *et al*., 1993). As the described strains are isogenic and differ in CSH, they are a good set of strains for studying the importance of this characteristic in relation to the retention of individual cells in the microbial community of an activated sludge floc.

The question arises whether changes such as those observed in strains A3 and AE176R are common. Moreover, the environmental factors that trigger such changes in the activated sludge and in the defined culture matings, respectively, and the underlying molecular principles need to be identified.

Experimental procedures

Strains

Strains AE815 and AE176 are derivatives of the metal-resistant *Ralstonia eutropha*-like strain CH34, isolated from a decantation tank of a zinc factory (Mergeay *et al*., 1985; Brim *et al*., 1999). Strain AE815 was obtained by curing strain CH34 from its two large heavy metal resistance plasmids pMOL28 and pMOL30, and making it rifampicin resistant (Springael *et al*., 1993). Strain AE176 is lysine auxotrophic (lys −176) and carries only one of the two large plasmids of CH34, i.e. pMOL28. This plasmid is non-conjugative, has a size of 180 kb and codes for resistance to cobalt, nickel, mercury and chromate (Mergeay *et al*., 1985; Collard *et al*., 1994). Both strains also grow in the presence of 300 p.p.m. kanamycin. Strains A3 and AE176R are autoaggregative variants of AE815 and AE176 respectively (this work). Using the *E. coli* strain CM120(RP4) (Top *et al*., 1995) as donor, the IncP plasmid RP4 (Datta *et al*., 1971) carrying genes for tetracycline, kanamycin and ampicillin resistance was introduced in the *R. eutropha*-like strains A3 and AE176R by conjugation, yielding transconjugants A3(RP4) and AE176(RP4). Strain AE815(RP4) was obtained in the same way in a previous study (Top *et al*., 1995).

Media

All strains were grown in LB broth (Luria broth) or in M9 minimal medium (6 g l^{-1} Na_2HPO_4, 3 g l^{-1} KH_2PO_4, 0.5 g l^{-1} NaCl, 1 g l^{-1} NH_4Cl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, pH 7.4) containing azelaic acid (2 g l^{-1}) as carbon source (E-medium) supplemented with lysine (40 mg l^{-1}) where appropriate. Antibiotics were added to a final concentration of 300, 20, 200 and 200 mg l^{-1} for kanamycin, tetracycline, rifampicin and cycloheximide respectively. The dye Congo red was added to LB to a final concentration of 66 mg l^{-1} (LBCR). Congo red was found specifically to stain the autoaggregative strains. Nickel was added as $NiSO_4$ to LB and to M9 minimal medium to final

concentrations of 5 and 2 mM respectively. The medium YEPD, used for co-culturing yeast and bacteria, contained yeast extract (10 g l^{-1}), bacto peptone (20 g l^{-1}) and glucose (20 g l^{-1}).

BIOLOG

Strains were compared for their ability to use 95 different carbon sources using BIOLOG GN microtitre plates as described previously (Bossier and Verstraete, 1996).

Genetic and molecular genetic techniques

Conjugations were performed by mixing and spotting 10 μl of donor and recipient strain onto LB plates, and leaving overnight at 28°C. Transconjugants were selected on appropriate selective media. Plasmids were extracted from the strains with a modified Kado and Liu method (Fujita and Ike, 1994).

Templates for REP-PCR fingerprinting were obtained by growing the bacterial strains overnight in LB broth, diluting them 25 times in filter-sterilized water (Sigma) and boiling and freezing three times consecutively. The REP-PCR fingerprinting patterns were further obtained with 2 μl of this template in a total volume of 25 μl, using REP primers and the PCR protocol according to the method of de Bruijn (1992), except that the PCR products were run on a 3.5% polyacrylamide gel for ≈3 h at 180 V in a DCode apparatus (Bio-Rad).

Plasmid incompatibility group was determined by PCR, using one set of the specific IncP primers (*trfA1*) (Götz *et al*., 1996) and subsequently hybridizing these amplified fragments with a digoxygenin (DIG)-labelled *trfA1* PCR fragment of the IncP plasmid RP4, obtained through PCR amplification with the same primers. Labelling was performed according to the manufacturer's instructions (Boehringer Mannheim), and hybridizations were carried out as reported previously (Fulthorpe *et al*., 1995).

The isolated strains were analysed by the genomic DNA AFLP fingerprinting technique. The concept of the AFLP technique consists essentially of three steps: (i) digestion of total genomic DNA with two restriction enzymes; (ii) ligation of double-stranded restriction half-site specific adapters to all restriction fragments; and (iii) selective amplification of these modified restriction fragments or templates with primers that contain, in addition to the same sequence as the corresponding adapters, one or more selective bases at their 3′ ends (Vos *et al*., 1995). Total bacterial DNA was extracted and purified as described by Pitcher *et al*. (1989). All protocols relating to the preparation of DNA templates for AFLP analysis were performed essentially as reported previously (Janssen *et al*., 1996). In short, 1 μg of DNA was digested with the restriction endonucleases *Apa*I and *Taq*I (Pharmacia Biotech), and adapters were ligated to all restriction fragments using T4 ligase (Pharmacia Biotech). Adapters were prepared by mixing equimolar amounts of the partly complementary oligonucleotides 5′-TCGTAGACTGCGTACAGGCC-3′ and 5′-TGTACGCAGTCTAC-3′ (for *Apa*I adapter) and 5′-GACGATGAGTCCTGAC-3′ and 5′-CGGTCAGGACTCAT-3′ (for *Taq*I adapter). These adapters served as binding sites for PCR primers A01 (5′-GACTGCGTACAGGCCCA-3′) and A04 (5′-GACTGCGTACAGGCCCT-3′), and for T01 (5′-CGATGAGTCCTGACCGAA-3′) and T03 (5′-GATGAGTCCTGACCGAG-3′) respectively (selective nucleotides are underlined). All amplification procedures have been described by Janssen *et al*. (1996). Primers A01 and A04 were labelled at their 5′ end in a T4 kinase (Pharmacia Biotech) assay using ^{32}P-labelled ATP (Amersham International) as described previously (Vos *et al*., 1995). PCR products were electrophoretically separated in a denaturing 5% polyacrylamide matrix (SequaGel; National Diagnostics) and visualized autoradiographically by exposure to Hyperfilm-MP (Amersham International) (Vos *et al*., 1995). Autoradiographs were scanned using a high-resolution densitometric scanner RayVen RSU1 (X-Ray Scanner Corporation). Transmission image data were stored as TIFF files and processed further by the GELCOMPAR software version 3.1 (Applied Maths).

Scanning electron microscopy (SEM)

Cultures of the strains AE815 and A3 were grown overnight on LB, pelleted and washed once with physiological solution (8.5 g l^{-1} NaCl). The pellets were fixed with 4% formaldehyde overnight and subsequently dehydrated using a gradual series of ethanol concentrations (30%, 50%, 75% and 95%). After a final overnight dehydration step in 100% ethanol, pellets passed a CO_2 critical point drying procedure. These dried pellets were sputter coated with gold and observed by scanning electron microscopy.

Contact angle measurement

The hydrophobicity of the cells was estimated by measurement of the water contact angle on a lawn of cells (Mozes and Rouxhet, 1987). A lawn of washed cells, about 30 layers thick, was prepared on a filter (4.5 cm diameter) with pore diameter 0.45 μm (MF-type, HAWP, Millipore). In order to standardize the moisture content, the filters with the cells were placed on a gel of 1% (w/v) agar containing 10% (v/v) glycerol (t_0) for 30 min. The filters were mounted on a microscope slide using double-sided adhesive tape. Contact angles were measured at several time intervals using 0.3 μl of water (purified by Milli-Q Plus system; Millipore). The values reported (t_0 and t_{60} after 0 and 60 min drying in air respectively) are the average of measurements from 10 different places on the filter.

Acknowledgements

This work was supported by a grant (VLAB/TOP-017) from the Flemish Biotechnology Action Program (IWT, Flanders, Belgium) and in part by FWO grant S2/5-AV-E89. E. M. Top is indebted to the Fund for Scientific Research Flanders (F.W.O.-Vlaanderen) for a position as Research Associate. The support of the Foundation for Training in Industrial and Agricultural Research (FRIA) and of the National Fund for Scientific Research (FNRS) is gratefully acknowledged. We thank S. Maertens and W. Dejonghe for technical and editorial help.

References

Andrup, L., Damgaard, J., and Wassermann, K. (1993) Mobilization of small plasmids in *Bacillus thuringiensis*

subsp. *israelensis* is accompanied by specific aggregation. *J Bacteriol* **175:** 6530–6536.

Bar-Or, Y. (1990) Hydrophobicity in the aquatic environment. In *Microbial Cell Surface Hydrophobicity*. Doyle, R.J., and Rosenberg, M. (eds). Washington, DC: American Society for Microbiology Press, pp. 211–228.

Bartlett, D.H., Wright, M.E., and Silverman, M. (1988) Variable expression of extracellular polysaccharide in the marine bacterium *Pseudomonas atlantica* is controlled by genome rearrangement. *Proc Natl Acad Sci USA* **85:** 3923–3927.

Bossier, P., and Verstraete, W. (1996) *Comamonas testosteroni* colony phenotype influences exopolysaccharide production and co-aggregation with yeast cells. *Appl Environ Microbiol* **62:** 2687–2691.

Brim, H., Heyndrickx, M., De Vos, P., Wilmotte, A., Springael, D., Schlegel, H.G., *et al*. (1999) Amplified rDNA restriction analysis and further genotypic characterisation of metal-resistant soil bacteria and related facultative hydrogenotrophs. *Syst Appl Microbiol* **22:** 258–268.

de Bruijn, F.J. (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. *Appl Environ Microbiol* **58:** 2180–2187.

Collard, J.M., Corbisier, P., Diels, L., Dong, Q., Jeanthon, C., Mergeay, M., *et al*. (1994) Plasmids for heavy metal resistance in *Alcaligenes eutrophus* CH34: mechanisms and applications. *FEMS Microbiol Rev* **14:** 405–414.

Collinson, S.K., Emödy, L., Trust, T.J., and Kay, W.W. (1992) Thin aggregative fimbriae from diarrheagenic *Escherichia coli*. *J Bacteriol* **174:** 4490–4495.

Collinson, S.K., Doig, P.C., Doran, J.L., Clouthier, S., Trust, T.J., and Kay, W.W. (1993) Thin, aggregative fimbriae mediate binding to *Salmonella enteritidis* to fibronectin. *J Bacteriol* **175:** 12–18.

Dalsgaard, I., Nielsen, B., and Larsen, J.L. (1994) Characterisation of *Aeromonas salmonicida* subsp. *salmonicida*: a comparative study of strains of different geographic origin. *J Appl Bacteriol* **77:** 21–30.

Darfeuille-Michaud, A., Jallat, C., Aubel, D., Sirot, D., Rich, C., Sirot, J., *et al*. (1992) R-plasmid-encoded adhesive factor in *Klebsiella pneumoniae* strains responsible for human nosocomial infections. *Infect Immun* **60:** 44–45.

Datta, N., Hedges, R.W., Shaw, E.J., Sykes, R.B., and Richmond, M.H. (1971) Properties of an R factor from *Pseudomonas aeruginosa*. *J Bacteriol* **108:** 1244–1249.

Fernández, A.I.G., Pérez, M.J., Rodríguez, L.A., and Nieto, T.P. (1995) Surface phenotype characteristics and virulence of Spanish isolates of *Aeromonas salmonicida* after passage through fish. *Appl Environ Microbiol* **61:** 2010–2012.

Fujita, M., and Ike, M. (1994) *Wastewater Treatment Using Genetically Engineered Microorganisms* Lancaster, PA, USA: Technomic Publishing Company.

Fulthorpe, R.R., McGowan, C., Maltseva, O.V., Holben, W.E., and Tiedje, J.M. (1995) 2,4-dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. *Appl Environ Microbiol* **61:** 3274–3281.

Götz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschäpe, H., *et al*. (1996) Detection and characterisation of broad-host-range plasmids in environmental bacteria by PCR. *Appl Environ Microbiol* **62:** 2621–2628.

Hermansson, M., Kjelleberg, S., Korhonen, T.K., and Stenstrom, T.A. (1982) Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesion to an air–water interface. *Arch Microbiol* **131:** 308–312.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., *et al*. (1996) Evaluation of the DNA fingerprinting method AFLP™ as a new tool in bacterial taxonomy. *Microbiology* **142:** 1881–1893.

Kampfer, P., Erhalt, R., Beimfohr, C., Bohringer, J., Wagner, M., and Amann, R. (1996) Characterisation of bacterial communities from activated sludge: culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA targeted oligonucleotide probes. *Microb Ecol* **32:** 101–121.

Katupitiya, S., Millet, J., Vesk, M., Viccars, L., Zeman, A., Lidong, Z., *et al*. (1995) A mutant of *Azospirillum brasilense* Sp7 impaired in flocculation with a modified colonization pattern and superior nitrogen fixation in association with wheat. *Appl Environ Microbiol* **61:** 1987–1995.

Mergeay, M., Niels, D., Schlegel, H.G., Gerits, J., Charles, P., and Van Gijsegem, F. (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J Bacteriol* **162:** 328–334.

Moxon, E.R., Rainey, P.B., Nowak, M.A., and Lenski, R.E. (1994) Adaptive evolution in highly mutable loci in pathogenic bacteria. *Curr Biol* **4:** 24–34.

Mozes, N., and Rouxhet, P.G. (1987) Methods for measuring hydrophobicity of microorganisms. *J Microbiol Methods* **6:** 99–112.

Pitcher, D.G., Saunders, N.A., and Owen, R.J. (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8:** 151–156.

Rainey, P.B., Moxon, E.R., and Thompson, I.P. (1993) Intraclonal polymorphism in bacteria. *Adv Microb Ecol* **13:** 263–300.

Rosenberg, M., and Doyle, R.J. (1990) Microbial cell surface hydrophobicity: History, measurement and significance. In *Microbial Cell Surface Hydrophobicity*. Doyle, R.J., and Rosenberg, M. (eds). Washington, DC: American Society for Microbiology Press, pp. 1–37.

Savarino, S.J., Fox, P., Yikang, D., and Nataro, J.P. (1994) Identification and characterisation of a gene cluster mediating enteroaggregative *Escherichia coli* aggregative adherence fimbria I biogenesis. *J Bacteriol* **176:** 4949–4957.

Smalley, J.W., Birss, A.J., McKee, A.S., and Marsh, P.D. (1995) Congo red binding by *Porphyromonas gingivalis* is mediated by a 66 kDa outer-membrane protein. *Microbiology* **141:** 205–211.

Springael, D., Kreps, S., and Mergeay, M. (1993) Identification of catabolic transposon, Tn*4371*, carrying biphenyl and 4-chlorobiphenyl degradation genes in *Alcaligenes eutrophus* A5. *J Bacteriol* **175:** 1674–1681.

Top, E.M., De Rore, H., Collard, J.-M., Gellens, V., Slobodkina, G., Verstraete, W., *et al*. (1995). Retromobilization of heavy metal resistance genes in unpolluted and heavy metal polluted soil. *FEMS Microbiol Ecol* **18:** 191–203.

Urbain, V., Block, J.C., and Manem, J. (1993) Bioflocculation in activated sludge: an analytical approach. *Water Res* **27:** 829–838.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., *et al*. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23:** 4407–4414.

Zita, A., and Hermansson, M. (1994) Effects of ionic strength on bacterial adhesion and stability of flocs in a wastewater activated sludge system. *Appl Environ Microbiol* **60:** 3041–3048.

Zita, A., and Hermansson, M. (1997) Effects of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. *Appl Environ Microbiol* **63:** 1168–1170.